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DATE DEPOSITED
19 February 2024

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The Influence of Common Genetic Variations on the Acute and Long-Term Effects of Caffeine on Cognitive Performance

Thesis submitted by:

Angeliki Kapellou

For the award of Doctor of Philosophy

Faculty of Sport, Allied Health and Performance Science
St Mary's University Twickenham, London
September 2023

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Acknowledgments

Primarily, I express my profound gratitude to my esteemed supervisors, Dr Yiannis Mavrommatis and Dr Leta Pilic. Their guidance, expertise and unwavering belief in my abilities have been instrumental in shaping my research ethos. Their patience, insightful feedback and encouragement have been a constant source of motivation throughout my PhD journey.

To the members of TeamGene, Ammi and Cat, I extend my sincere thanks. Your collaboration, shared insights and camaraderie have enriched this research in more ways than I can express. Collaborating with you both has been a privilege and I am grateful for the support and encouragement you provided to me.

I am deeply indebted to St Mary's University for providing a nurturing academic environment that facilitated my intellectual growth and allowed me to pursue this research with passion and dedication. I also extend my appreciation to the participants who generously contributed to this work. Your valuable cooperation has been fundamental in shaping this research and I am honoured to have had your involvement in this study.

To my cherished family, I express my heartfelt gratitude. My husband, George, has been a source of support, understanding and encouragement throughout this journey. His love and belief in me have given me the strength to overcome challenges and reach for my goals. To my sister, Georgia, and my nieces, Stella and Nikki, thank you for your love and inspiration. Your presence in my life has been a constant reminder of the importance of perseverance and family bonds.

To my dear friends, Natalia, Lynn, Anna, Sofia and Vasilis, you have been the pillars of support and the bright lights that illuminated this journey. I am fortunate to have you by my side as friends and I cherish each of you dearly.

Finally, I dedicate this thesis to my late parents and most especially, to my beloved father. His untimely departure during the initial stages of my PhD left an irreplaceable void. His belief in my potential and his unwavering support were the foundation upon which I built my academic pursuits. I hope, from wherever he may be, that he is proud of what I have accomplished. Every achievement in this thesis is a tribute to his memory.

Angeliki Kapellou - The Influence of Common Genetic Variations on the Acute and Long-Term Effects of Caffeine on Cognitive Performance

PhD Health Sciences, St Mary's University, London

30/09/2023

Abstract

Caffeine, a potent psychostimulant widely consumed worldwide, has a controversial impact on cognition. Genetic factors related to caffeine metabolism and physiological effects may contribute to research variability. Therefore, this research explores the interactions between genetic variations implicated in caffeine pharmacokinetics and pharmacodynamics and habitual and acute caffeine intake on performance across all key domains of cognition.

The present research consists of three studies. The first study involved a systematic review of genetics studies on caffeine and brain-related outcomes. The second study was an online population-based study (n = 131) assessing a) habitual caffeine intake from all sources, b) cognitive performance in social cognition, memory, executive function and attention) and c) genes associated with caffeine, sleep quality and cognitive performance. The methodology from the second study was transferred to the final study, a double-blind cross-over randomised trial (n = 12), involving a 4-week protocol of long-term caffeine/placebo intake and four experimental sessions of 3 mg/kg body mass acute caffeine/placebo supplementation. The cognitive test battery was performed during each session at baseline and 1-, 3- and 6-h post-supplementation.

Significant gene x caffeine interactions were observed for the domains of social cognition, F (2, 123) = 5.848, p = 0.004 and executive function, F (2, 109) = 3.690, p = 0.028. 'Fast' metabolisers had a lower performance in social cognition compared with 'slow' metabolisers, among high caffeine consumers (p = 0.004), while 'slow' metabolisers had a lower performance in executive function compared with 'fast' metabolisers among moderate caffeine consumers (p = 0.002). No other gene x caffeine interactions were observed. The present research has introduced, for the first time in genetics studies, a previously used protocol designed to control for caffeine withdrawal, a major limitation in caffeine research. Future genetics studies are recommended to incorporate this protocol to delineate how genetics can influence the effects of caffeine on cognition and other aspects of human health.

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Publications and Conference Presentations

Kapellou, A., Pilic, L. and Mavrommatis Y. 2023. Habitual Caffeine Intake, Cognition and DNA. Poster presentation at NuGOweek 2023 19th edition, September 5-8, 2023, Senigallia, Italy.

Kapellou, A., Pilic, L. and Mavrommatis Y. 2023. Habitual Caffeine Intake, Cognition and DNA. Oral presentation at the SAHPS Festival of Research, May 23-26, 2023, St Mary's University Twickenham.

Kapellou, A., King, A., Graham, C.A.M., Pilic, L. and Mavrommatis Y. 2023. Genetics of caffeine and brain-related outcomes - a systematic review of observational studies and randomized trials. Nutr Rev. 2023 Apr 8:nuad029. doi: 10.1093/nutrit/nuad029.

Kapellou, A., Pilic, L. and Mavrommatis, Y. 2022. The Genetics of Caffeine on Brain-related Effects and Neurodegeneration. Oral presentation at the 2nd International Rehabilitation Conference, 4-5 November 2022, Athens, Greece.

Kapellou, A., King, A., Graham, C.A.M., Pilic, L. and Mavrommatis Y. 2022. The Genetics of Caffeine on Brain-related Outcomes – A Systematic Review. Poster presentation at NuGOweek 2022 18th edition LIVE, August 29 – September 1, 2022, Tarragona, Spain. *The poster was awarded the prize for second best poster presentation.*

Kapellou, A., King, A., Graham, C.A.M., Pilic, L. and Mavrommatis Y. 2022. The Genetics of Caffeine on Brain-related Outcomes – A Systematic Review. Mini-oral presentation at the SAHPS Festival of Research, May 23-27, 2022, St Mary's University Twickenham.

Kapellou, A., Silva, G., Pilic, L., & Mavrommatis, Y. 2021. Nutrition knowledge, food choices and diet quality of genotyped and non-genotyped individuals during the COVID-19 pandemic. Nutrition And Health, 026010602110268. https://doi.org/10.1177/02601060211026834.

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Abbreviations

AAMU	5-acetylamino-6-amino-3-methyluracil
AD	Alzheimer's Disease
ADA	Adenosine Deaminase
ADAM12	ADAM Metallopeptidase Domain 12
ADHD	Attention Deficit Hyperactivity Disorder
ADORA2A	Adenosine Receptor A2a
AFMU	5-acetylamino-6-formylamino-3-methyluracil
AHR	Aryl Hydrocarbon Receptor
ANOVA	Analysis of variance
APOE	Apolipoprotein E
ATP	Adenosine triphosphate
AUC	Area under the curve
BBB	Blood-brain-barrier
BD	Bipolar disorder
BDNF	Brain-Derived Neurotrophic Factor
BMI	Body mass index
cAMP	cyclic adenosine monophosphate
CCB	Caffeine-containing beverages
CCQ-R	Caffeine Consumption Questionnaire
CI	Confidence interval
CMSG	Genetic caffeine metabolism score
CNS	Central Nervous System
COMT	Catechol-o-methyltransferase
COPD	Coronary Obstructive Pulmonary Disease
CPT	Continuous Performance Task
CR	Cognitive Reserve
CRN	Council for Responsible Nutrition
CT	Computed Tomography
CYP1A2	Cytochrome P450 1A2
CYP2D6	Cytochrome P450 2D6
CYP2E1	Cytochrome P450 2E1
CYP3A4	Cytochrome P450 3A4
CYP3A5	Cytochrome P450 3A5
CPT	Continuous performance task
DAT1	Dopamine transporter 1
DHA	Docosahexaenoic acid
DMXL2	Dmx Like 2
DSM-5	Diagnostic and Statistical Manual for Mental Disorders
EEG	Electroencephalogram
EFSA	European Food Safety Authority
EMIT	Enzyme Multiplied Immunoassay Technique
EPA	Eicosapentaenoic acid
ERT	Expression Recognition Task
FDA	Food and Drug Administration
FFQ	Food Frequency Questionnaire
FI	Fluid Intelligence
fMRI	Functional Magnetic Resonance Imaging
GABA	Gamma-aminobutyric acid
GCs	Glucocorticoids
GCKR	Glucokinase Regulator
GDR	Genotype-Driven Recruitment
GPD2	Glycerol-3-Phosphate Dehydrogenase 2
GRADE	Grading of Recommendations, Assessment, Development & Evaluations
GWAS	Genome Wide Association Studies

HD	Huntington's disease
HPA	Hypothalamic-pituitary-adrenal
HPLC	High-performance liquid chromatography
HWE	Hardy Weinberg Equilibrium
IPAQ	International Physical Activity Questionnaire
ISSN	International Society of Sports Nutrition
ITIH3	Inter-Alpha-Trypsin Inhibitor Heavy Chain H3
IQ	Intelligence Quotient
IQR	Interquartile range
K_a	Absorption rate constant
K_e	Elimination rate constant
K_i	Inhibition rate constant
KDEFS	Karolinska Directed Emotional Face System
KSS	Karolinska Sleepiness Scale
LD	Linkage Disequilibrium
LSNR	Log of the signal-to noise ratio
MAF	Minor Allele Frequency
MLXIPL	MLX Interacting Protein Like
MMSE	Mini-Mental State Exam
MoCA	Montreal Cognitive Assessment
MS	Multiple Sclerosis
MTCH2	Mitochondrial Carrier 2
NAT2	N-acetyltransferase-2
NDA	Nutrition and allergies
NO	Nitric Oxide
NPSR	Neuropeptide S receptor gene
NRSIs	Non-randomised Studies of interventions
PAL	Physical Activity Level
PD	Parkinson's disease
PET	Positron Emission Tomography
PICOS	Population, Intervention, Comparison, Outcome, Study design
PN	Personalised Nutrition
POR	Cytochrome P450 Oxidoreductase
PRISMA	Preferred Reporting Items for Systematic Review and Meta-Analyses
PSQI	Pittsburgh Sleep Quality Index
PVT	Psychomotor Vigilance Task
RCT	Randomised Controlled Trial
REM	Rapid-Eye Movement
RoB-2	Risk of Bias in randomised trials
ROBINS-I	Risk Of Bias in Non-randomised Studies of interventions
RT	Reaction Time
SD	Standard Deviation
SDS	Symbol Digit Substitution
SES	Socioeconomic status
SLC6A4	Solute Carrier family 6-member 4
SNP	Single Nucleotide Polymorphism
SPPL2C	Signal Peptide Peptidase Like 2C
SSRIs	Selective Serotonin Reuptake Inhibitors
TBI	Traumatic brain injury
TOT	Time on task
TSD	Total Sleep Deprivation
TST	Total Sleep Time
WNT4	Wnt Family Member 4
XO	Xanthine Oxidase
ΔRT	Change in RT

Chapter 1. Literature Review

This chapter represents the literature review that provides the basis of the present research. The first section defines the brain, known as the cognitive organ, and explores the key domains of human cognition, together with the several factors that enhance or hinder cognitive function. In the second section, the most widely used enhancer of cognitive performance, caffeine, is discussed. In this section, caffeine pharmacokinetics and pharmacodynamics, as well as the factors influencing them are explained. The third section constitutes an overview of one of the key factors affecting caffeine pharmacokinetics and pharmacodynamics: genetics. At the end of this chapter, the aims and objectives of this programme of research are introduced.

1.1. Cognition

1.1.1. Brain – the cognitive organ

Cognition is defined as the mental action or process of acquiring knowledge and understanding through thought, experience and the senses (Dhakal and Bobrin, 2023). The brain is an organ composed of nervous tissue and is responsible for executing all cognitive processes such as movement, senses, emotions, language, communication, thinking and memory (Maldonado and Alsayouri, 2023). Together with the spinal cord, they comprise the central nervous system (CNS), the most complex of all biological systems in the human body (Thau, Reddy and Singh, 2023).

The brain, despite comprising only 2% of body weight, has a substantial metabolic demand. It accounts for 15% of the cardiac output, 20% of total body oxygen and 20% of the total energy supply (Rolfe and Brown, 1997; Siegel *et al.*, 1999). While glucose serves as the primary energy source for the brain, it can adapt in response to low glucose levels to use ketone bodies and can switch to lactate utilisation during physical activity (Owen *et al.*, 1967; Courchesne-Loyer *et al.*, 2017). Although research has made significant progress in understanding this remarkable organ, the complex structure and functions of the brain remain a subject of ongoing exploration and understanding (McGilchrist, 2010).

1.1.1.1 Brain anatomy

The three main parts of the human brain include the cerebrum, the cerebellum and the brainstem. The cerebrum is the largest part of the brain and is responsible for a wide range of complex functions, including consciousness, perception, thinking and memory (Maldonado and Alsayouri, 2023). It is divided into two hemispheres (left and right), each of which is associated with specific cognitive functions (Jawabri and Sharma, 2023). The cerebral cortex, the outer layer of the cerebrum, is responsible for integrating and interpreting sensory information from different parts of the body, allowing us to perceive and respond to our environment (Chayer and Freedman, 2001). The cerebrum also plays a key role in emotion regulation, motivation and social behaviour (Sporns, Tononi and Kötter, 2005; McGilchrist, 2010).

The cerebellum is a small part of the brain that plays a crucial role in motor coordination and control (Manto *et al.*, 2012). It receives input from the sensory systems, spinal cord and other parts of the brain, and integrates this information to fine-tune and modulate movements, including balance and posture (Asan, McIntosh and Carmel, 2022). The cerebellum also contributes to cognitive processes such as attention, language and memory, as well as emotional regulation (Jimsheleishvili and Dididze, 2023).

The brainstem is the part of the brain that connects the spinal cord to the rest of the brain (Maldonado and Alsayouri, 2023). It is responsible for many vital functions, including regulating heart rate, breathing, blood pressure and consciousness (Angeles Fernández-Gil *et al.*, 2010). It also controls basic reflexes such as swallowing, vomiting and coughing (Van Essen *et al.*, 1998).

1.1.1.2. Neurons – a communication network

The adult brain is made up of over 100 billion neurons, which are cells that process information and communicate throughout the nervous system by connecting with neighbouring neurons (Brinkmann *et al.*, 2008; Stiles and Jernigan, 2010). These specialised cells receive, process and transmit electrochemical signals, enabling the brain to perform a wide range of functions throughout the body (Lovinger, 2008). Nevertheless, despite the significant progress in

Neurosciences, the human cognitive function that emerges from neuronal structure and dynamics is not entirely understood (Sporns, Tononi and Kötter, 2005).

Despite sharing many characteristics with other cells, neurons are specialised in a way that allows them to fulfil their distinct function in the nervous system (Lee *et al.*, 2019). One such unique feature is their shape. Neurons have thin branches known as dendrites and axons in addition to the cell body, or soma (Lovinger, 2008). The brief, branch-like structures called dendrites are responsible for receiving signals from other neurons. The axon is a long, thin structure that sends messages to neighbouring neurons, muscles and glands (Lee *et al.*, 2019). The small, bulb-like structures known as axon terminals are found at the end of axons and are responsible for releasing neurotransmitters, which are chemical messengers used to connect with other neurons or muscle cells (Figure 1.1) (Guillamón-Vivancos, Gómez-Pinedo and Matías-Guiu, 2015).

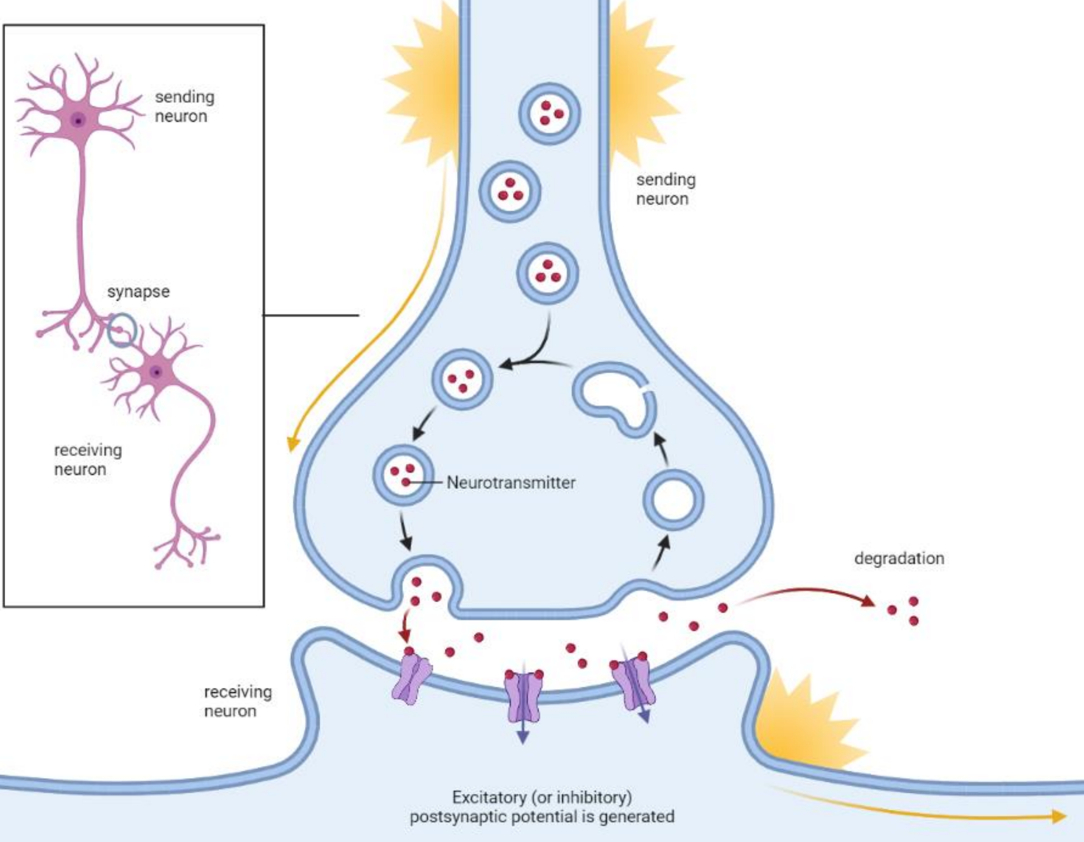

Figure 1. 1 Neurons during synaptic transmission. At the synaptic junction, the presynaptic neuron (above) releases neurotransmitters (small red circles) into the synaptic cleft. These neurotransmitters travel across the gap and bind to receptors on the postsynaptic neuron (below), generating electrical signals.

Communication between neurons is facilitated via the release of neurotransmitters into the synaptic space, a 20-50 nm area between neurons (Lovinger, 2008). The neurotransmitter then travels to the postsynaptic neuron and binds to receptors to influence its activity (Südhof, 2013). Each of the steps that lead to release can be regulated during a variety of short- and long-term presynaptic plasticity processes, thus modulating the properties of neural networks and underlying multiple forms of information processing in the brain (Regehr, 2012).

1.1.1.3 Neurotransmitters

Cognitive function involves the participation of many different neurotransmitters in a variety of brain areas (Rizo, 2018). Neurotransmitters are chemical messengers that carry the signals between neurons and target cells throughout the body. These target cells may be in glands, muscles, or other neurons (Herlenius and Lagercrantz, 2004). Billions of neurotransmitter molecules constantly work to keep the brain and the entire body functioning, from breathing, heartbeat and learning, to psychological functions such as fear, anxiety and mood (Sheffler, Reddy and Pillarisetty, 2023).

When a neurotransmitter binds to a specific receptor, it can trigger a cascade of chemical events inside the postsynaptic neuron that ultimately lead to changes in its activity (Jones *et al.*, 2017). Neurotransmitters are implicated in a variety of physiological and psychological processes and each one has a unique impact on the postsynaptic neuron (Sheffler, Reddy and Pillarisetty, 2023). Sometimes they bind to receptors and increase the likelihood that the neuron will produce an action potential (excitatory effect) (Niyonambaza *et al.*, 2019). In other cases, the neurotransmitter can block the signal from continuing, preventing the message from being delivered (inhibitory effect) (Murley and Rowe, 2018). Modulatory neurotransmitters, also known as neuromodulators, can diffuse more broadly and act on multiple neurons to produce longer-lasting effects on neural activity (Nadim and Bucher, 2014; Avery and Krichmar, 2017). Based on their chemical properties, neurotransmitters are categorised in classes (Teleanu *et al.*, 2022) and these are listed below with some examples.

1.1.1.3.1 Amino acids

Glutamate and gamma-aminobutyric acid (GABA) are the major amino acid neurotransmitters. Glutamate is the principal excitatory neurotransmitter used in the brain and the primary mediator of nervous system plasticity and is implicated in memory (Gross, 2006; Zhou and Danbolt, 2014). GABA is the major inhibitory neurotransmitter and accounts for approximately 40% of the inhibitory processing in the brain (Bowery and Smart, 2006).

1.1.1.3.2 Monoamines

Monoamines include dopamine, epinephrine and norepinephrine. Dopamine plays an essential role in several brain functions including learning, motor control, reward and emotion (Wise, 2004; Ko and Strafella, 2012). Epinephrine, also known as adrenaline, plays a key role in stress response by increasing heart rate, blood pressure and blood glucose levels (Molina, 2005). Norepinephrine release in the brain exerts effects on a variety of processes, including stress, sleep, attention and focus (O'Donnell *et al.*, 2012).

1.1.1.3.3 Peptides

Endorphins are the major peptide neurotransmitters. They bind to opioid receptors and produce a feeling of euphoria and pain relief and are released during exercise, stress and other activities (Chaudhry and Gossman, 2023). They have been implicated in modulating the reward and pleasure pathways of the brain (Holden, Jeong and Forrest, 2005).

1.1.1.3.4 Purines

Adenosine is the major purine and plays important roles in the regulation of synaptic transmission and neuronal activity in the CNS by inhibiting neurotransmitter release and decreasing neuronal excitability (Cunha, 2001). Thus, adenosine influences several brain functions such as sleep and arousal, cognition and memory, neuronal damage and degeneration (Porkka-Heiskanen, 1999; de Mendonça and Ribeiro, 2001; Ribeiro, Sebastião and de Mendonça, 2002).

1.1.1.3.5 Gasotransmitters

Nitric oxide (NO), the principal gasotransmitter, is involved in a wide range of physiological processes, including cardiovascular function and immune response

(Dawson and Snyder, 1994). In the brain, NO is involved in synaptic plasticity and has been implicated in learning and memory (Garthwaite, 2008).

1.1.1.3.6 Cholinergic neurotransmitters

Acetylcholine is the most widely studied in this class. Acetylcholine is found in both the central and peripheral nervous systems and it is the primary neurotransmitter associated with motor neurons, as well as memory and learning (Picciotto, Higley and Mineur, 2012).

In summary, the brain, the control centre of the human body, consists of billions of interconnected neurons. Neurons are specialised cells that transmit information through electrical and chemical signals. This communication occurs via molecules called neurotransmitters, which travel between neurons to facilitate the transfer of information. The intricate network of neurons and neurotransmitters forms the foundation of cognitive processes, which will be discussed in the following section.

1.1.2. Classes and domains of human cognition

Cognition includes all the conscious and unconscious processes involved in thinking, perceiving and reasoning (Lezak, Howieson and Loring, 2004). The term 'cognitive function' refers to the performance in objective tasks requiring conscious mental effort that enables humans to exert control over their environment and is critical for survival (Lamport *et al.*, 2014; Taylor *et al.*, 2016). Characterisation and classification of cognition in Neuropsychology refers to classes and domains of cognitive performance. Within each class and domain there are subdomains, which refer to component ability processes within the larger constructs (Harvey, 2019).

The classes of cognitive function have their analogues in the computer operations of input, storage, processing (sorting, combining, relating data in various ways) and output (Matlin, 1989). Each functional class comprises many discrete activities. Although each function constitutes a distinct class of behaviours, normally they work in a close, interdependent concert (Lezak, Howieson and Loring, 2004). These classes of functions share basic neuroanatomical and psychometric relationships within the functional system, yet they differ in their neuroanatomical organisation and behavioural expression (Lezak, Howieson and Loring, 2004). Despite the seeming ease with which the classes of cognitive functions can be

distinguished conceptually, they are not just interconnected; they are intrinsically linked, representing different facets of the same cognitive activity (Harvey, 2019).

The conceptualisation of cognitive domains was originally linked to the brain regions where these processes were observed (Babcock, 1930). This strategy is based on lesion studies and characterise functions as originating, for example, from the frontal lobe, hippocampus, or other structures (Harvey, 2019). Over time, another approach has been employed to conceptualise cognitive ability domains. This includes classification by the general process involved, such as memory or attention (Lezak, Howieson and Loring, 2004).

An additional organisational structure is hierarchical and based on the complexity of the operations. The task-control system of the brain is thought to consist of functionally diverse regions that are anatomically separate from downstream processing systems (Posner and Petersen, 1990). Often referred to as top-down versus bottom-up, the idea is that basic sensory and perceptual operations are least complex and reasoning and problem solving, referred to as executive functioning, are most complex (Al-Aidroos, Said and Turk-Browne, 2012). Therefore, cognitive functions are categorised as simple or complex; simple tasks are those which require very simple perceptual motor skills (e.g., attention tasks), whereas complex tasks require a greater effort, such as multiple/dual tasks (e.g., memory and executive function tasks) (Harvey, 2019).

As highlighted by Harvey (2019), while there is consensus on the nature of most cognitive domains, clear inconsistencies exist in the literature. These inconsistencies are predominantly found in domains that encompass multiple component processes, and it is frequently uncertain whether these processes should be classified within the broader domains considered separately (Harvey, 2019). The American Psychiatric Association's Diagnostic and Statistical Manual for Mental Disorders (DSM-5) identifies six core components of neurocognitive function: a) perceptual-motor function, b) attention and processing speed, c) executive function, d) memory, e) language and f) social cognition (Figure 1.2) (Sachdev *et al.*, 2014; Harvey, 2019).

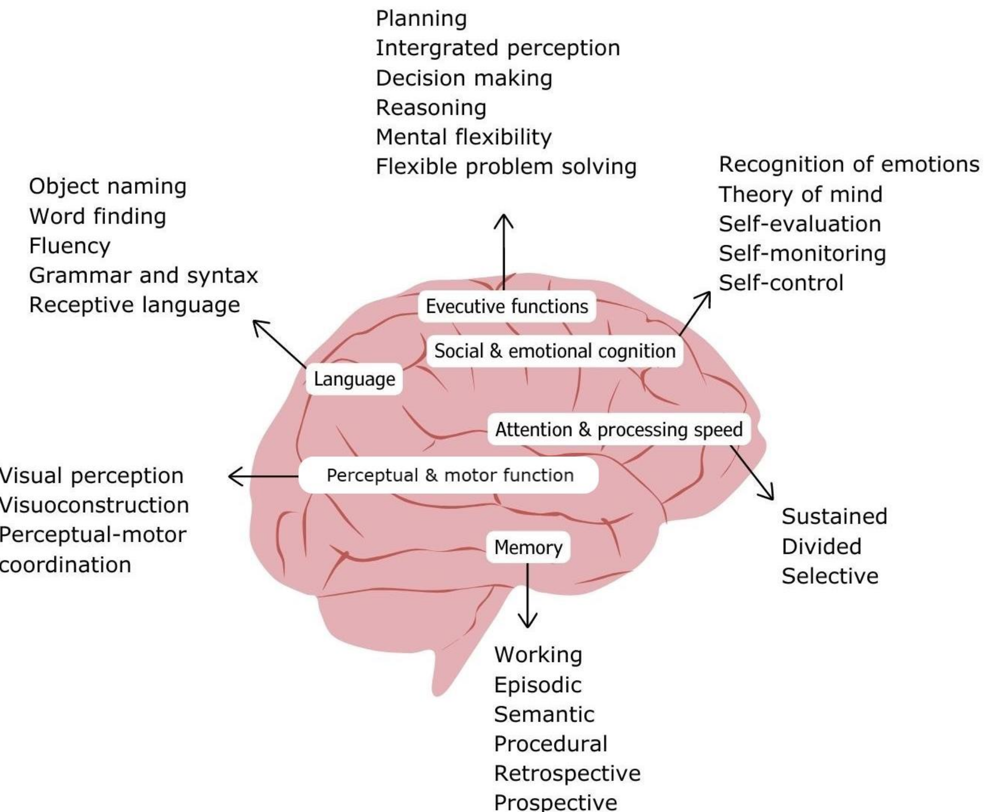

Figure 1. 2 An overview of the major domains (in white frame) and subdomains (indicated by arrows) of cognitive function.

1.1.2.1. Perceptual – motor function

Perceptual – motor function refers to the ability to process and interpret sensory stimuli and then use motor skills to respond to that information (Censor, Sagi and Cohen, 2012). This function includes skills such as hand-eye coordination, balance and spatial awareness (Rattanavichit *et al.*, 2022). The ability to identify a stimulus from the five sensory modalities respectively, i.e., visual, auditory, tactile, gustatory, and olfactory senses, falls under the domain of perception (Zhong, Ahrens and Huang, 2023). Within the domain of perception, sensory data are both processed and synthesised (Harvey, 2019). One fundamental aspect of perception pertains to the recognition of objects based on prior sensory input (Censor, Sagi and Cohen, 2012).

Motor skills are actions carried out when the CNS and muscles work together. It is a function that involves the precise movement of muscles with the

intent to perform a specific act and involves various brain regions (Mink, 1996; Shadmehr and Holcomb, 1997). Motor skills are categorised into gross and fine motor skills. Gross motor skills are involved in movement and coordination of the arms and legs in actions such as balance, running and swimming (Schmidt *et al.*, 2018). Fine motor skills are required in smaller movements that involve the wrists, hands, fingers and toes and include more precise actions such as picking up objects between the thumb and finger (Grissmer *et al.*, 2010). Fine motor skills are shown to be involved in the development of cognitive abilities in typically developing children (Martzog, Stoeger and Suggate, 2019) and to correlate strongly with attention and executive function (Roebers and Kauer, 2009; Michel *et al.*, 2011).

1.1.2.2. Attention and processing speed

The domain of attention covers the ability to selectively attend to specific information (Chun, Golomb and Turk-Browne, 2011). Attention is a fundamental element for information processing and is closely linked with the domain of memory - the more one's attention is directed toward information, the more likely they will be able to retrieve it from memory at a later stage (Oberauer, 2019).

The attention system has various subdivisions. Based on the theory of Petersen and Posner, attention is divided into sustained and selective attention (Posner and Petersen, 1990; Petersen and Posner, 2012). Sustained attention refers to the ability to maintain performance on an attention-requiring task over an extended period, especially in a low-arousal context (Oken, Salinsky and Elsas, 2006; Silva and Lee, 2021). Sustained attention overlaps with vigilance, a term that refers to the ability to sustain attention to a task over time (Oken, Salinsky and Elsas, 2006). Additionally, it is associated with arousal, a term referring to the nonspecific brain activation in relation to sleep-wake states (Oken, Salinsky and Elsas, 2006). Selective attention entails prioritising certain information while ignoring others by focusing on specific aspects within a scene (Carrasco, 2011).

Another related concept in attentional processes is divided attention, which describes the attention to two or more stimuli at a time and has been proposed as separate from selective attention (Posner and Petersen, 1990). However, brain imaging studies have failed to demonstrate clear differences in functional activation of brain areas during selective and divided attention tasks, suggesting that these two types of attention involve common processing mechanisms (Hahn *et al.*, 2008).

Processing speed is considered a core component of attention because most tests of attention are speed-dependent (Silva and Lee, 2021). Processing speed relates to the ability to direct mental focus to relevant information, facilitating rapid and efficient processing, as well as enabling appropriate decision-making in line with external rules and internal knowledge (Schneider and Shiffrin, 1977). Processing speed refers to cognitive processing assessments that require rapid performance of tasks that range from simple to complex (Lim and Dinges, 1987). Psychomotor speed refers to the speed at which information can be perceived, understood, and responded to with physical movements, e.g., the click of a button (Silva and Lee, 2021).

1.1.2.3. Memory

Memory, which is among the most complex cognitive domains, refers to the ability to store information (Harvey, 2019). There are three key components of memory and these include encoding, storage and retrieval (Zlotnik and Vansintjan, 2019). Encoding refers to the process of taking in information and converting it into a form that can be stored for longer-term storage. The effectiveness of encoding can be influenced by a variety of factors, including attention and repetition (Schacter, 2013). Storage refers to the process of retention of information after encoding, which can be recalled at long post-encoding time periods. It has been argued that all information ever learned is stored and that failures to access are entirely due to retrieval failures (Zlotnik and Vansintjan, 2019). Retrieval is the process of accessing and retrieving stored information and can happen in several different ways (Karpicke and Grimaldi, 2012). Factors that can influence the retrieval of information from memory include the strength and quality of the original encoding, the context in which the information was learned, the emotional significance of the information and the level of rehearsal or repetition of information (Squire, 2009).

Memory can be categorised as short-term (retrieval occurs within 30 s of stimulus) or long-term (retrieval occurs after 30 s). Additionally, it can be explicit, involving conscious retrieval or implicit, involving unconscious retrieval (Cowan, 2008). There is evidence of specialised CNS processing for object, location, action, verbal and spatial memory (Henson, Burgess and Frith, 2000; Ranganath, Johnson and D'Esposito, 2003; Wagner *et al.*, 2005; Simons *et al.*, 2006; Spiers and

Maguire, 2007; Voss and Paller, 2008). The main subdomains of memory are presented below.

1.1.2.3.1. Working memory

Working memory involves the temporary maintenance of information in consciousness for adaptive use, requiring the control of attentional resources (Burgess, 1997; Baddeley, 2012). Working memory includes two separable components - maintenance and manipulation of information (Baddeley and Logie, 1999). Maintenance working memory refers to the ability to hold information over brief periods of time. Some examples of tasks that rely on maintenance working memory include remembering a phone number long enough to dial it, recalling a list of items to buy at the grocery store, etc. (Nyberg and Eriksson, 2016; Jablonska *et al.*, 2020). Unlike maintenance working memory, which is passive storage of information, manipulation working memory involves actively updating, rearranging, or transforming information in real-time to achieve a specific goal or task (Nyberg and Eriksson, 2016). Manipulation working memory is involved in a wide range of cognitive tasks, including problem-solving and language comprehension (Jablonska *et al.*, 2020).

Information can be transferred from working memory to longer-term storage, but that process requires attention, active processing, or salience of the stimulus in working memory for encoding (Baddeley, 2012). Functional neuroimaging work shows that working memory is not localised in a single brain region, but is rather described as an interaction between the prefrontal cortex and the rest of the brain (D'Esposito, 2007). Evidence also suggests that the network of brain regions recruited for the active maintenance of task-relevant information depends on the type of information being maintained (Curtis, Rao and D'Esposito, 2004; D'Esposito, 2007). The observation that numerous elements of working memory appear to be subject to higher-level control has prompted some to argue that working memory is a component of executive function (McCabe *et al.*, 2010). Considering the broad definition of executive functioning, the manipulation aspect of working memory can be conceptualised as such. However, it is worth noting that maintenance working memory can be measured even in organisms like drosophila, whose executive functioning is clearly limited (Harvey, 2019).

1.1.2.3.2. Episodic memory

Episodic memory, a subtype of long-term memory, involves the ability to recollect specific personal events or experiences that occurred at a particular time and place (Dickerson and Eichenbaum, 2010). The ability to describe the specifics of a recent office meeting or a holiday gathering that occurred in the preceding weeks or months, for example, relies on intact episodic memory function (Pause *et al.*, 2013). Episodic memory involves multiple brain regions, including the hippocampus, amygdala and prefrontal cortex (Aggleton and Brown, 1999).

1.1.2.3.3. Semantic memory

Semantic memory refers to the process of long-term storage of general knowledge such as concepts, meanings and categories (Schendan, 2012). It is the mental storehouse of our knowledge of language, concepts and facts that we have acquired throughout our lifetime and is essential for everyday functioning such as understanding language and making decisions. Semantic memory appears to remain intact over the lifespan and continues to accrue new information even into late life (Czaja *et al.*, 2006).

1.1.2.3.4. Procedural memory

Procedural memory involves a network of interconnected brain structures primarily located within the frontal and basal ganglia (Ullman, 2016). It refers to the learning and remembering how to perform different skills or actions such as riding a bike or playing a musical instrument (Henke, 2010). This type of memory is formed gradually over time through repeated practice and repetition and is considered a type of implicit memory, which means it does not require deliberate attention or conscious effort (Oudman *et al.*, 2015).

1.1.2.3.5. Retrospective and prospective memory

Contrary to retrospective memory, which refers to retrieving past events, prospective memory is the ability to remember to perform a planned action at a specific time or in response to a specific cue (Papagno, 2018). Prospective memory encompasses the ability to remember to perform a task in the future, such as taking medication at a designated time, making it one of the most important cognitive domains in everyday life (Twamley *et al.*, 2008).

Prospective memory, often referred to as memory for the future, is a form of episodic memory and shares many characteristics with retrospective memory

(Kondo *et al.*, 2010). Neuroimaging findings indicate that specific regions of the parietal lobe exhibit increased cerebral blood flow when participants were anticipating a prospective memory cue (Burgess, Quayle and Frith, 2001).

1.1.2.4. Executive function

Executive function is a domain that includes complex (or higher-order) thinking and decision-making processes that are distinguishable from processing speed, memory and attention (Diamond, 2013). It is also commonly referred to as reasoning and problem solving. The global concept of executive functioning is the set of processes that manifest control over other component cognitive abilities, such that cognitive resources can be effectively utilised to solve problems efficiently and make plans (Harvey, 2019).

This domain includes processes such as inhibition (the ability to suppress automatic and habitual responses or behaviours), strategic memory search (conscious, controlled retrieval of structured information), planning, mental flexibility (consideration of new strategies and rapid rejection of failed efforts), updating, multitasking and initiation and monitoring of actions (Burgess, 1997; Baddeley, 1998; Miyake *et al.*, 2000; Leh, Petrides and Strafella, 2010; Diamond, 2013).

Executive function is the definitional set of top-down processes, because simple cognitive abilities are required for real-world adaptive success (Harvey, 2019). In fact, all of the above processes are dependent on working memory and are controlled by attentional resources (Baddeley, 2012). Neuroimaging studies suggest that the prefrontal cortex and striatum interact to perform specific executive functions, and that distinct brain regions are recruited for different executive functions (Robbins, 2007). For instance, the left inferior frontal gyrus in the prefrontal cortex is recruited in verbal fluency tasks (Costafreda *et al.*, 2006), whereas the right inferior frontal gyrus shows greater activation in tasks measuring both shifting and inhibition (Robbins, 2007).

1.1.2.5. Language

Language is a complex cognitive domain that encompasses several abilities, including speech perception, comprehension, production and written language

(Fedorenko, 2014; Harvey, 2019). Language involves the use of a system of symbols (words, gestures, or written symbols) to communicate and share information (Vallotton and Ayoub, 2010). Language is one of few cognitive abilities unique to humans and is critical for social interaction, education and occupational performance. However, studies suggest that language does not exist in isolation from other cognitive functions (Campbell and Tyler, 2018).

While neuropsychological research has long argued for a primarily left hemisphere language system, more recent data have drawn attention to the wider neural context of language (Dronkers *et al.*, 2004; Tyler *et al.*, 2011). This wider context views language as a coalition of domain-specific and domain-general neural systems (Blank and Fedorenko, 2017). Indeed, some neural networks are specialised to perform language-specific functions such as syntax (Zaccarella and Friederici, 2015), whereas other functions are general and require the coactivation of language-specific regions and cognitive domains such as attention and memory (Fedorenko, 2014). In addition, processing language under difficult conditions such as noisy environments can spontaneously recruit domain-general networks (Davis *et al.*, 2014; Campbell *et al.*, 2016; Campbell and Tyler, 2018).

1.1.2.6. Social & emotional cognition

Social cognition refers to an array of operations involving cognitive ability related to interpersonal contacts and to the perception of oneself and others in the social environment (Nuechterlein *et al.*, 2008; Penn, Sanna and Roberts, 2008). Hence, social cognition refers to all cognitive processes that enable an individual to understand the behaviour of others as a requirement for engaging in social interactions (Frith and Frith, 2007; Pinkham *et al.*, 2014). Social cognition was introduced in the American Psychiatric Association's DSM-5 as one of six key domains of cognition in 2016 (Harvey *et al.*, 2016).

The three key subdomains of social cognition include: 1) social perception, 2) social understanding and 3) social decision-making (Penn, Sanna and Roberts, 2008; Harvey and Penn, 2010). Social perception refers to the ability to distinguish between objects and individuals (characterised by emotions, intentions and motivations, which make their behaviour unpredictable) (Vogeley, 2017). Social understanding encompasses the ability to decode emotions (e.g., facial expressions, tone of voice), mental states and intentions of others (referred to as

'Theory of Mind') (Korkmaz, 2011; Heyes and Frith, 2014). Drawing inferences about the behaviour of other individuals in terms of mental states such as beliefs, desires, intentions, experiences and emotions is a critical step for predicting their future actions (Korman *et al.*, 2015). Social decision-making refers to making appropriate decisions consistent with social norms on the awareness of their consequences for both ourselves and others in a variety of social contexts (Ruff and Fehr, 2014).

Social cognitive skills are critical for successful communication and, consequently, mental health and wellbeing. Social abilities emerge as early as 14 months and remain crucial for the lifespan (Slaughter *et al.*, 2015; Scott and Baillargeon, 2017). Their centrality in everyday life is highlighted in conditions in which a social cognitive impairment results in a variety of adverse outcomes, e.g., mental and physical deficits, functional disability, unemployment and poor quality of life (Holt-Lunstad, Smith and Layton, 2010; Phillips *et al.*, 2010; Henry *et al.*, 2016; Jones *et al.*, 2017). Failures of social cognition most often present as poor Theory of Mind, reduced empathy, impaired social perception, or abnormal social behaviour (Arioli, Crespi and Canessa, 2018).

In conclusion, cognitive function encompasses a diverse array of domains, each characterised by unique complexities. These domains include a) perceptual-motor function, which is responsible for interactions with the physical world; b) attention and processing speed, which determine the ability to focus and react quickly; c) memory, where information is stored and retrieved; d) executive function, which guides higher-level thinking and decision-making; e) language, enabling communication; and f) social and emotional cognition, influencing human interactions and relationships. The complexity of cognitive function arises from the dynamic interplay among these domains and sub-domains, collectively shaping thoughts, behaviours and intellectual capacity. In the following section, the main factors that can either enhance or impede cognitive function will be discussed, providing insights into the determinants of these vital mental processes.

1.1.3. Enhancers and depressors of cognition

Cognitive performance is a complex construct that is influenced by several factors. The most crucial factors influencing cognition will be briefly explained in this section and presented in Figure 1.3.

1.1.3.1. Age

Research has highlighted the importance of preserving cognitive function during ageing (Rivera *et al.*, 2015; Fjell *et al.*, 2017). Normal ageing is accompanied by predictable and reproducible changes in cognition, especially in the domains of attention, executive function and memory (Salthouse, 2010, 2012; Harada, Natelson Love and Triebel, 2013; Tremblay *et al.*, 2016; Adólfsdóttir *et al.*, 2017). Results from reviews show that these normal ageing brain changes result from relatively mild but important changes at the molecular, biochemical and structural level (Peters, 2006; Nikhra, 2017; Blinkouskaya and Weickenmeier, 2021; Lee and Kim, 2022).

At the molecular level, changes in gene expression have been demonstrated starting in middle age, with a notable downregulation of genes related to synaptic functions of memory and learning (Yankner, Lu and Loerch, 2008). At the biochemical level, it has been revealed that levels of major neurotransmitters such as catecholamine, norepinephrine, dopamine and serotonin undergo alterations in several regions of the brain as part of the ageing process (Nikhra, 2017). Finally, the brain undergoes various morphological changes with ageing, such as brain atrophy and volume loss (Blinkouskaya and Weickenmeier, 2021). Brain volume and weight are shown to decrease with age at a rate of approximately 5% per decade after 40 years of age, while the rate of decline may acutely increase after 70 years of age (Peters, 2006).

Several studies have shown that the older the age, the lower the performance in cognitive tasks (Alexandre *et al.*, 2014; Rivera *et al.*, 2015; Maruya *et al.*, 2018). Nevertheless, it needs to be considered that investigations in cognitive changes during ageing are subject to limitations (Murman, 2015). In cross-sectional study designs, cohort bias may occur because of differences between age cohorts that are not due to ageing but due to other differences, such as culture or nutrition and education during childhood and adolescence (Harada, Natelson Love and Triebel, 2013). Furthermore, these investigations are susceptible to misclassification bias, wherein individuals displaying early signs and symptoms of dementia are categorised as having normal cognitive function (Ritchie, Terrera and Quinn, 2015). In such cases, the cognitive test scores of these participants would exaggerate the extent of cognitive decline attributed to normal ageing, introducing

a misclassification bias into the studies (Harada, Natelson Love and Triebel, 2013). These types of bias may influence test performance beyond normal cognitive ageing and potentially overestimate the effects of ageing on cognition (Murman, 2015).

In longitudinal research, cognitive performance is assessed over time to gain insights into how ageing impacts cognition. However, there are two potential limitations inherent to these studies: the practice effect bias and attrition or survival bias (Murman, 2015). Over time, study populations may experience attrition, and since the individuals most likely to remain in the study often tend to be the healthiest, best-educated, and more financially advantaged, they often exhibit the highest cognitive test scores (Van Beijsterveldt *et al.*, 2002). Moreover, these studies can be influenced by practice effects because participants are required to repeat the same tests on multiple occasions, potentially enabling them to enhance or sustain their test scores despite cognitive decline (Salthouse, 2010; Abner *et al.*, 2012). Therefore, both learning and attrition biases tend to underestimate the degree of cognitive decline observed with ageing (Murman, 2015).

1.1.3.2. Sex

There is evidence indicating that cognitive abilities exhibit differences between males and females. In general, females appear to outperform males in areas such as verbal fluency, perceptual speed, accuracy and fine motor skills, while males outperform females in memory, visuospatial abilities and problem-solving (Sherwin, 2003; Torres *et al.*, 2006; Zaidi, 2010). However, meta-analyses of sex differences across various cognitive functions support that males and females are largely similar in most, but not all, cognitive aspects (Hyde, 2016).

It has been demonstrated that mental skills in females can vary during different phases of the menstrual cycle (Phillips and Sherwin, 1992). Specifically, the high levels of progesterone during the luteal phase of the menstrual cycle may enhance cognitive abilities compared to the follicular phase (Hampson, 1990). A study assessing females during both phases revealed that male cognitive functions were comparable to those of females in the follicular phase. However, during the luteal phase, females showed advantages in executive tasks and disadvantages in attentional tasks compared to males (Upadhayay and Guragain, 2014).

Additionally, data suggest that education plays a role in amplifying cognitive disadvantages in females in middle-income compared with high-income countries, as shown in a study involving over 70,000 participants aged 60 and older (Jablonska *et al.*, 2020). This study revealed that females had lower educational levels than males across all countries and adjusting for education mitigated the observed sex differences in cognitive performance.

Moreover, sex differences in cognitive function may vary depending on the region and age group under investigation. For example, a study reported that females had higher cognitive scores than males in the 50–59 age group but slightly lower scores in the 80–89 age group (Ahrenfeldt *et al.*, 2019). Regional differences were also reported in the same study, with Southern Europe showing a male advantage in cognitive function from ages 60 to 89. Hence, it remains unclear whether reported sex differences in cognition can be attributed to hormonal fluctuations during the menstrual cycle, educational levels, regional disparities, or the decades over which such differences may manifest.

1.1.3.3. Genetics

Data from twin and family studies show that genetics can largely (50%–80%) explain interindividual differences in cognition (Bouchard and McGue, 2003; Lenroot and Giedd, 2008; Goriounova and Mansvelder, 2019). Despite this, the identification of genes linked to cognitive ability remains incomplete. While research has pinpointed 1,041 genes in this context, they collectively explain less than half of the overall heritability (21%-22%) (Lam *et al.*, 2017; Trampush *et al.*, 2017; Savage *et al.*, 2018; Coleman *et al.*, 2019). Notably, these genetic influences stem from numerous genes, with 95% residing in non-coding regions that potentially regulate gene activity and only a fraction (1.4%) in protein-coding regions (Savage *et al.*, 2018).

Genetic factors exert a growing influence on cognition from infancy to adulthood, with their influence being most pronounced in socioeconomically advantaged settings (Mollon *et al.*, 2021). Transactional models suggest that individuals in high-opportunity environments actively seek and engage in positive learning experiences aligned with their genetic predispositions, reciprocally shaping cognition (Tucker-Drob, Briley and Harden, 2013). As a consequence of this dynamic interplay between environment and genetic predisposition, the same set of

genes acquires an increasing impact on intelligence with age (Plomin and von Stumm, 2018).

The genes associated with cognitive function are implicated in synaptic communication and neuronal excitability from early development (Lam *et al.*, 2017; Trampush *et al.*, 2017). Certain candidate genes influence signalling pathways crucial for synaptic communication such as the Dmx Like 2 (*DMXL2*), the Signal Peptide Peptidase Like 2C (*SPPL2C*) and the Wnt Family Member 4 (WNT4) gene (Coleman *et al.*, 2019). Additionally, many protein-coding genes in cognitive ability are involved in cell-to-cell communication, exemplified by genes including the Inter-Alpha-Trypsin Inhibitor Heavy Chain H3 (*ITIH3*) and ADAM Metallopeptidase Domain 12 (*ADAM12*) (Zabaneh *et al.*, 2018). Lastly, high-frequency neuronal excitability is shown to be influenced by genes involved in energy supply, including the Glycerol-3-Phosphate Dehydrogenase 2 (*GPD2*) and Mitochondrial Carrier 2 (*MTCH2*) genes (Savage *et al.*, 2018).

The most studied gene in relation to cognition up to date is the Apolipoprotein E (*APOE*) gene, which codes for a protein involved in the metabolism of lipids and has been implicated in cognitive function and risk of Alzheimer's Disease (AD) (Corder *et al.*, 1993; Wisdom, Callahan and Hawkins, 2011; Lambert *et al.*, 2013; Davies *et al.*, 2014). A meta-analysis of 38 studies on healthy individuals indicated significant *APOE*-ε4 group differences for global cognitive functioning, episodic memory and executive functioning, in favour of non-ε4 carriers. In addition, older age and *APOE*-ε4 heterozygosity were associated with smaller ε4-related impairments (Small *et al.*, 2004). A cohort study of 5,561 participants during a mean follow-up of 20.2 years involving *APOE* genotyping and repeated cognitive tests for reasoning, memory and semantic fluency also showed that ε4 homozygotes had poorer global cognitive scores compared with non-ε4 carriers starting from 65 years of age (Gharbi-Meliani *et al.*, 2021).

1.1.3.4. Education

According to the cognitive reserve (CR) hypothesis, individuals with higher reserve are better equipped to withstand the age-associated cognitive decline (Stern, 2003; Whalley *et al.*, 2004). This theory posits that early-life mental training, primarily through education, offers an added cognitive capacity to compensate for later-life decline (Stern, 2003). Thus, individuals with more mental

stimulating experience are able to maintain better cognitive function when brain pathology is taking place (Stern, 2003; Whalley *et al.*, 2004).

Educational attainment is a major contributor to interindividual variability in cognitive function, where individuals with higher levels of education are at a lower risk of developing cognitive impairments (Livingston *et al.*, 2020) and demonstrate superior cognitive performance than those with lower educational attainment, even in advanced stages of ageing (Chen *et al.*, 2019; Lövdén *et al.*, 2020; Seblova, Berggren and Lövdén, 2020; Stern *et al.*, 2020). Higher levels of education are shown to predict better cognitive performance across all age groups, with varying effects on different cognitive domains (Guerra-Carrillo, Katovich and Bunge, 2017).

A meta-analysis from 42 data sets involving over 600,000 participants revealed consistent and positive effects of education on cognitive abilities of approximately 1 to 5 intelligence quotient (IQ) points per additional year of education. Moderator analyses indicated that the effects persisted across the life span and were present on all categories of cognitive ability studied (Ritchie and Tucker-Drob, 2018). Interestingly, new learning opportunities have been shown to reduce performance gaps related to educational history (Guerra-Carrillo, Katovich and Bunge, 2017).

However, the underlying mechanism of the protective effects of education on cognition remains elusive. The brain reserve theory suggests that having sufficient neural resources (i.e., greater brain volume) can withstand more brain deficits before reaching a clinical threshold (Stern, 2009). Longer education duration is reportedly associated with positive changes in various brain functional magnetic resonance imaging (fMRI) measures in older individuals, such as greater cerebral (Foubert-Samier *et al.*, 2012) or grey matter volume (Arenaza-Urquijo *et al.*, 2013) and potentially more favourable brain structure (Cox *et al.*, 2016).

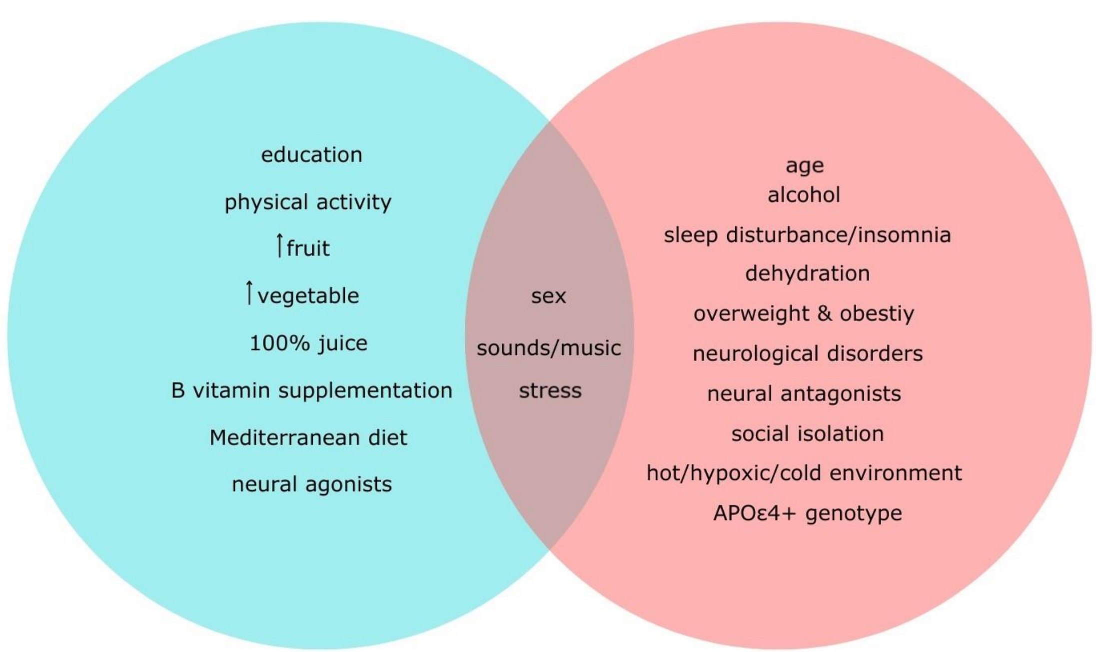

Figure 1. 3 Enhancers (left) and depressors (right) of human cognitive function. The overlapping area between the two circles represents factors that can have both enhancing and depressing effects on cognition, depending on various contexts.

1.1.3.5. Stress

Acute stress refers to a brief and recent encounter with a single stressor (Shields, Sazma and Yonelinas, 2016). The impact of perceived stress on cognitive performance can vary, as mild stress might enhance cognitive function, especially when the cognitive load is not excessive (Luethi, Meier and Sandi, 2009; Hidalgo *et al.*, 2012). High levels of acute stress, however, can impair complex cognitive functions (Wang *et al.*, 2005; Lupien *et al.*, 2009), yet the extent of impairment varies due to interindividual differences in stress response (Sweis *et al.*, 2013; Kohn, Hermans and Fernández, 2017; Tsai, Eccles and Jaeggi, 2019). A meta-analysis on the topic indicated that acute stress may impair cognitive flexibility and interference control and enhance response inhibition (Shields, Sazma and Yonelinas, 2016).

Chronic stress, a form of prolonged distress, can adversely affect mental health and cognitive function (Koolhaas *et al.*, 2011; Koenen *et al.*, 2017). Long-term exposure to various stressors leads to physiological and psychological

responses, including distraction, insomnia and declined cognitive abilities, especially in executive function and working memory (Qin *et al.*, 2009; Kasimay Cakir *et al.*, 2017; Jackowska, Fuchs and Klaperski, 2018; Luettgau, Schlagenhauf and Sjoerds, 2018; Han *et al.*, 2019; Christensen *et al.*, 2023). Moreover, individuals under prolonged stress, including academic stress, tend to exhibit deficits in attention and neural efficiency compared to non-stressed individuals (Lupien *et al.*, 1998; Duan *et al.*, 2015).

The influence of stress on cognition is mediated through exposure to elevated levels of Glucocorticoids (GCs), notably cortisol (de Souza-Talarico *et al.*, 2011). GCs are stress hormones released by the hypothalamic-pituitary-adrenal (HPA) axis (de Kloet *et al.*, 2019) and cross the blood-brain-barrier (BBB) to bind to receptors found in key brain regions including the amygdala, prefrontal cortex and hippocampus (Mason *et al.*, 2010). Considering the pivotal roles of these brain regions in processing emotions, executive functions and memory (Anand and Dhikav, 2012; Funahashi and Andreau, 2013; Šimić *et al.*, 2021), this rationale supports further exploration of the role of stress on cognitive functions associated with these regions.

Prior imaging studies have confirmed that stress alters cerebral blood flow and induces structural and functional changes in the prefrontal cortex, affecting both its architecture and function (Wang *et al.*, 2005; Hinwood *et al.*, 2013; McEwen and Morrison, 2013; Arnsten, 2015). Notably, the effects of GCs on cognitive performance depend on various factors, including the duration of exposure to high GC levels, thus delineating acute from chronic effects (Diorio, Viau and Meaney, 1993).

1.1.3.6. Alcohol

There is a broad scientific consensus on the acute negative effect of alcohol on cognitive indices such as attention, psychomotor speed, memory and cognitive flexibility (Matthews and Silvers, 2004; Fillmore, 2007; Mintzer, 2007; Dry *et al.*, 2012). The acute impact of alcohol on cortical function involves the modulation of inhibitory and excitatory receptor function in neuronal processes (Valenzuela, 1997), resulting in an immediate systemic depressant effect (Yaka *et al.*, 2003; Lobo and Harris, 2008).

Excessive alcohol consumption is considered neurotoxic and long-term alcohol abuse in adults leads to neurodegeneration (Clark, Thatcher and Tapert, 2008). Additionally, fMRI and computed tomography (CT) have revealed associations between long-term alcohol consumption and changes in brain structure, such as brain shrinkage and the presence of brain lesions (Charness, 1993; Pfefferbaum *et al.*, 1995). Particularly affected brain regions include the prefrontal cortex and hippocampus (Oscar-Berman and Marinković, 2007), although structural and functional atrophy has been observed in other brain regions as well (de la Monte and Kril, 2014).

This neurodegeneration can result in functional deficits, such as impairments in executive and motivational functions that influence self-regulation and goal-directed behaviour (Oscar-Berman and Marinković, 2007). Longitudinal studies following binge-drinking adolescents into adulthood have indicated that prolonged excessive drinking is associated with negative effects on working memory, verbal memory and learning, in contrast to non-binge-drinking adolescents (Nguyen-Louie *et al.*, 2016; Carbia *et al.*, 2017).

While there is ongoing debate regarding the potential neuroprotective effects of light to moderate alcohol consumption on cognitive impairment and dementia (Chikritzhs *et al.*, 2015; Brennan *et al.*, 2020; Zhang *et al.*, 2020), understanding the impact of chronic alcohol use on cognition is complicated. This complexity arises from various factors, including age, concurrent drug use and disorders such as alcohol dementia, which are directly linked to long-term alcohol abuse and can complicate our understanding of the individual effects of alcohol on cognitive function (Costin and Miles, 2014; Staples and Mandyam, 2016).

1.1.3.7. Sleep

Most adults need 8 h of sleep / day, while sleeping less than 6 h / day is linked to impaired daytime functioning, poorer general health and an increased risk of metabolic disorders (Drake *et al.*, 2001; Roca *et al.*, 2012; Watson *et al.*, 2015). Individuals with insomnia exhibit performance impairments for several cognitive functions, including working memory, episodic memory and some aspects of executive functioning (Fortier-Brochu *et al.*, 2012). In fact, sleep restriction (3 h / day) has been found to affect processing speed on tasks measuring vigilance and

executive function, while tasks assessing orienting of attention were largely unaffected (Cunningham *et al.*, 2018).

Individuals whose circadian rhythm is disturbed, such as night or shift workers, have demonstrated changes in alertness and cognitive efficiency. In fact, long-term exposure to shift work may impair cognitive functioning, with neuropsychological performance declining as exposure duration increases (Rouch *et al.*, 2005). Moreover, it has been found that students who regularly sleep 2-3 h less than recommended for their age experience significant neurobehavioral deficits in alertness and mood when exposed to partial sleep deprivation (Lo *et al.*, 2016).

The question of how sleep deprivation impacts brain function has been investigated using fMRI (Drummond *et al.*, 1999, 2000; Elvsåshagen *et al.*, 2015, 2017; Huang *et al.*, 2022). Early neuroimaging studies revealed that sleep deprivation leads to dynamic shifts in brain activation patterns (Drummond *et al.*, 1999, 2000). These changes can manifest as increased or decreased activity within brain regions typically involved in specific tasks (Drummond *et al.*, 1999), while attempts to compensate for the negative effects of sleep deprivation on cognition appear to recruit entirely new brain regions (Drummond *et al.*, 2000). More recent neuroimaging research has also identified significant alterations in white matter and cortical thickness following 23 h of acute sleep deprivation compared with a rested wakefulness state (Elvsåshagen *et al.*, 2015, 2017; Huang *et al.*, 2022).

In summary, in addition to shifts in brain activation, acute sleep deprivation induces alterations in the functional architecture of the brain. These structural changes may be linked to performance changes in multiple cognitive domains.

1.1.3.8. Physical activity

Physical activity has been extensively studied for its impact on cognitive function, as evidenced by several meta-analyses (Hamer and Chida, 2009; McMorris and Hale, 2012; Verburgh *et al.*, 2014; Ludyga *et al.*, 2016). In healthy adults, aerobic exercise interventions have shown improvements in cognition, with the most significant effects observed in motor function and auditory attention, and moderate effects on response speed and attention (Angevaren *et al.*, 2008). Additionally, exercise has been found to enhance verbal learning and memory after a 12-week intervention (Pereira *et al.*, 2007). The positive association between

physical activity and cognitive function extends to children aged 4 to 18 years, suggesting a link between physical activity and cognitive development. Specifically, physical activity has been associated with improved performance in perceptual and verbal skills, problem-solving and memory (Sibley and Etnier, 2003).

The results from meta-analyses have indicated that acute exercise has small to moderate positive effects on cognition across various age groups, with more substantial impact on executive function tasks compared to tasks requiring recall or alertness/attention (McMorris and Hale, 2012; Verburgh *et al.*, 2014; Ludyga *et al.*, 2016). Finally, a meta-analysis of prospective studies found that regular physical exercise reduced the relative risk of dementia by 28%, suggesting protective long-term effects of physical activity in cognitive decline (Hamer and Chida, 2009).

Research also highlights the mood-altering effects of short bouts of exercise, including increased positive mood states (Liao, Shonkoff and Dunton, 2015; Basso and Suzuki, 2017). Effects have been observed immediately after and lasting up to 30 min after acute exercise sessions (Reed and Ones, 2006). However, the direction and extent of mood changes after acute exercise can vary and are influenced by factors such as exercise duration and intensity (Berger *et al.*, 2016). Moreover, the impact of exercise on cognition and mood may be contingent on the level of fitness or physical activity of individuals. Some studies suggest that acute exercise is most beneficial for cognition in individuals who are physically fit or more active (Budde *et al.*, 2012; Tsai *et al.*, 2016), underscoring the significance of considering physical activity levels in this context.

The exact mechanisms underlying the cognitive improvement seen in relation to acute and long-term exercise are not fully understood. Hypothesised mechanisms include direct effects on the brain, such as increased vasculature and cerebral blood flow and production of neurotrophic factors, which may promote neuronal repair, neuronal growth and plasticity (Lopez-Lopez, LeRoith and Torres-Aleman, 2004; Fang *et al.*, 2013; Basso and Suzuki, 2017).

1.1.3.9. Nutrition

Nutrition is an essential modulator for brain development (Cusick and Georgieff, 2016), which commences during the foetal period and extends into adulthood (Aubert-Broche *et al.*, 2013; Dennison *et al.*, 2013; Ducharme *et al.*,

2016; Vijayakumar *et al.*, 2016). Both specific nutrients and dietary patterns can exert substantial influence on distinct cognitive functions, including memory, processing speed, as well as in the delay of cognitive decline (Klimova, Dziuba and Cierniak-Emerych, 2020).

Research suggests that high consumption of fruits, vegetables, and 100% fruit juices is associated with cognitive benefits (Lamport *et al.*, 2014). These benefits may be attributed to the high fibre content in these food groups. Importantly, a systematic review has demonstrated that dietary fibre can influence the composition of the gut microbiome, which, in turn, may affect cognitive functions (Askarova *et al.*, 2020). These associations may also be mediated by the so-called 'neurotropic' B vitamins, which are essential for brain function (Calderón-Ospina and Nava-Mesa, 2019). For instance, maintaining adequate vitamin B12 levels, especially during pregnancy and early childhood, is vital for neural myelination and brain development (Venkatramanan *et al.*, 2016). Furthermore, B vitamin supplementation has been linked to delay in cognitive decline, especially in populations receiving prolonged early interventions. In particular, a higher intake of dietary folate is linked to a reduced risk of incident dementia in non-dementia aged populations (Zhibin Wang *et al.*, 2022).

Moreover, adherence to the Mediterranean diet during middle age is reported to protect neurocognition later in life (Gauci *et al.*, 2022). A key feature of this dietary pattern is the fat content, ranging from 28% to 40%, primarily comprising polyunsaturated fatty acids (PUFAs) and low saturated fats (Aridi, Walker and Wright, 2017). PUFAs regulate the function and structure of neurons, endothelial cells and glial cells in the brain (McNamara *et al.*, 2018), while low omega-3 PUFA intake may contribute to memory loss (Spencer *et al.*, 2017). Nonetheless, although there is biological plausibility for an association between PUFAs and performance in attention, executive function, psychomotor speed and language, a meta-analysis failed to confirm this link, possibly due to small sample sizes in the included trials (Lehner *et al.*, 2021). A review also reported an association between a high-saturated fat diet and impaired cognitive function in healthy adults (Francis and Stevenson, 2013). It has been proposed that consumption of a high-fat diet stimulates the hippocampus to initiate a neuro-inflammatory response, resulting in memory deficits (Spencer *et al.*, 2017).

1.1.3.10. Hydration

Research has supported the hypothesis that cognitive performance and mood could be impaired by dehydration and improved by rehydration. In these studies, cognitive deficits in domains such as attention, memory and perceptual abilities appear to be modest, while more complex cognitive functions such as executive function appear to be relatively preserved (Cian *et al.*, 2000, 2001; Lieberman *et al.*, 2005; Petri, Dropulić and Kardum, 2006; D'anci *et al.*, 2009; Ganio *et al.*, 2011; Lindseth *et al.*, 2013; Zhang *et al.*, 2019).

Moreover, a prospective analysis of a cohort of older adults with metabolic syndrome and overweight or obesity showed that reduced hydration status was associated with greater declines in global cognitive function over a 2-year period (Nishi *et al.*, 2023). Studies examining self-reported changes in mental state have also identified links between dehydration and feeling of fatigue, mood disturbances and perceived difficulties in cognitive tasks (Szinnai *et al.*, 2005; Baker, Conroy and Kenney, 2007; D'anci *et al.*, 2009; Ganio *et al.*, 2011; Armstrong *et al.*, 2012; Zhang *et al.*, 2019).

However, it is worth noting that some investigations have not observed significant impairments in cognitive performance following dehydration (Adam *et al.*, 2008; Bandelow *et al.*, 2010; Ely *et al.*, 2013; Wittbrodt *et al.*, 2015). In these particular studies, heat stress, physical activity, or a combination of these factors were employed instead of water deprivation, and these additional variables could have influenced the cognitive responses observed (Tashiro *et al.*, 2001; Secher, Seifert and Van Lieshout, 2008; Hwang *et al.*, 2016; Mazlomi *et al.*, 2017).

1.1.3.11. Overweight & obesity

While there is compelling evidence in the literature establishing a negative association between overweight and obesity and cognitive performance in children and adults (Gunstad *et al.*, 2007; Li *et al.*, 2008; Lokken *et al.*, 2009; Nilsson and Nilsson, 2009), the link between body weight and cognition in older individuals is not yet fully understood (Smith *et al.*, 2011).

A review of the existing research on this topic presented evidence from some studies supporting an 'obesity paradox' in cognition, according to which

higher weight may preserve cognition in old age (Assuncao *et al.*, 2018). However, it needs to be considered that most studies in older populations are cross-sectional, while it is preferable to rely on longitudinal studies for drawing causal conclusions when appropriate methods are employed (Kronschnabl *et al.*, 2021). In fact, there is unanimous support from prospective studies that being overweight or obese in midlife is positively associated with poorer cognitive performance in late life (Gustafson *et al.*, 2003; Rosengren *et al.*, 2005; Whitmer *et al.*, 2005; Kronschnabl *et al.*, 2021). Moreover, the mixed results observed in studies involving older populations may be attributed to difficulties in disentangling the contribution of obesity to brain impairments from the effects of normal ageing or obesity-related comorbidities (e.g., insulin resistance, metabolic dysregulation and hypertension) (West and Haan, 2009; Dixon *et al.*, 2015).

Imaging studies investigating the effects of overweight and obesity on brain structure have reported decreased brain volume (Ward *et al.*, 2005) and decreased grey matter density (Taki *et al.*, 2008; Walther *et al.*, 2010; Shefer, Marcus and Stern, 2013). Moreover, they have observed cerebral inflammation and changes in white matter volume, with proinflammatory cytokines potentially serving as a contributing mechanism (Sellbom and Gunstad, 2012; Bolzenius *et al.*, 2013; Ronan *et al.*, 2016). Importantly, obesity is characterised by a reduction in adiponectin (Kaser *et al.*, 2008), which is known for its protective role against inflammation and the upregulation of pro-inflammatory adipokines, which may lead to a chronic inflammatory state and metabolic disease (Ouchi *et al.*, 2011; Nigro *et al.*, 2014).

In addition to structural alterations in the obese brain, neuroimaging studies have revealed altered patterns of functional activity, including decreased blood flow to the prefrontal cortex (Willeumier, Taylor and Amen, 2011). The reduced functional activity in cortical areas is associated with lower performance in tasks related to episodic and working memory, executive function and attention (Gonzales *et al.*, 2010; Diamond, 2013; Prickett, Brennan and Stolwyk, 2015; Cheke *et al.*, 2017; Yang *et al.*, 2018).

1.1.3.12. Diseases

There are many conditions that can affect cognitive performance and these mainly belong in the spectrum of neurological disorders. The main disorders associated with cognitive function are briefly explained in this section.

1.1.3.12.1. Alzheimer’s disease

AD is a progressive neurodegenerative disease frequently associated with memory impairment and cognitive decline (DeTure and Dickson, 2019). Neuropathological alterations in the cerebral cortex and limbic system lead to deficits in learning, memory, language, and visuospatial skills (Corey-Bloom, 2002). The extent of cognitive decline is influenced by the distribution of pathological changes in AD and differs across the spectrum of disease severity (Corey-Bloom, 2002).

1.1.3.12.2. Parkinson's disease

Parkinson's disease (PD) ranks second after AD in the common progressive neurodegenerative disorders (Kalia and Lang, 2015). Mild-moderate cognitive impairment tends to be present in early stage of the disease, which accounts for around 40% of overall PD patients (Hely *et al.*, 2008), while more than 80% of PD individuals do evolve into dementia in later stages (Pfeiffer *et al.*, 2014).

1.1.3.12.3. Multiple Sclerosis

Cognitive impairment is an important feature of Multiple Sclerosis (MS) and may affect everyday activities of patients (Deloire *et al.*, 2011). Slowed cognitive processing speed and episodic memory decline are the most common cognitive deficits in MS, with observed difficulties in executive function, verbal fluency and visuospatial analysis (Rao *et al.*, 1991; Benedict *et al.*, 2006; Deloire *et al.*, 2011).

1.1.3.12.4. Huntington's disease

Huntington's disease (HD) is an inherited disorder that causes degeneration of brain cells and can affect cognition and movement (Say *et al.*, 2011). Early symptoms of HD include deficits in emotional recognition, which is significantly different from healthy controls (Paulsen, 2011). Moreover, it manifests with impairments in processing speed, accuracy, learning and working memory (Paulsen, 2011; Say *et al.*, 2011).

1.1.3.12.5. Traumatic brain injury

Traumatic brain injury (TBI) is a condition characterised by changes in brain function resulting from external forces, leading to brain damage (Menon *et al.*, 2010). Common causes of TBI are falls, motor vehicle accidents and assaults (Viano *et al.*, 2017). Globally, TBI is a significant contributor to both mortality and disability (Esterov *et al.*, 2021). Survivors of moderate to severe TBI frequently suffer from long-lasting cognitive deficits (Barman, Chatterjee and Bhide, 2016). These include impairments in different aspects of cognition such as memory, attention and executive function (Arciniegas, Held and Wagner, 2002; Cristofori and Levin, 2015).

1.1.3.12.6. Stroke

A stroke is a brain event that is caused either by bleeding or by reducing blood and oxygen supply to the organ (Al-Qazzaz *et al.*, 2014). It is a significant cause of long-term physical disabilities in adults and ranks as the second most common cause of cognitive impairment and dementia. Stroke is also the third leading cause of death, following cardiovascular diseases and cancer (Cumming, Marshall and Lazar, 2013). Approximately 30% of stroke patients develop dementia within 1 year of stroke onset. Memory, language and orientation are commonly impaired by stroke, with attention and executive function being particularly affected, even shortly after diagnosis (Cullen *et al.*, 2007; Al-Qazzaz *et al.*, 2014; Sun, Tan and Yu, 2014).

1.1.3.12.7. Depression

Cognition is closely linked to emotions (Millan *et al.*, 2012). Depression, a prevalent mood disorder affecting approximately 5% of adults globally, is characterised by persistent sadness and a loss of interest in once-enjoyable activities (Chakrabarty, Hadjipavlou and Lam, 2016). In terms of cognition, depression often leads to cognitive deficits in attention, executive function, memory and processing speed domains (Millan *et al.*, 2012; McIntyre *et al.*, 2015). These deficits are reported to be present in 85% to 94% of depressive episodes and can persist in 39% to 44% of cases during remission (Conradi, Ormel and de Jonge, 2011).

1.1.3.12.8. Schizophrenia

Schizophrenia is a chronic psychiatric illness characterised by delusions, hallucinations and disorganised speech (Haddad *et al.*, 2021). It is frequently manifested with mild to moderate deficits in attention, verbal fluency, motor skills, working memory and processing speed and severe deficits in executive functioning (Talreja, Shah and Kataria, 2013; McCutcheon, Keefe and McGuire, 2023). Some impairments are present even before disease diagnosis, while moderate to severe impairments are detectable at the time of the first episode and remain stable until middle age (Bowie and Harvey, 2006; Talreja, Shah and Kataria, 2013; McCutcheon, Keefe and McGuire, 2023).

1.1.3.12.9. Bipolar disorder

Bipolar disorder (BD) is a psychiatric condition that has a significant impact on the lives of most patients, with over 6% of individuals dying by suicide within two decades of diagnosis (Anderson, Haddad and Scott, 2012). BD is associated with impairment in cognitive function not only during acute mood episodes but also during remission periods (Sadana *et al.*, 2019). Meta-analyses have provided evidence indicating that in comparison with healthy controls, BD patients show impairments in various cognitive domains such as attention, memory and executive function (Arts *et al.*, 2008; Mann-Wrobel, Carreno and Dickinson, 2011; Bourne *et al.*, 2013).

1.1.3.13. Medications

Numerous medications have been shown to influence cognitive performance. Among these, some neuro-acting medications are categorised as agonists, increasing the effects of specific neurotransmitters, while others are classified as antagonists, inhibiting neurotransmission (Nevado-Holgado *et al.*, 2016). These substances can exert either direct or indirect effect. Those that have a direct effect work by mimicking neurotransmitters due to their similar chemical structure, while those with indirect effects work by acting on synaptic receptors (Hilditch and Drew, 1984; Hruby, 2002). A list of medications which have been associated with cognitive performance is provided below.

1.1.3.13.1. Antidepressants

A meta-analysis of 33 studies revealed that antidepressants have a modest, positive effect on divided attention, executive function, immediate memory, processing speed, recent memory and sustained attention in depressed participants (Prado, Watt and Crowe, 2018). Selective Serotonin Reuptake Inhibitors (SSRI's) were found to have the greatest positive effect on cognition for depressed participants, as compared to the other classes of antidepressants analysed. Nevertheless, antidepressant use is shown to counterbalance the side-effects in patients with depression who experience cognitive impairments and were not found to be associated with cognitive function among cognitively normal patients with depression (Fang *et al.*, 2013), nor to significantly affect cognitive function in non-depressed individuals (Prado, Watt and Crowe, 2018).

1.1.3.13.2. Antipsychotics

Antipsychotics are the first-line evidence-based treatment for schizophrenia and other primary psychotic disorders (Lally and MacCabe, 2015). Antipsychotic drugs are shown to improve the cognitive deficits caused in individuals with psychotic disorders. In fact, medications in this class were found to improve verbal learning, attention, executive function, working memory, processing speed and overall cognition (Baldez *et al.*, 2021). However, it needs to be considered that there are reported cases of antipsychotic medications leading to sedation, which might potentially exacerbate cognitive impairments (Stroup and Gray, 2018).

1.1.3.13.3. Benzodiazepines

Benzodiazepines, commonly prescribed for anxiety and sleep disorders, are known to have a depressant effect on the CNS (Edinoff *et al.*, 2021). Chronic use of benzodiazepines was previously shown to cause small, yet significant changes in fluid intelligence and to be positively associated with cognitive decline (Bierman *et al.*, 2007). A recent study also demonstrated that more than 20.7% of chronic benzodiazepine users exhibited cognitive impairment across all cognitive domains (Zetsen *et al.*, 2022). The largest effects were observed in processing speed and sustained attention, with women generally displaying lower performance, an effect which appeared to be moderated by anxiety symptoms.

1.1.3.13.4. Antihistamines

Histamine antagonists are often prescribed to treat allergies and common side-effects include sleepiness and cognitive deficits. These agents can cause sedative effects that may interfere even with next-day performance and safety (Simon and Simons, 2008). A previous review showed that individuals treated with first-generation antihistamines experienced significant performance deficits in attention, working memory, vigilance and psychomotor speed. In contrast, individuals treated with second-generation antihistamines performed similarly to those treated with placebo (Kay, 2000). A more recent report, however, suggests that treatment with second- or third-generation antihistamines may also be associated with cognitive impairment of episodic working memory, particularly in elderly patients (Adler and Baumgart, 2020).

1.1.3.13.5. Opioids

Opioids are prescribed to manage pain, however, they may lead to several adverse effects, including cognitive impairment (Sá Santos *et al.*, 2016). Older reports show that opioids can lead to a reduced attention span, time disorientation, hallucinations and delirium (Vella-Brincat and Macleod, 2007). Nevertheless, a systematic review reported mixed results with both improvements and impairments in cognition in studies with higher mean opioid doses (Pask *et al.*, 2020). These associations were evident in attention, language, orientation, psychomotor function, verbal and working memory.

1.1.3.13.6. Contraceptives

Behavioural effects of hormonal contraceptives have been shown in cognitive tasks such as mental rotation (ability to imagine how an object seen from one perspective would look if it were rotated) and verbal fluency (Beltz, Hampson and Berenbaum, 2015; Griksiene *et al.*, 2018). A systematic review of neuroimaging studies also reported structural and functional changes in brain areas involved in cognitive processing such as the prefrontal cortex and the amygdala (Brønnick *et al.*, 2020).

1.1.3.13.7. Nootropics

Nootropics are a heterogeneous group of compounds which are considered to enhance thinking, learning and memory, especially in cases where these functions are impaired (Malik *et al.*, 2007). Most of these substances are naturally

derived and can be found as dietary supplements (Vyas, Kothari and Kachhwaha, 2019). Their suggested mechanisms of action include improved glucose and oxygen supply to the brain, promotion of neuronal protein and nucleic acid synthesis and elimination of oxygen free radicals (Nicholson, 1990; McDaniel, Maier and Einstein, 2003; Malik *et al.*, 2007). Some common nootropics include Lecithin, Guarana and Ginkgo Biloba (Malík and Tlustoš, 2022). Nonetheless, there is not enough evidence regarding their effectiveness and safety in the case of long-term use, especially for the synthetic variants of these drugs (Malík and Tlustoš, 2022).

1.1.3.13.8. Stimulants

Stimulants are a broad class of sympathomimetic drugs that stimulate the CNS, leading to increased arousal, alertness, attention, vigour, wakefulness and energy levels (Mehendale, Bauer and Yuan, 2004; Wood *et al.*, 2007). The main psychostimulants include cocaine, amphetamine, modafinil and caffeine (Wood *et al.*, 2014) and, with the exception of caffeine, they are commonly used to treat conditions such as attention deficit hyperactivity disorder (ADHD) and narcolepsy (Arnsten, 2006).

Psychostimulant action can be understood as a continuum: at low doses, stimulants can enhance cognition. As dose increases, hyperlocomotion is seen, with an increased sense of power (Wood *et al.*, 2014). Subsequently, individuals may experience euphoria, or a drug-induced high (Boutrel and Koob, 2004). Importantly, these effects are outside of the range of cognitive enhancement; often leading to cognitive deficits and disrupted thinking (Carrillo-Mora *et al.*, 2022). In cases of overdose, symptoms such as agitation, confusion, and psychosis may manifest. At extremely high doses, stimulants can induce typical toxic effects, including coma, circulatory collapse and even death (Wood *et al.*, 2014).

1.1.3.14. Social Isolation

Research indicates that social isolation and perceived loneliness may contribute to poorer cognitive performance (Cacioppo *et al.*, 2000; Cardona and Andrés, 2023; Duan *et al.*, 2023; Lammer *et al.*, 2023) and higher cognitive decline on follow-up (Tilvis *et al.*, 2004; Wilson *et al.*, 2007; Lara *et al.*, 2019; Lammer *et al.*, 2023).

Cognitive abilities associated with social isolation include executive function (Cacioppo *et al.*, 2000; Lammer *et al.*, 2023), memory (Lara *et al.*, 2019; Lammer *et al.*, 2023), verbal fluency (Lara *et al.*, 2019), processing speed (Lammer *et al.*, 2023), as well as overall cognition (Lara *et al.*, 2019; Ingram, Hand and Maciejewski, 2021). Notably, a study investigating the impact of COVID-19-induced social isolation on cognitive function found that lockdown conditions during the pandemic were linked to poorer cognitive performance (Ingram, Hand and Maciejewski, 2021). Conversely, the easing of restrictions, which allowed for more social interaction, coincided with improvements in overall cognitive scores.

The results from a longitudinal population-based fMRI study revealed that baseline social isolation and changes in social isolation over a 6-year follow-up were associated with reductions in hippocampal volume and cortical thickness, indicative of brain atrophy (Lammer *et al.*, 2023). Finally, a recent systematic review emphasised that while depression might be an important mediator between loneliness and cognitive decline of ageing, the lack of cognitive stimulation during social isolation might exert a more pronounced influence on cognition (Cardona and Andrés, 2023).

1.1.3.15. Environmental conditions

Research has reported that different environmental conditions may impact cognitive function. Previous trials have shown that hot (Parker, Bussey and Wilding, 2005; Morley *et al.*, 2012), cold (Spitznagel *et al.*, 2009; Muller *et al.*, 2012) and hypoxic (Ando *et al.*, 2013; Neuhaus and Hinkelbein, 2014) exposures can impair cognitive processes in humans. Nevertheless, it is difficult to conclude whether environmental exposure per se has an adverse effect on cognitive function, or is a result of, for example, heat-induced dehydration (Gaoua, 2010).

In a study on temperature exposure based on residential location, adverse cognitive associations with cold temperatures in traditionally warm regions and improved cognition in summer and fall seasons were observed, indicating the importance of climate change on cognitive health (Khan *et al.*, 2021). As reviewed by Taylor and colleagues, alterations in blood flow, hyperhomocysteinaemia and a decrease in catecholamine availability combined with psychological factors may underlie the reductions in cognitive function during hot, hypoxic and cold exposure, respectively (Taylor *et al.*, 2016).

1.1.3.16. Sounds / background music

The impact of background music on cognitive performance is inconclusive. As highlighted in a review, some studies suggest that background music enhances performance, while others find it detrimental or show no effect (Waterhouse, 2006). In research studies where background music was found to enhance attention compared to silence or other types of auditory background noise (Angel, Polzella and Elvers, 2010; Thompson, Schellenberg and Letnic, 2012), the beneficial influence of music may be explained by feelings of pleasantness and increased arousal (Thompson, Schellenberg and Husain, 2001; Perlovsky, 2012).

However, other studies found that the silent condition led to better performance or no differences compared to the music conditions (Furnham and Strbac, 2002; Dobbs, Furnham and McClelland, 2011). Researchers have suggested that music that is perceived as too stimulating and arousing may interfere with cognition, while preferred or familiar music can divert attention from the task (Perham and Vizard, 2011; Nemati *et al.*, 2019). Therefore, background music can attract the attention of the listener away from a target task and competes for attention, hindering inhibitory processes (Perham and Vizard, 2011).

In summary, cognition is subject to the influence of several enhancers and depressors, with their collective interaction playing a pivotal role in shaping human cognitive performance. In the following section, the emphasis will shift towards the assessment of the methodologies employed by researchers to a) quantify cognition, examining the links between the discussed effectors and performance and b) evaluate the effectiveness of nutrients or drugs in optimising cognitive performance.

1.1.4. Assessment of cognitive function

In this section, the commonly employed methods for evaluating cognitive function and their mode of delivery in research will be explored. The review of these methods within this thesis is crucial for identifying the most suitable approaches for the upcoming studies. This process will guarantee that the present research is built upon robust and fitting cognitive assessment methodologies.

1.1.4.1. Purpose and mode of delivery

Cognitive assessment involves the objective measurement of cognitive abilities (Verhagen *et al.*, 2019; Chennaoui, Léger and Gomez-Merino, 2020). Development of tests to measure cognitive function date back over a century. In 1884, Francis Galton administered the first cognitive test to thousands of individuals during the international health exhibit in London (Wasserman, 2018). Since then, cognitive assessments have been devised for a variety of purposes in research and in healthcare. Some tests have been designed and validated to screen cognitive development in children (Di Cesare, Di Cesare and Di Carlo, 2021). Others are tailored to assess different cognitive functions, providing insights into the cognitive potential of healthy adult populations across various domains (Lamport *et al.*, 2014). Additionally, certain tests are designed to detect neurological disorders such as AD and dementia (Woodford and George, 2007).

Cognitive performance was traditionally assessed using paper-and-pencil tests administered face to face. However, in the past decades there has been a shift towards computerised test batteries that offer customised tests (Collerton *et al.*, 2007). The adoption of computerised administration offers potential advantages compared to paper-and-pencil tests, including precise measurements down to millisecond timing, automated scoring and greater standardisation of presentation across test sessions (Collerton *et al.*, 2007; Björngrim *et al.*, 2019). Cognitive tests often take several minutes to administer and are performed in the presence of a professional in minimum distraction, controlled environments (Björngrim *et al.*, 2019).

Nevertheless, the laboratory testing conditions differ from everyday environments, which could potentially lead to cognitive tests having only moderate ecological validity in predicting everyday cognitive function (Chaytor and Schmitter-Edgecombe, 2003). Recent findings support that cognitive tasks performed in uncontrolled naturalistic settings (e.g., at home or at work) yield measurements with comparable reliability to assessments conducted in tightly controlled settings (Sliwinski *et al.*, 2018). Everyday life is comprised of multi-sensory elements such as distracting sounds, smells, lights, or tactile stimuli (Dijk, Duffy and Czeisler, 1992). Consequently, to enhance our understanding of everyday cognition, research may need to consider the influence of numerous intrapersonal factors like

mood, age and sleepiness, as well as contextual factors like location (Verhagen *et al.*, 2019). This suggests that assessments may need to occur in natural daily environments (Bouvard *et al.*, 2018; Verhagen *et al.*, 2019).

Furthermore, cognition is known to fluctuate over the day, influenced by factors such as the level of alertness or food intake (Dijk, Duffy and Czeisler, 1992). It has been argued that assessing individual cognitive test scores from a single occasion may not adequately encompass the full range of within-individual performance variations that contribute to typical cognitive functioning of individuals (Vaughan and Birney, 2023). Therefore, engaging in repetitive cognitive tasks within everyday environments, which take several minutes to complete, could offer valuable insights into daily cognitive function and allow for the study of how different daily situations influence cognition (Verhagen *et al.*, 2019).

1.1.4.2. Cognitive assessment tools

In cognitive research, there is a tendency to either employ comprehensive cognitive measurements from neuropsychological batteries or choose cognitive measurements based on prior use (Masento *et al.*, 2014). There are thousands of cognitive assessment tools available (de Jager *et al.*, 2014), ranging from those designed to evaluate a single cognitive domain, to cognitive function batteries assessing more than one domain or all key domains of cognition (Gonzalez Kelso and Tadi, 2022). Nonetheless, cognitive tests are recommended to be characterised by: a) accurate, standardised and robust methodology; b) demonstrated construct validity and retest reliability; c) high sensitivity and specificity of cognitive measure for the outcome being assessed and d) established levels of confidence for target populations (Mayeux, 2004; de Vries *et al.*, 2013). Examples of cognitive assessment tools by cognitive domain are discussed below.

Tasks related to perceptual – motor function typically impose minimal cognitive demands and are primarily used to identify the ability to comprehend instructions and fundamental motor skill issues (Harvey, 2019). These functions are commonly evaluated in the context of cognitive development assessments for children (van der Fels *et al.*, 2015) and cognitive impairment in the elderly (Liu *et al.*, 2021). In assessments of cognitive function in healthy populations, perceptual-motor skills play a crucial role, as they serve as a foundational requirement for

accurate evaluations of more complex cognitive abilities, particularly in timed computerised tests (Grissmer *et al.*, 2010; Lin *et al.*, 2017).

Tasks assessing attention and processing speed involve the detection of simple stimuli presented infrequently within a set of other stimuli, with the continuous performance task (CPT) serving as a common example (Conners, 1985). For measuring processing speed, tasks like simple reaction time (RT) tests have been utilised since the 19th century, requiring fast responses to visual stimuli (Lim and Dinges, 2008). The primary metric in attention and processing speed tasks is RT in milliseconds (ms) (Kyllonen and Zu, 2016). Cognitive speed is important for various tasks, even those without explicit speed requirements, as stimuli are typically presented at a fixed rate, such as one word or digit per second in memory tasks (Harvey, 2019). Sustained attention assessments are influenced by motivation, and prolonged testing can lead to decreased attention and disengagement (Carriere *et al.*, 2010).

Research has employed a wide variety of memory tests, mostly due to the range of subdomains in memory function (Harvey, 2019). Nonetheless, the use of markedly different tasks to assess the same process or the use of the same name for two potentially distinct processes has been reported, limiting the internal consistency of memory research (Cheke and Clayton, 2013). A good example of a working memory test is the n-back task (Owen *et al.*, 2005). Participants are typically instructed to monitor a series of stimuli and respond when the stimulus presented matches the one presented n trials earlier (Owen *et al.*, 2005). Most studies report response latencies and accuracy (%); as the task difficulty increases, RTs usually increase and accuracy decreases (Miller *et al.*, 2009; Schmidt *et al.*, 2009).

Executive function is assessed using various cognitive measures that encompass different functions such as inhibition, planning, problem-solving, decision-making, tracking and abstract reasoning (de Jager *et al.*, 2014). The Stroop test is commonly employed to evaluate the ability to inhibit cognitive interference, i.e., the ability to suppress or control irrelevant or interfering thoughts (Stroop, 1935). Considering that executive function covers a range of higher-order processes and skills, these functions do not all correlate specifically (Harvey, 2019). Thus, care is needed in selecting appropriate tests and interpreting results,

especially when comparing studies using different test constructs in this domain (Muslimović *et al.*, 2009).

Among the most widely used tasks for assessing social cognition is the facial expression test, which assesses the ability of individuals to recognise emotions expressed on faces among the six basic emotions, namely, happiness, sadness, anger, fear, disgust and surprise, which are common across cultures and races (Ekman and Friesen, 1971). Finally, language can be assessed through tasks such as naming, repetition, comprehension, reading and writing (Harvey, 2019). Language assessment measures fluency (e.g., name as many animals as possible), object naming and response to instructions and are commonly used in the diagnosis of developmental and neurological disorders such as brain damage, stroke, or dementia and AD (Paulsen, 2011; Charidimou *et al.*, 2014).

Nonetheless, categorisation of cognitive tasks remains somewhat problematic as each task activates different regions of the brain (Taylor *et al.*, 2016). Although a particular task might be identified as having a primary neuropsychological focus such as executive function or working memory, such measures are not 'task pure' (Burgess, 1997). For this reason, test batteries have been designed to cover the full range of cognitive domains, aiming to capture the overall spectrum of cognition, also known as global cognitive function (de Jager *et al.*, 2014). Prototypical tests of global cognitive function include the Mini-Mental State Exam (MMSE) and the Montreal Cognitive Assessment (MoCA) (Nasreddine *et al.*, 2005). These tests are suitable for screening cognitive impairment in older individuals, detecting dementia and cognitive impairment and measure broad cognitive changes over time in longitudinal studies (Letenneur *et al.*, 2007).

1.1.4.3. Drug/nutrient effects on cognitive performance

Cognitive tests have also been developed or adapted to measure the different aspects of cognitive function in relation to drug or nutrient intake (Benton, Kallus and Schmitt, 2005). Yet, there is no standardised cognitive test or battery that has been consistently applied to examine such associations following either acute or long-term intake of specific agents (de Jager *et al.*, 2014). For example, a meta-analysis identified 66 different cognitive tests employed in 19 trials of vitamin B supplementation (Ford and Almeida, 2012), while a review of 31 macronutrient

intervention studies reported 132 cognitive outcomes (Hoyland, Lawton and Dye, 2008).

When evaluating drug/nutrient effects on cognitive performance, several issues need to be considered. For example, while two tests may claim to measure the same cognitive domain, variations in the specific cognitive processes recruited by the task may render one test more sensitive than the other in detecting the effects of a drug/nutrient manipulation (Dye, Lluch and Blundell, 2000; Lieberman, 2003; Hoyland, Lawton and Dye, 2008). Additionally, it is important to distinguish between screening tests designed to identify cognitive impairment and those intended to assess changes in cognitive functions in healthy individuals (Wesnes, 2010).

Therefore, when assessing a relationship between a drug/nutrient and cognitive performance, a few factors need to be considered: a) there is a known or hypothesised relationship between the drug/nutrient and a particular cognitive domain; b) the selected test is validated and widely used in research; c) there is a known or hypothesised task sensitivity to the drug/nutrient, i.e., the test score demonstrates a response to the intervention; d) there is biological plausibility, i.e., an underlying biological mechanism of action of the drug/nutrient related to cognition (Dye and Blundell, 2002; Lieberman, 2007; Hoyland, Lawton and Dye, 2008; Hoyland, Dye and Lawton, 2009; de Jager *et al.*, 2014). Importantly, such assessments need to employ tasks in multiple cognitive domains to acquire a deeper understanding for domain-specific associations between cognition and the drug/nutrient of interest. For example, previous reviews have reported stronger positive effects of polyphenol consumption for memory function compared to attention (Lamport *et al.*, 2014).

To summarise, numerous methods have been proposed to evaluate cognitive function and to ascertain the effectiveness of nutrients or drugs in enhancing cognitive abilities. However, the sensitivity and validity of these assessment tasks are of paramount importance to ensure reproducible results. Currently, there is a scarcity of standardised comparative methods across research studies, highlighting the need for comprehensive and consistent approaches to better understand the impact of various enhancers and depressors on cognition and the effectiveness of various agents in improving performance.

1.2. Caffeine

1.2.1. Chemical structure and sources

Caffeine (1,3,7-trimethylxanthine) is a plant alkaloid with a chemical structure of $C_8H_{10}N_4O_2$ and a molecular weight of 194.19. Caffeine is an aromatic ring compound which belongs to the class of purines and chemically resembles adenosine (Tavagnacco, Corucci and Gerelli, 2021). In its pure form, it is a bitter white powder. In general, caffeine is a hydrophobic molecule. However, the oxygen molecules and the non-methylated nitrogen (Figure 1.4) interact weakly with water, and hence caffeine tends to considerable self-association in aqueous medium with a solubility of 16 mg/ml in water (Tavagnacco *et al.*, 2011).

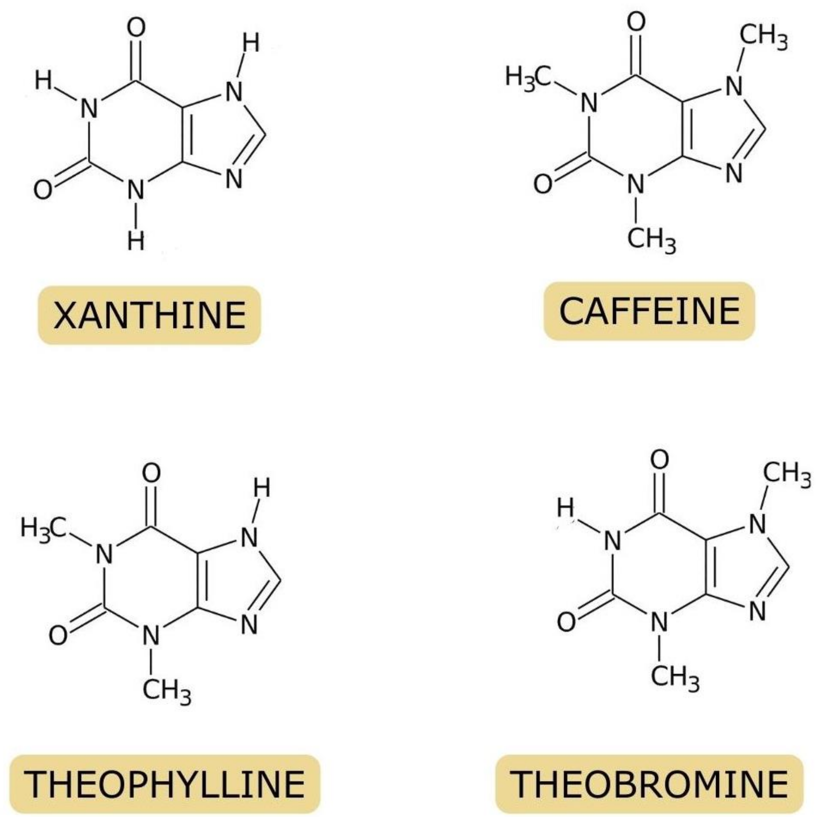

Figure 1. 4 Chemical structure of methylxanthines.

Caffeine is naturally found in the leaves, nuts and beans of more than 60 plant species native to South America and East Asia, such as tea leaves (*Camellia*

sinensis), coffee beans (*Coffea Arabica*), cocoa beans (*Theobroma cacao*), kola nuts (*Cola acuminata*), guarana and mate (Frary, Johnson and Wang, 2005; Heckman, Weil and De Mejia, 2010; Fitt, Pell and Cole, 2013). Synthetic forms of caffeine are used in soda, stimulant (energy) drinks, as well as dietary supplements and medications (Finnegan, 2003; Gurley, Steelman and Thomas, 2015). Table 1.1 shows the average caffeine content in common products.

Table 1. 1 Average caffeine content in foods, beverages, and medications.

Product	**Quantity**	**Caffeine (mg)**
Coffee		
Instant	1 cup (200ml)	100
Filter coffee	1 cup (200ml)	90
Espresso	60ml	80
Brewed decaffeinated	1 cup (200ml)	5.6
Instant decaffeinated	1 cup (200ml)	3.3
Tea		
Black tea (strong infusion)	1 cup (220ml)	49.5
Black tea (weak infusion)	1 cup (220ml)	36.3
Green tea	1 cup (220ml)	33.2
Iced tea	1 can (330ml)	14
Cola & Energy drinks		
Monster	1 can (500ml)	160
Red Bull	1 can (250ml)	80
Lucozade Original Energy	1 bottle (500ml)	60
Regular / low calorie cola (mean of most popular brands)	1 can (330ml)	35.6
Chocolate		
Candy bar	1 small	25
Dark chocolate	1 bar (50g)	25
Cocoa drink	1 cup (180ml)	13
Milk chocolate	1 bar (50g)	10
Medications		
Excedrin (acetaminophen + acetylsalicylic acid + caffeine)		200
Vivarin		200
Nodoz		100
Vanquish (acetaminophen + acetylsalicylic acid + caffeine)	1 tablet	33
Anacin		32
Midol (acetaminophen + pyrilamine maleate + caffeine)		32
Dristan (acetylsalicylic acid + caffeine)		16
Dexatrim (phenylpropanolamine + caffeine)		16

Adapted from: (Fitt, Pell and Cole, 2013; Cappelletti *et al.*, 2015; European Food Safety Authority, 2015).

1.2.2. Clinical features

Caffeine is a stimulant known to have generally dose-dependent effects with positive or desirable effects at lower doses and undesirable effects at higher doses, with substantial inter-individual variation (Smith, 2002). Desirable effects include increased alertness, arousal, concentration and well-being (Kaplan *et al.*, 1997; Smith, 2002), whereas adverse effects include but are not limited to irritability, nervousness, anxiety, nausea and dizziness (Kaplan *et al.*, 1997).

High doses of caffeine can lead to acute toxicity with different effects in adults, both short- and long-term (Willson, 2018). These adverse effects have been reviewed and were reported to include dizziness, flushing, irritability, headache, insomnia, nervousness, anxiety, fever, loss of appetite and analgesia (Alsabri *et al.*, 2018). Various cardiovascular-specific effects have been reported, starting with hypertension early on and followed by hypotension, palpitations, tachycardia and arrhythmias (Alsabri *et al.*, 2018; Murray and Traylor, 2023). Additionally, toxic effects of caffeine on the gastrointestinal system may include nausea and vomiting, diarrhoea, epigastric pain and peptic ulcers (Alsabri *et al.*, 2018). In children, acute caffeine toxicity is manifested by agitation, tachycardia, severe vomiting and diuresis (Meltzer *et al.*, 2008).

Caffeine toxic effects begin to manifest after a dose of around 1 g (Bonsignore *et al.*, 2014). Toxicity from coffee or tea ingestion is extremely rare, because of the excessive amount of fluids that would have to be ingested to reach toxic levels (Gummin *et al.*, 2017). Caffeine toxicity in adults can be encountered in cases of overdose of medications such as decongestants, bronchodilators, weight-loss aids, or stay-awake pills (Bioh, Gallagher and Prasad, 2013; Bonsignore *et al.*, 2014). Conversely, caffeine toxicity in children and adolescents is typically caused by accidental ingestion (Meltzer *et al.*, 2008; Bigard, 2010). Lethal doses of caffeine although rare, have been reported at blood concentrations of 80-100 µg/ml, which can be reached with caffeine ingestion of approximately 10 g or greater (Cappelletti *et al.*, 2018).

Based on extensive review of the evidence, Health Canada, The United States Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA) have concluded that for healthy adults habitual caffeine consumption levels up to 400 mg/day do not raise health safety concerns (Health

Canada, 2010; European Food Safety Authority, 2015). According to the same authorities, children and adolescents should not consume more than 2.5 – 3 mg/kg body mass/day, while for pregnant women, maximum daily intake levels of caffeine are set at 200 mg/day (Health Canada, 2010; European Food Safety Authority, 2015).

1.2.3. History and intakes

Caffeine has been consumed for thousands of years with evidence for tea consumption in China 2,100 years ago during the Western Han Dynasty (Fredholm, 2011; Lu *et al.*, 2016) and the first use of coffee infusions with boiling water around the year 1000 (Fredholm, 2011). However, caffeine was isolated as the active constituent of coffee in 1819 followed by its first total synthesis in 1895 (Waldvogel, 2003). By the 14th century the process of roasting of coffee beans had been discovered and by the 16th century coffee consumption and commercialisation had become widespread in coffee houses in Constantinople and Arabia (Fredholm, 2011; Cappelletti *et al.*, 2015). By the 17th century, consumption of coffee and tea had become more common in Europe and later spread to North America (Heckman, Weil and De Mejia, 2010; Fredholm, 2011; Cappelletti *et al.*, 2015). Tea and coffee have since served as the major beverage sources of caffeine. However, in the late 1800s, caffeinated soda emerged in the market and gained significant popularity during the latter half of the 20th century (Heckman, Weil and De Mejia, 2010).

Today, caffeine is the most widely consumed drug, being used habitually by more than 80% of the world population (Ogawa and Ueki, 2007; Heckman, Weil and De Mejia, 2010; EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2015). On average, adults consume 227 mg of caffeine daily, which is approximately two regular 125-ml cups of coffee . The consumption of caffeine-containing beverages (CCB) per country has been extensively reviewed (Reyes and Cornelis, 2018). In Africa and in Asian-Pacific countries, the most consumed CCB include tea and carbonated soda. Carbonated soda and coffee are the most sold CCB in Latin America and the Caribbean. The US consumes mostly coffee and carbonated sodas and, in fact, the most carbonated soda and energy drinks per capita than any other country in the world. In Europe, coffee and carbonated sodas are the top CCB sold (Reyes and Cornelis, 2018). Based on recent data, the Netherlands consumes the largest volume of coffee per capita than any other

country in the world, followed by Finland and Sweden (*Statista Market Forecast*, 2020).

The most recent data from the United Kingdom showed that in all age groups, total caffeine intake was 122-143 mg/day. Coffee (49.5 mg/day) and tea (36.2 mg/day) were the greatest contributors of daily caffeine intake, followed by sodas and energy drinks combined (34.5 mg/day) (Fitt, Pell and Cole, 2013).

1.2.4. Behavioural aspects

1.2.4.1. Tolerance

Originally, drug tolerance was characterised by behavioural plasticity, indicating a diminished response to repeated drug exposure (Kalant, 1998). Caffeine tolerance thus arises when the physiological, behavioural and subjective effects of caffeine diminish after repeated administration, such that: a) the same dose no longer produces positive effects; b) a gradually higher dose of caffeine is required to produce the desired effects and c) tolerance to some of the undesirable effects of caffeine may occur, leading individuals to tolerate higher doses over time (Meredith *et al.*, 2013; Nehlig, 2018). Studies have reported tolerance to the subjective effects of caffeine (Evans and Griffiths, 1992), as well as tolerance to other physiological effects such as diuresis, oxygen consumption, sleep disturbance and increase in blood pressure (Bonnet and Arand, 1992; Griffiths and Mumford, 1996). At doses of 300 mg/day, caffeine is more likely to produce only partial and not complete tolerance (Juliano and Griffiths, 2004).

1.2.4.2. Withdrawal

Contrary to the well-known stimulating effects of caffeine, caffeine withdrawal refers to a time-limited syndrome that develops following cessation of chronic caffeine administration and can be summarised as a common CNS depression (Ammon, 1991). Caffeine withdrawal has been well documented in humans (Juliano and Griffiths, 2004; Juliano, Evatt, *et al.*, 2012) and common symptoms include irritability, headache, fatigue, nervousness, difficulty concentrating, loss of energy and dysphoric mood (Griffiths, Bigelow and Liebson, 1989; Griffiths *et al.*, 1990; Juliano and Griffiths, 2004; Juliano, Huntley, *et al.*, 2012). The incidence or severity of symptoms is shown to increase with increases

in daily dose; abstinence from doses as low as 100 mg/day can produce symptoms (Juliano and Griffiths, 2004) and these symptoms have been demonstrated to be suppressed by low caffeine doses (Evans and Griffiths, 1999).

1.2.4.3. Subjective effects

Low to moderate caffeine consumption has been found to increase self-reported drug liking (Griffiths, Bigelow and Liebson, 1989) as well as other positive subjective effects such as increased alertness, focus, energy/stimulation, well-being and sociability (Griffiths *et al.*, 1990; Evans and Griffiths, 1992), while lowering the negative subjective effects of caffeine, namely anxiety, jitteriness and nervousness (Evans and Griffiths, 1999). Although caffeine has favourable subjective benefits in non-habitual users as well, these benefits are more profound in habitual users, probably as a result of the suppression of withdrawal symptoms (Juliano, Ferré and Griffiths, 2014).

1.2.4.4. Reinforcement

An important behavioural process influencing rates of operant behaviour, including drug use, is reinforcement. When drug administration increases the future likelihood of drug use, this drug is considered a reinforcer (Meredith *et al.*, 2013). Research has shown that caffeine can function as a reinforcer at low to moderate doses (Griffiths, Reissig and First, 2008); however, it is more likely to function as a reinforcer among individuals with a history of heavy caffeine use (Meredith *et al.*, 2013). It has also been shown that the main drive in the reinforcing effects of caffeine in habitual users is avoidance of caffeine withdrawal symptoms (Hughes *et al.*, 1993; Liguori, Hughes and Grass, 1997). Naturally, caffeine reinforcement correlates with the positive subjective effects of the drug: habitual caffeine consumers tend to report positive subjective effects after drug administration, while non-consumers tend to report more negative subjective effects (Evans and Griffiths, 1992).

1.2.4.5. Conditioned taste preference

Through responder training, a neutral stimulus can acquire reinforcing properties when repeatedly paired with a reinforcer (Pavlov’s theory of conditioning) (Rehman *et al.*, 2023). As a result, when caffeine is repeatedly

combined with a new flavour in experiments using a conditioned flavour preference paradigm, a liking for the paired flavour can develop (Rogers, Richardson and Elliman, 1995; Richardson, Rogers and Elliman, 1996; Yeomans, Spetch and Rogers, 1998). In fact, ratings of how much individuals like a novel flavoured beverage significantly increase when the beverage is paired with caffeine compared with placebo (Yeomans *et al.*, 2000). Suppression of withdrawal symptoms plays an important role in the development of caffeine flavour liking (Yeomans, Spetch and Rogers, 1998; Yeomans, Pryke and Durlach, 2002; Tinley, Durlach and Yeomans, 2004).

1.2.5. Caffeine pharmacokinetics

1.2.5.1. Absorption and Distribution

Within 60 min of oral intake, 99% of ingested caffeine is absorbed from the gastrointestinal tract (Blanchard and Sawers, 1983; Graham, 2001); approximately 20% at the level of the stomach and the largest part in the small intestine, with no significant hepatic first-pass effect (i.e., the liver does not appear to remove caffeine as it passes from the gut to the circulation) (Chvasta and Cooke, 1971; Bonati *et al.*, 1982; Mandel, 2002; Kot and Daniel, 2008). Caffeine absorption rate constant (K_a) is approximately 0.33/min and time to reach the maximum plasma concentration is varying between 15 and 120 min (Blanchard and Sawers, 1983). Healthy adults absorbing 5-8 mg/kg caffeine have been shown to reach peak plasma caffeine concentration (8-10 mg/L) in 30-75 min (Blanchard and Sawers, 1983; Kot and Daniel, 2008).

Because of its hydrophobic, water-soluble characteristics, caffeine can pass through all biological membranes (Kot and Daniel, 2008). Caffeine readily crosses the BBB by simple diffusion and carrier-mediated transport. It is also shown that caffeine binds reversibly to plasma proteins, and protein-bound caffeine accounts for 10-30% of the total plasma pool (Kot and Daniel, 2008). Caffeine is distributed throughout the body fluids and tissues (Fredholm *et al.*, 1999) with a distribution volume of 0.5-0.75 L/kg, a value suggesting that it distributes freely into the intracellular tissue water without any signs of accumulation in any specific tissue (Blanchard and Sawers, 1983a; Kot and Daniel, 2008). Table 1.2 summarises caffeine pharmacokinetics.

Table 1. 2 Caffeine pharmacokinetics

Absorption rate constant (K_a)	~ 0.33/min
Volume of distribution	0.5-0.75 L/kg
Plasma-protein binding	10-30%
Half-life ($t_{1/2}$)	2-12 h
Clearance rate	1-3 mg/kg/min
Elimination rate constant (K_e)	0.09-0.33/h

Adapted from (Institute of Medicine (US) Committee on Military Nutrition Research, 2001; Alsabri *et al.*, 2018).

1.2.5.2. Metabolism and excretion

Caffeine elimination follows the first-order kinetics (i.e., reaction depends linearly on reactant concentration) in a one-compartment open model system (i.e., free movement and distribution of drug in all body fluids) (Bonati *et al.*, 1982; Blanchard and Sawers, 1983). Nevertheless, some studies have noted that caffeine may follow non-linear kinetics if the dose is high enough to saturate its metabolism, meaning that the metabolising enzymes become overwhelmed, leading to decreased caffeine elimination rate (Denaro *et al.*, 1990). In fact, it has been reported that caffeine metabolism can saturate at levels as low as 1-4 mg/kg, or between 70 and 300mg of caffeine intake (Cheng *et al.*, 1990; Begas *et al.*, 2007).

Caffeine is metabolised in the liver by Cytochrome P450 1A2 (CYP1A2), the enzyme responsible for > 95% of the biochemical reactions pertaining to caffeine biotransformation and xenobiotic metabolism (Begas *et al.*, 2007; Kot and Daniel, 2008; Grzegorzewski *et al.*, 2021). Ingested caffeine is broken down through phase I oxidation reactions mainly to paraxanthine (1,7-dimethylxanthine), theobromine (3,7-dimethylxanthine), and theophylline (1,3-dimethylxanthine) followed by phase II conjugation (Begas *et al.*, 2007). Caffeine biotransformation by CYP1A2 averages 81.5% for paraxanthine, 10.8% for theobromine and 5.4% for theophylline formation (Gu *et al.*, 1992).

Paraxanthine is further demethylated primarily by CYP1A2, acetylated by N-acetyltransferase-2 (NAT2), and oxidised by Xanthine Oxidase (XO) to form 1-methylxanthine, 1-methyluric acid, 5-acetylamino-6-formylamino-3-methyluracil, and 1,7-dimethyluric acid, which are the major caffeine metabolites in urine (Begas *et al.*, 2007). Overall, more than 25 metabolites have been identified in humans after caffeine administration, demonstrating a rather complex metabolism (Carrillo

and Benitez, 2000). Additional CYP isoenzymes such as CYP2D6, CYP2E1, and CYP3A4/3A5 become more active at higher concentrations, which are typically not reached with typical caffeine consumption (Arnaud, 2011). CYP2D6 catalyses caffeine demethylation and 8-hydroxylation, while CYP2E1 plays a more minor role in these pathways and is predominantly responsible for the formation of theophylline and theobromine (Gu *et al.*, 1992). CYP3A4 predominantly mediates 8-hydroxylation and may be involved in the *in vivo* synthesis of 1,3,7-trimethylurea (Carrillo and Benitez, 2000) (Figure 1.5).

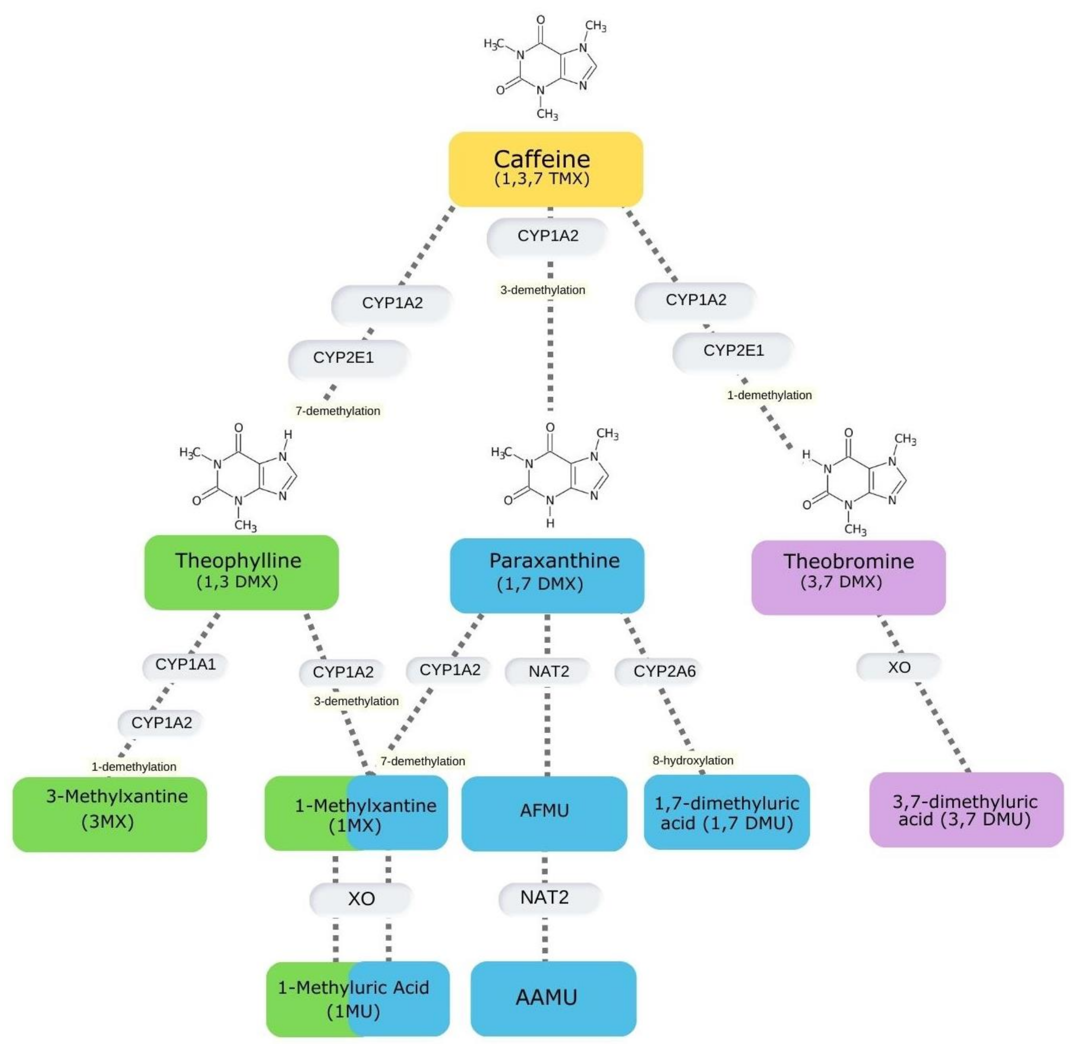

Figure 1. 5 Primary pathways and enzymes involved in the degradation of caffeine. The colour scheme aids in distinguishing different compounds: caffeine is depicted in orange, theophylline in green, theobromine in purple and paraxanthine, the principal caffeine metabolite, in blue. AAMU: 5-acetylamino-6-amino-3-methyluracil; AFMU: 5-acetylamino-6-formylamino-3-methyluracil; CYP:

cytochrome P450, followed by the number corresponding to each specific isoform; NAT2: N-acetyltransferase-2; XO: xanthine oxidase.

In healthy individuals, the elimination rate constant (K_e) for caffeine is ranging between 0.09 and 0.33/h (Alsabri *et al.*, 2018). Caffeine is cleared more quickly than paraxanthine, therefore 8-10 h after caffeine ingestion, paraxanthine levels exceed caffeine levels in plasma (Institute of Medicine (US) Committee on Military Nutrition Research, 2001; Cappelletti *et al.*, 2015). Caffeine clearance ranges between 1 and 3 mg/kg/min (Alsabri *et al.*, 2018). Although caffeine half-life ($t_{1/2}$) is reported at 4-6 h (Institute of Medicine (US) Committee on Military Nutrition Research, 2001), it demonstrates significant interindividual variability and may range between 2 and 12 h (Blanchard and Sawers, 1983; Cappelletti *et al.*, 2015). Caffeine half-life ($t_{1/2}$) is defined by the time from the peak of caffeine concentration in plasma to the time when the concentration reaches 50% of the maximal level (Alsabri *et al.*, 2018; Lin *et al.*, 2022). This wide range in the plasma mean half-life of caffeine is due to both innate individual variations, and a variety of physiological and environmental characteristics that influence caffeine absorption and metabolism, which will be discussed in detail in the next section.

Less than 5% of ingested caffeine is excreted unchanged (Carrillo and Benitez, 2000). Once caffeine has been filtered by the glomeruli, it is reabsorbed in the renal tubules and only 3% of caffeine dose is excreted unchanged by the kidneys in urine (Begas *et al.*, 2007; Kot and Daniel, 2008). In the faeces, approximately 5% of ingested caffeine is excreted over 48 h primarily as uric acid compounds and unchanged caffeine (Callahan *et al.*, 1982).

1.2.5.3. Factors affecting caffeine pharmacokinetics

Caffeine pharmacokinetics are affected by various endogenous and exogenous factors such as caffeine dose, administration vehicle, age, sex, liver diseases, pregnancy, lifestyle factors, medications and genetics. Genetics will be extensively discussed in a following section and will not be included here. The enhancers and depressors of caffeine pharmacokinetics are summarised in Table 1.3 and discussed below.

Table 1. 3 Enhancers and depressors of caffeine absorption and metabolism.

Enhancers (↑)	**Depressors (↓)**
Nutrition & Lifestyle	**Physiological**
high habitual caffeine intake	1st and 2nd trimester of life
caffeine capsules and gums	pregnancy
chronic and strenuous exercise	
tobacco smoking	**Nutrition & Lifestyle**
fluid intake	apiaceous vegetables
36 h fasting	curcumin
cruciferous vegetables	turmeric
	grapefruit juice (?)
	fibre
	Diseases
	alcohol-associated liver diseases
	cirrhosis
	Medications
	oral contraceptives
	exogenous oestrogens
	fluvoxamine
	idrocilamide
	methoxsalen
	tryptamine (?)
	olanzapine (?)
	furafylline
	theophylline
	enoxacin (?)
	ciproflaxin (?)
	norfloxacin
	pipedemic acid
	Herbal supplements
	Hydrastis canadensis
	Piper methysticum
	Cimicifuga racemose

Listings with (?) indicate that there is no conclusive evidence in the literature about the significance of these factors.

1.2.5.3.1. Age & sex

Caffeine clearance rate is slow in neonates because of their immature metabolising enzymes, while it increases linearly with postnatal age, reaching a plateau during the second trimester of life (Pons *et al.*, 1988). Nevertheless, the pharmacokinetics of caffeine in healthy young and elderly men are similar

(Blanchard and Sawers, 1983). Furthermore, as reviewed by Arnaud (2011), although CYP1A2 enzyme appears to be more active in males than in females, no sex difference has been documented in caffeine metabolism based on urinary metabolites (Arnaud, 2011).

1.2.5.3.2. Habituation, source and dose

Previous studies have revealed that the half-life of caffeine is higher when higher levels of caffeine are ingested (Denaro *et al.*, 1990; Kaplan *et al.*, 1997). On the contrary, habitual heavy coffee consumption (i.e., three cups/day), appears to stimulate CYP1A2 enzyme, suggesting that caffeine intake can act as an inducer for CYP1A2 activity (Tantcheva-Poór *et al.*, 1999; Djordjevic *et al.*, 2008).

Moreover, studies suggest that caffeine absorption in caffeine-containing beverages is similar and irrespective of drink temperature or rate of administration (Mumford *et al.*, 1996; White *et al.*, 2016). Nonetheless, caffeine absorption is reduced by 25% in chocolate and cola drinks compared with caffeine capsules, most probably because of delayed gastric emptying from these sources (Mumford *et al.*, 1996). Further, caffeine absorption from gum formulation has been found significantly faster than capsules and may indicate absorption via the buccal mucosa (Kamimori *et al.*, 2002).

1.2.5.3.3. Nutrition & lifestyle

Compared to a standard diet, a diet containing cruciferous vegetables (broccoli, cabbage, cauliflower, brussels sprouts, radish and watercress) was shown to increase caffeine clearance through induction of CYP1A2 enzyme (Kall, Vang and Clausen, 1996; Lampe *et al.*, 2000). On the contrary, adding apiaceous vegetables (carrots, celery, parsley, caraway, fennel, etc.) (Lampe *et al.*, 2000) and herbs such as curcumin and turmeric to the diet is reported to downregulate CYP1A2 enzyme activity (Chen *et al.*, 2010). Although there is evidence that grapefruit juice downregulates CYP enzyme activity (Murray, 2006), only one study has reported that grapefruit juice (1.2 L/day) decreases caffeine clearance by 23% and prolongs caffeine half-life by 31% (Fuhr, Klittich and Staib, 1993), yet such volume may not be clinically relevant.

The absorption of caffeine can also be delayed when dietary components, such as fibre, are ingested, as they may delay gastric emptying (Arnaud, 1987). In addition to fibre, fluid intake may also modify caffeine clearance, provided that

caffeine clearance is urine flow-dependent, i.e., there is a positive association between concentrations of several urinary caffeine metabolites and urine flow rate (Tang-Liu, Williams and Riegelman, 1983; Wu and Chen, 2020). Two studies have also shown that in healthy individuals, short-term (36 h) fasting increased oral caffeine clearance by 20% (Lammers *et al.*, 2015) and 17% (Lammers *et al.*, 2017) compared to an overnight fast.

Caffeine elimination time is decreased by smoking, an effect mediated by an acceleration of demethylation reactions (Parsons and Neims, 1978). This finding was recently verified by a metabolic phenotyping meta-analysis (Grzegorzewski *et al.*, 2021). Cessation of smoking is shown to restore caffeine demethylation capacity within three weeks of cessation (Swanson *et al.*, 1997). Alcohol exhibits an inhibitory effect on CYP1A2 activity - 50 g alcohol/day prolong caffeine half-life by 72% and decrease caffeine clearance rate by 36% (George *et al.*, 1986).

Changes in hepatic blood flow during exercise may influence metabolism and clearance of drugs from the liver (Niederberger and Parnham, 2021). There is evidence that 30 days of vigorous physical exercise increased CYP450 enzyme activity by 50% (Vistisen, Loft and Poulsen, 1991), while 1-h exercise has been shown to reduce caffeine half-life by 1.7 h compared with at rest (Collomp *et al.*, 1991). Moreover, chronic exercise has been linked to upregulation of metabolising enzymes, leading to potential increase in caffeine clearance rate (Yiamouyiannis *et al.*, 1992).

1.2.5.3.4. Hormones

Earlier studies suggested that caffeine clearance may fluctuate across the menstrual cycle, with elimination rate being up to 25% higher in the luteal phase, an effect related to levels of progesterone (Balogh *et al.*, 1987; Lane *et al.*, 1992). Later studies, however, indicate no significant effects on caffeine pharmacokinetics across phases of the menstrual cycle in healthy women who are not using oral contraceptives (Kamimori *et al.*, 1999; McLean and Graham, 2002), therefore such associations warrant further study.

The half-life of caffeine is on average 8.3 h longer during pregnancy and may be as much as 18 h longer toward the end of pregnancy compared with pre-pregnancy caffeine half-life (Knutti, Rothweiler and Schlatter, 1982; Brazier *et al.*, 1983). A prospective pharmacokinetic study showed a significant increase in the

dose-normalised concentrations of caffeine in serum and urine when comparing the first and third trimesters, confirming that caffeine metabolism decreases during the last 3 months of pregnancy (Yu *et al.*, 2016).

1.2.5.3.5. Liver disease

Since caffeine is predominantly metabolised by the liver, liver diseases of varying degrees can result in a reduction of demethylation reactions responsible for caffeine biotransformation to paraxanthine (Desmond *et al.*, 1980). The most profound reduction is observed in cirrhotic liver disease, correlating with the degree of hepatic impairment (Rodopoulos, Wisen and Norman, 1995; Park *et al.*, 2003; Jodynis-Liebert *et al.*, 2004; Tripathi *et al.*, 2015). A recent metabolomics analysis measured the concentration of caffeine and its metabolites in urine from patients with alcohol-associated liver disease. The results showed that the concentrations of 1-methylxanthine, paraxanthine, and 5-acetylamino-6-amino-3-methyluracil are markedly decreased with increased disease severity (Xu *et al.*, 2023).

1.2.5.3.6. Medications

The CYP enzymes participate in the metabolism of caffeine and several medications and xenobiotics (Bhatt *et al.*, 2022). Various drugs have been reported to be potent inhibitors of CYP enzymes and this has important clinical implications, since drugs that are metabolised by the same CYP enzyme have a high potential for pharmacokinetic interactions (Zhao *et al.*, 2021).

Several investigations have shown that healthy women on long-term oral contraceptive use have a CYP1A2-mediated increase in caffeine half-life up to two-fold (Arnaud, 2011), mainly during the second part of the cycle, the luteal phase (Lane *et al.*, 1992). A 2.8-fold higher plasma caffeine-to-paraxanthine ratio in oral contraceptive users compared with controls confirms that this increase is mediated through inhibition of CYP1A2 (Tantcheva-Poór *et al.*, 1999; Rasmussen *et al.*, 2002; Granfors *et al.*, 2005), with a consistent and reproducible effect in over more than 50 years of pharmacokinetic research (Grzegorzewski *et al.*, 2021). Considering that CYP1A2 is the enzyme responsible for the hydroxylation of the main oestrogens, estrone and oestradiol (Lee *et al.*, 2003), it is suggested that exogenous oestrogen in healthy postmenopausal women receiving hormone replacement therapy decreases caffeine clearance rates (Pollock *et al.*, 1999).

The clearance of caffeine was shown to decrease by 80% and caffeine half-life to increase by 500% during concomitant intake of fluvoxamine, an SSRI used in depression (Jeppesen *et al.*, 1996; Grzegorzewski *et al.*, 2021). Psychiatric medications such as tryptamine and olanzapine slightly reduce caffeine clearance. However, the inhibition of CYP by these substances might be clinically relevant and warrants further investigation (Dinger *et al.*, 2016).

Quinolone antibiotics exert a competitive and dose-dependent inhibitory effect on CYP1A2 enzyme (Fuhr *et al.*, 1990; Kinzig-Schippers *et al.*, 1999). A significant decrease in plasma clearance of caffeine has been documented during concomitant intake of caffeine and a number of quinolones, including enoxacin, ciprofloxacin, norfloxacin and pipemidic acid (Staib *et al.*, 1987; Stille *et al.*, 1987; Carbó *et al.*, 1989; Fuhr *et al.*, 1992; Granfors *et al.*, 2004), however data have been confirmed only for norfloxacin and pipemidic acid using pharmacokinetics methods (Grzegorzewski *et al.*, 2021).

Furafylline and theophylline are bronchodilators commonly used to treat asthma, and they are known to have inhibitory effects on CYP1A2 (Sato *et al.*, 1993). Studies in healthy volunteers have demonstrated that the administration of these bronchodilators can lead to an excessive accumulation of caffeine from typical dietary intake (Tarrus *et al.*, 1987; Sato *et al.*, 1993). In some volunteers, plasma caffeine concentrations and half-life increased more than 10-fold (Tarrus *et al.*, 1987). The concomitant intake of dietary caffeine and theophylline led to a decreased elimination of both compounds in healthy men, with mean serum concentrations of caffeine increasing by 158% (Sato *et al.*, 1993).

The myorelaxant idrocilamide inhibits the biotransformation of caffeine (mainly acting through CYP1A2 inhibition) in habitual consumers, leading to a 9-fold increase in half-life (Brazier *et al.*, 1980). Medications used to treat psoriasis (psoralens) are also potent inhibitors of the metabolism of caffeine, with clearance being shown to decline markedly by 70% after the use of methoxsalen in patients with psoriasis (Mays *et al.*, 1987; Apseloff *et al.*, 1990; Bendriss *et al.*, 1996).

According to a review, herbal supplements that inhibit CYP enzyme activity and have the potential to interfere with caffeine metabolism include: a) goldenseal (*Hydrastis canadensis*), used in common cold; b) Kava kava (*Piper methysticum*),

an anxiolytic and c) black cohosh (*Cimicifuga racemosa*) which alleviates menopausal and premenstrual syndrome symptoms (Tsai *et al.*, 2012).

Summarising all the above, caffeine pharmacokinetics are influenced by a range of factors, each capable of either accelerating or decelerating caffeine absorption and metabolism within the human body. The interplay among factors such as nutrition, diseases and concurrent medication use plays a crucial role in determining individual responses to caffeine. In the present research, it is crucial to understand the intricacies of these interactions governing caffeine pharmacokinetics. This understanding will enable us to differentiate between interindividual differences in caffeine metabolism attributable to genetic factors and those influenced by other variables.

1.2.6. Caffeine pharmacodynamics

As indicated by the review from Cappelletti and colleagues (2015), caffeine and its metabolites are biologically active and exert effects on the CNS and the cardiovascular, respiratory and renal systems through various mechanisms (Cappelletti *et al.*, 2015).

Caffeine has been attributed unique enhancing properties regarding cognitive function and physical performance (Rogers *et al.*, 2010; Cappelletti *et al.*, 2015; Renda *et al.*, 2015). The acute effects of caffeine on the cardiovascular system in individuals who have not developed tolerance include a slight increase in blood pressure and tachycardia (Turnbull *et al.*, 2017). In the respiratory system, caffeine ingestion has been shown to elevate oxygen consumption, increase respiratory rates in healthy individuals and increase ventilation in patients with coronary obstructive pulmonary disease (COPD) (Robertson *et al.*, 1978; Woodcock *et al.*, 1981). In the urinary system, caffeine increases sodium excretion through enhancing renin release from the kidneys; however, this effect is shown to be mild (Zhang *et al.*, 2015).

All the above caffeine actions are thought to be mediated via several mechanisms: a) the release of calcium from intracellular stores, b) inhibition of phosphodiesterases and c) antagonism of adenosine. These are discussed below.

1.2.6.1. Calcium Mobilisation

Calcium mobilisation refers to the release of calcium from intracellular storage pools (sarcoplasmic reticulum) and inhibition of calcium reuptake in skeletal and cardiac muscle (Endo, 1977; Supinski, Deal and Kelsen, 1984). Intracellular calcium release as a result of binding to and activating calcium-release channels was the first mechanism to be proposed as a potential mechanism for caffeine action (Sawynok and Yaksh, 1993; Carrillo and Benitez, 2000; Magkos and Kavouras, 2005; Porta *et al.*, 2011). As a result, endothelial NO synthase is activated and higher quantities of the neurotransmitter NO are produced, with subsequent increase in contractility during submaximal contractions (Goodman and Synder, 1982).

Nevertheless, calcium mobilisation requires concentrations that are unlikely to be achieved from typical everyday caffeine consumption. For example, it has been reported that at least 250 μmol (approximately 48.5 mg/L) are required to cause any increase in calcium release while concentrations between 5 and 20 mM (approximately 971-3884 mg/L) are required for substantial increases (Sawynok and Yaksh, 1993; Carrillo and Benitez, 2000; Porta *et al.*, 2011). Thus, calcium mobilisation is unlikely to play a significant role in the mechanism of caffeine, except perhaps in cases of toxic, if not lethal doses.

1.2.6.2. Inhibition of Phosphodiesterases

Caffeine has been referred to as a phosphodiesterase inhibitor, a mechanism thought to mediate caffeine stimulatory effects on the cardiovascular system and which are accompanied by increased coronary blood flow (Echeverri *et al.*, 2010). Caffeine increases intracellular concentrations of cyclic adenosine monophosphate (cAMP) by inhibiting phosphodiesterase enzymes in skeletal muscle and adipose tissues (Umemura *et al.*, 2006). Increased cAMP promotes lipolysis via the activation of hormone-sensitive lipases, with the release of free fatty acids and glycerol and has a vital role in the adrenaline cascade. The increased availability of these fuels in skeletal muscle spares the consumption of muscle glycogen (Chasiotis, Sahlin and Hultman, 1983). It also activates protein kinase A, which in turn phosphorylates several enzymes implicated in glucose and lipid metabolism (Graham, 2001).

Similarly to the case of calcium mobilisation, caffeine is only able to interact with this molecular target at concentrations that greatly exceed those achieved with

typical caffeine consumption (Sawynok and Yaksh, 1993; Fredholm *et al.*, 1999; Carrillo and Benitez, 2000; Magkos and Kavouras, 2005). For example, the inhibition rate constant (K_i) value, which measures the affinity for phosphodiesterase by caffeine, is 480 µmol (approximately 93.2 mg/L) (Sawynok and Yaksh, 1993; Carrillo and Benitez, 2000; Magkos and Kavouras, 2005). Thus, phosphodiesterase inhibition is unlikely to play any role in caffeine's mechanisms except in cases where highly toxic and potentially lethal doses have been ingested.

1.2.6.3. Antagonism of adenosine

The only proposed molecular target of caffeine at clinically relevant concentrations are the adenosine receptors (Sawynok and Yaksh, 1993; Carrillo and Benitez, 2000; Magkos and Kavouras, 2005). Because of their similar structure, caffeine competitively antagonises adenosine by blocking its binding with adenosine receptors (Fredholm, 1995; Cappelletti *et al.*, 2015; Nehlig, 2018). Adenosine receptors are G-protein coupled receptors or seven transmembrane receptors with four subtypes: A1, A2a, A2b, and A3, which activate G-proteins in the cell leading to various effects upon signalling molecules such as cAMP (Fredholm *et al.*, 2000; Kobilka, 2007).

Specifically, caffeine is a non-selective adenosine receptor antagonist with K_i of 44 and 40 µmol (around 8.5 and 7.8 mg/L) for the adenosine A1 and A2a receptor subtypes, respectively, although others have reported even lower values (Sawynok and Yaksh, 1993; Carrillo and Benitez, 2000; Fredholm and Svenningsson, 2003; Magkos and Kavouras, 2005). The A1 subtype is mainly localised in the brain, spinal cord, eye, adrenal gland, heart, skeletal muscle and the adipose tissue (Fredholm *et al.*, 2000, 2001; Magkos and Kavouras, 2005; Jacobson and Müller, 2016). The A2a subtype is mainly localised in the spleen, thymus, striatopallidal GABAergic neurons and to a lesser degree the heart, lung and blood vessels (Fredholm *et al.*, 2000, 2001; Magkos and Kavouras, 2005; Jacobson and Müller, 2016). Although caffeine also acts as an antagonist at the A2b receptor, its tissue expression (cecum, colon, bladder and bronchial smooth muscle) does not seem to be as toxicologically relevant as compared to the other receptor subtypes (Fredholm *et al.*, 2000, 2001; Magkos and Kavouras, 2005; Jacobson and Müller, 2016). Finally, caffeine does not appear to have a high affinity for the A3 receptor subtype (Magkos and Kavouras, 2005).

Due to the blocking of adenosine inhibitory effects in neuronal function, caffeine indirectly stimulates the release of dopamine, norepinephrine, serotonin, acetylcholine, glutamate and GABA neurotransmitters, thus promoting alertness and wakefulness (Fredholm *et al.*, 1999; Fredholm and Svenningsson, 2003). Up-regulation of the adenosine system after chronic caffeine administration appears to be a neurochemical mechanism underlying caffeine withdrawal syndrome. This mechanism results in increased functional sensitivity to adenosine during caffeine abstinence, and it likely plays an important role in the behavioural and physiological effects produced by caffeine withdrawal (Griffiths, Reissig and First, 2008).

In conclusion, although a few suggested mechanisms of action have been identified for caffeine, the antagonism of adenosine remains the most plausible mechanism with the typical daily caffeine intake of individuals.

1.2.7. Caffeine and cognitive function

Caffeine has notable enhancing properties in cognitive function, which explain its popularity, especially in shift workers, students and anyone generally seeking to overcome fatigue or prolong their capacity to complete everyday activities (Cappelletti *et al.*, 2015; Renda *et al.*, 2015; Carswell *et al.*, 2020). In general, caffeine improves performance with minimal side effects across a wide range of cognitive functions at doses easily attained from everyday consumption (McLellan, Caldwell and Lieberman, 2016). This is often accomplished by preventing decrements in alertness and attention caused by suboptimal arousal, such as during fatiguing circumstances or sleep deprivation (Lieberman *et al.*, 2002; Cappelletti *et al.*, 2015; McLellan, Caldwell and Lieberman, 2016).

In this section, the domain-specific associations between caffeine and performance are discussed, followed by an exploration of the methodological challenges encountered in caffeine-related cognitive research.

1.2.7.1. Perceptual – motor function

As mentioned in the previous section, perceptual-motor skills are implicitly assessed in more complex functions, especially in timed computerised tests (Grissmer *et al.*, 2010; Lin *et al.*, 2017). Therefore, the association between caffeine and other cognitive domains is discussed below.

1.2.7.2. Attention and processing speed

Caffeine is known to enhance aspects of attention, as shown with behavioural measures, as well as using Electroencephalogram (EEG) studies (Ruxton, 2008; Tieges *et al.*, 2009; Brunyé, Mahoney, Lieberman and Taylor, 2010; Serra-Grabulosa *et al.*, 2010; Foxe *et al.*, 2012). Even small caffeine doses (32 mg or ~0.5 mg/kg) are shown to have beneficial effects, often regardless of whether individuals are sleep-deprived or well-rested (Nehlig, 2010; Einöther and Giesbrecht, 2013).

Reviews have noted that caffeine reliably enhances vigilance independent of gender, age and typical daily intake levels (at least up to 400 mg) (Smith, 2002). While there is some debate about the impact of caffeine on simple versus complex attention tasks, a review has concluded that caffeine has positive effects on both. Simple tasks benefitted from doses 12.5-400 mg (~0.2–5.5 mg/kg) and more complex tasks benefitted from doses in the range of 60–400 mg (~0.75–5.5 mg/kg) (Einöther and Giesbrecht, 2013).

1.2.7.3. Memory

Studies on the acute effects of caffeine on short-term memory have produced mixed results. Some studies found no significant effects of caffeine on memory after caffeine supplementation with 1-3.5 mg/kg (Amendola, Gabrieli and Lieberman, 1998; Warburton, Bersellini and Sweeney, 2001). However, studies in non-habitual caffeine consumers have reported dose-dependent, however contradicting effects of caffeine on aspects of memory. These include findings of improved discrimination between learned and false information (Borota *et al.*, 2014) and increased false memories (Mahoney *et al.*, 2012).

There is also data on a positive relationship between habitual (long-term) caffeine consumption and verbal memory, particularly in females and older adults (Jarvis, 1993; Hameleers *et al.*, 2000; Johnson-Kozlow *et al.*, 2002). However, it is worth noting that these associations could be influenced by the fact that older individuals who are healthier tend to consume more caffeine, while those with health concerns reduce their caffeine intake (Soroko, Chang and Barrett-Connor, 1996). Interestingly, in young adults, habitual caffeine consumption has not shown similar cognitive benefits (Harvanko *et al.*, 2015).

1.2.7.4. Executive function

Limited research has explored the impact of caffeine on executive function. A study in sleep-deprived individuals found that a single dose of 600 mg (~8.0 mg/kg) of caffeine ingested after 44 h of wakefulness failed to preserve executive function performance (Killgore *et al.*, 2009). Moreover, 200 mg (~2.5 mg/kg) of caffeine administered at 11 and 23 h of wakefulness did not mitigate sleepiness-related executive control (Gottselig *et al.*, 2006). In contrast, repeated 200 mg doses of caffeine administered across three nights of continuous wakefulness significantly improved executive performance. However, the authors suggested that improvement was driven by lower-level efficiency enhancements rather than better higher-order functioning (Killgore, Kamimori and Balkin, 2014).

Additionally, caffeine was found to improve executive function in both low and high habitual caffeine consumers, although a higher dose of caffeine was needed in the high-consumption group (200 mg vs 400 mg, respectively) (Brunyé, Mahoney, Lieberman, Giles, *et al.*, 2010). A more recent study found that even a low dose of caffeine (50 mg or ~0.7 mg/kg) enhanced executive function (Soar *et al.*, 2016). However, as highlighted in a review, the cognitive task used in this study may not be appropriate in assessing executive function (McLellan, Caldwell and Lieberman, 2016), which raises the question of task specificity for assessing executive function and sensitivity to caffeine supplementation (de Vries *et al.*, 2013; de Jager *et al.*, 2014).

1.2.7.5. Language and social cognition

In the only investigation up to date on language performance and caffeine, higher detection of spelling errors were found after 400 mg of caffeine and this relationship was related to subjective arousal states (Brunyé *et al.*, 2012). To the author's knowledge, there are currently no investigations on the associations between the domain of social cognition and caffeine, most probably because this domain has been recently introduced in the list of the core domains of cognition.

1.2.7.6. Methodological challenges

The scientific consensus regarding cognitive functions is that caffeine in doses 32-300 mg, i.e., at typically consumed doses, enhances fundamental aspects

of cognitive performance, such as attention and processing speed (Snel, Lorist and Tieges, 2004; Nehlig, 2010; McLellan, Caldwell and Lieberman, 2016). Still, research on caffeine and cognitive performance in memory and executive function remains controversial (Smith *et al.*, 2011; James, 2014; Cappelletti *et al.*, 2015), in part because there are fewer studies and those available vary greatly in methodologies employed (Cappelletti *et al.*, 2015; McLellan, Caldwell and Lieberman, 2016). Moreover, there are limited or no studies on caffeine and language or social cognition domains. In this section, two of the major methodological challenges in caffeine research, namely withdrawal reversal and the Yerkes-Dodson law, will be discussed.

1.2.7.6.1. Withdrawal reversal

Caffeine withdrawal and withdrawal reversal are subjects of controversy in research (Einöther and Giesbrecht, 2013; James, 2014). As discussed previously in this chapter, regular caffeine consumption leads to physical dependence, resulting in withdrawal symptoms when caffeine is discontinued (Hughes *et al.*, 1993). In research, participants who have abstained from caffeine since the previous evening may experience early stages of caffeine withdrawal during testing (typically 12–14 h since caffeine was last ingested) (James and Gregg, 2004; Juliano and Griffiths, 2004). This raises the question of whether improved performance (attributed to caffeine) reflects the actual effects of the stimulant, the reversal of withdrawal, or both.

Recruiting caffeine-naïve participants, who consume little or no caffeine and are not susceptible to withdrawal, is a common approach to address this issue (James, 2014). However, since most of the population consumes caffeine daily, caffeine-naïve individuals represent a small and self-selected minority (Rogers *et al.*, 2013). Consequently, the generalisability of findings from caffeine-naïve participants to regular consumers is questionable (Cappelletti *et al.*, 2015). Moreover, infrequent caffeine consumers often report negative reactions to caffeine (e.g., jitteriness) because of lack of tolerance to the drug (Bonnet and Arand, 1992; Griffiths and Mumford, 1996), which limits the ability to generalise between groups with varying levels of caffeine consumption (Rogers *et al.*, 2013).

This suggests that the classic drug-challenge protocol commonly employed in caffeine research may not be a suitable design for revealing the effects of a drug

that is both widely available and subject to development of withdrawal and tolerance (James and Rogers, 2005; James, 2014). Therefore, long-term withdrawal designs are promising in mitigating confounding due to reversal of caffeine withdrawal (James and Rogers, 2005). These designs can incorporate core features of the traditional drug-challenge protocol, including double-blinding and placebo control, combined with periods of abstinence. Because the time course of caffeine tolerance and withdrawal is 3–5 days (Griffiths, Bigelow and Liebson, 1986; Denaro *et al.*, 1990; Hughes *et al.*, 1993; James, 1994a), this duration would protect against confounding due to both.

In this research, a long-term withdrawal protocol has been selected to assess the acute effects of caffeine on cognition based on genetics. Therefore, topics related to caffeine tolerance, withdrawal and potential strategies to mitigate confounding in caffeine research will be thoroughly addressed in Chapter 4 of this thesis.

1.2.7.6.2. The Yerkes-Dodson law

Apart from stimulatory effects, caffeine has been reported to possess anxiogenic properties, which can subsequently affect cognitive performance (Rogers *et al.*, 2010; Shields, Sazma and Yonelinas, 2016). In fact, moderate doses (100–300 mg or ~1.5–3.0 mg/kg) typically increase alertness, while higher doses (> 400 mg or ~5.5 mg/kg) are more likely to result in anxiety and may impair performance, especially in non-habitual caffeine consumers (Stafford, Rusted and Yeomans, 2006; Nehlig, 2010; Smith *et al.*, 2011). Hence, caffeine appears to follow a U-shaped arousal continuum, where performance improves with increased arousal up to a certain point, after which further increases in arousal can lead to a decline in performance (Stafford, Rusted and Yeomans, 2006; McLellan, Caldwell and Lieberman, 2016).

This can be explained by the Yerkes-Dodson law. The Yerkes-Dodson law is a psychological principle which suggests that performance is an inverted U function of arousal, with a negative relationship between arousal and task difficulty (Yerkes and Dodson, 1908). This indicates that moderate arousal leads to optimal performance. When arousal levels become too high, performance decreases (Yerkes and Dodson, 1908). Indeed, cognitive performance on difficult tasks has been shown to improve in less aroused subjects, while performance first improved and

then deteriorated in more aroused subjects (Anderson, 1994; Watters, Martin and Schreter, 1997).

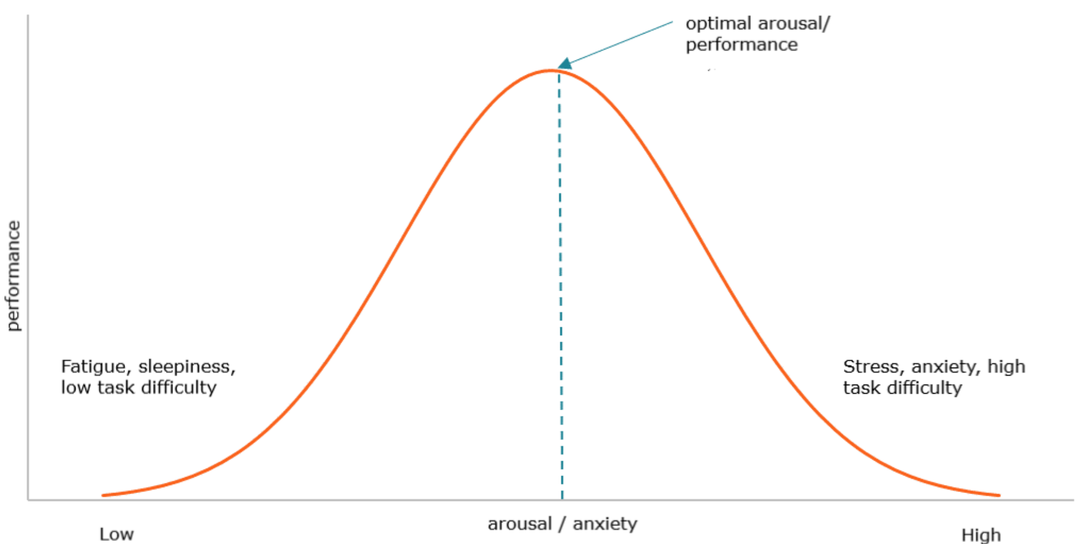

Figure 1. 6 The Yerkes-Dodson law. The inverted U-shaped curve represents how performance levels change based on varying degrees of arousal. Optimal performance occurs at an intermediate level of arousal (vertical blue line), as indicated by the peak of the curve. As arousal levels deviate from this optimal point, performance may either decline with excessive arousal or deteriorate due to insufficient arousal, resulting in a suboptimal performance.

Pre-existing arousal levels may influence the effects of caffeine, as it can either improve or degrade performance based on the individual's baseline arousal state, especially the extent to which subjects are sleep-deprived or fatigued versus well-rested (Wood *et al.*, 2014). Therefore, administration of a large dose of caffeine to an individual who is severely fatigued will likely improve performance because in this case, caffeine promotes a favourable arousal level. Conversely, administration of the same dose to someone who is well-rested and highly aroused may degrade performance because in this case, caffeine produces a state of over-arousal, which according to the Yerkes-Dodson law, will degrade cognition (Figure 1.7). In support of this hypothesis, evidence suggests that individuals use caffeine to achieve a self-perceived, peak state of arousal, as they modify caffeine usage until they reach their self-selected optimal level of arousal and cognitive performance (Harvanko *et al.*, 2015).

Still, it would be an oversimplification to assume that caffeine's effects precisely follow an inverted U-shaped function across all behaviours and individuals, or that caffeine does not possess a net effect on cognition. When assessing the associations between caffeine and cognition, several factors need to be considered. For example, the precise effects of caffeine on behaviour may vary across different domains and be differentially influenced by the difficulty of the task under investigation (Diamond, 2005). Other factors may include participant motivation (Diamond *et al.*, 2007), impulsivity and sociability (Anderson, 1994), as well as individual differences in caffeine metabolism and sensitivity due to genetic factors (Renda *et al.*, 2015). These genetic variabilities among individuals will be discussed in detail in the next section.

1.3. Genetics of caffeine

As previously stated, a number of environmental and biological factors have been identified to impact caffeine pharmacokinetics and especially caffeine CYP1A2-mediated metabolism (Gunes and Dahl, 2008). However, much of the variation in caffeine metabolism is heritable (Gunes and Dahl, 2008; Perera, Gross and McLachlan, 2012) and population-based Genome Wide Association Studies (GWAS) have been employed to identify the precise genetic factors.

GWAS aim to identify associations of genotypes with phenotypes by testing for differences in the allele frequency of genetic variants between individuals who are ancestrally similar but differ phenotypically (Uffelmann *et al.*, 2021). The most studied genetic variants in GWAS are Single Nucleotide Polymorphisms (SNPs) (Uffelmann *et al.*, 2021). GWAS typically report correlated SNPs that show a statistically significant association with the trait of interest and they are known as genomic risk loci (Jia *et al.*, 2011).

The effect allele is the allele to which the effect estimate refers, regardless of whether this estimate is increasing or decreasing (Kido *et al.*, 2018). The effect allele can be both the minor (less frequently found in the population) and major (most frequently found in the population) allele of a gene (Wootton and Sallis, 2020). Consequently, GWAS are hypothesis-generating studies that can be further studied in genetic association studies (Shaffer, Feingold and Marazita, 2012).

1.3.1. Genetics of habitual caffeine intake

Genetics are involved in individual variability in caffeine consumption, which occurs both at the pharmacodynamic and pharmacokinetic level (Laitala, Kaprio and Silventoinen, 2008; Yang, Palmer and de Wit, 2010). Heritability estimates for caffeine and coffee consumption range between 36% and 58% (Yang, Palmer and de Wit, 2010).

GWAS of habitual caffeine and coffee intake have identified variants near *CYP1A2* and Aryl Hydrocarbon Receptor (*AHR)* genes (Cornelis *et al.*, 2011; Sulem *et al.*, 2011; Amin *et al.*, 2012). Both the *AHR* and *CYP1A2* genes are biologically plausible candidates as CYP1A2 enzyme is responsible for ~95% of caffeine metabolism in humans and *AHR* plays a regulatory role in substrate-induced expression of target genes, including *CYP1A1* and *CYP1A2* (Le Vee, Jouan and Fardel, 2010; Larigot *et al.*, 2018; Granados *et al.*, 2022). In a GWAS meta-analysis of 47,341 individuals of European descent, the strongest associated SNP (rs4410790) was located near *AHR* gene*.* The second strongest associated SNP (rs2470893) mapped between the *CYP1A1* and *CYP1A2* genes. The C allele of rs4410790 is positively correlated with cerebellum *AHR* methylation, suggesting a role of AHR in motor or learning pathways that may trigger coffee consumption (Jorge-Nebert *et al.*, 2010). The rs2472297 T variant putatively weakens the binding of SP1, a co-activator in the Ahr-Arnt complex regulating CYP1 locus transcription (Swanson, 2002). The rs4410790 C and the rs2470893 T alleles were associated with higher caffeine consumption, with the crude weighted mean difference between homozygote genotypes (CC vs TT and TT vs CC, respectively) of 44 mg/day for rs4410790 and 38 mg/day for rs2470893. The two SNPs together, however, explained between 0.06% and 0.72% of the total variation in caffeine intake across studies (Cornelis *et al.*, 2011).

Two additional SNPs in the same region were associated with coffee intake in a separate study. Notably, the T allele of rs2472297, located between *CYP1A1* and *CYP1A2* genes and the T allele of rs6968865, located near *AHR,* were associated with coffee intake, with an effect of ~0.2 cups/day per effect allele for both SNPs (Sulem *et al.*, 2011). Further, in a GWAS meta-analysis on coffee consumption comprising eight cohorts of 18,000 individuals of European ancestry, rs2470893 and rs2472297 SNPs located between *CYP1A1* and *CYP1A2* genes

exceeded the genome-wide significance threshold of 5 x 10^{-8}. The best hit was rs2470893, while the two SNPs were in strong linkage disequilibrium (LD) (r^2 = 0.70). LD is the non-random association between alleles of different loci and may be extremely important in identifying the functional polymorphisms of complex traits, such as caffeine and coffee intake (Shifman *et al.*, 2003). The T allele of rs2470893 was consistently positively associated with coffee consumption across all cohorts, with effect estimates ranging from 0.013 to 0.169 (Amin *et al.*, 2012).

In a subsequent analysis, caffeine intake allele score at the level of *AHR* rs4410790 and *CYP12A* rs2470893 was associated with a 42% higher coffee intake, indicating the cooperative action of both loci (Nordestgaard and Nordestgaard, 2016). An additional variant in the *CYP1A2* gene, the rs762551 polymorphism was also shown to be associated with coffee intake. The rs762551 AA genotype may lead to higher coffee intake, especially in males, younger age groups and individuals of Caucasian ethnicity (Denden *et al.*, 2016).

In a GWAS meta-analysis of regular coffee consumption (cups/day) among coffee consumers of European and African American ancestry, eight loci demonstrated effect sizes of 0.03-0.14 cups/day per effect allele and can collectively explain ~1.3% of the phenotypic variance of coffee intake (Cornelis *et al.*, 2015). The intergenic loci near *AHR* and *CYP1A1/CYP1A2* remained the most prominent loci associated with coffee consumption in both ethnic backgrounds (Cornelis *et al.*, 2015).

Between the newly identified loci, two are likely implicated in caffeine metabolism. One polymorphism mapped the Cytochrome P450 Oxidoreductase (*POR*) gene, which encodes P450 oxidoreductase that is essential for all metabolic processes catalysed by CYP450 (Hu *et al.*, 2012). The rs17685 A variant, associated with higher coffee consumption, is linked to increased *POR* expression and potential subsequent faster caffeine metabolism (Rome *et al.*, 2009). The rs1481012 locus maps to adenosine triphosphate (ATP) binding cassette subfamily G member 2 (*ABCG2*) gene. This gene encodes xenobiotic efflux transporter proteins that facilitate transport molecules across cell membranes (Woodward *et al.*, 2013) and plays an important role in preventing accumulation of xenobiotic substrates such as caffeine in certain tissues (Klaassen & Aleksunes, 2010).

The Glucokinase Regulator (*GCKR*) and MLX Interacting Protein Like (*MLXIPL*) genes have also been associated with coffee intake, however the link is unclear. The *GKRP* rs1260326 T allele, associated with lower coffee intake, encodes a nonsynonymous change in the glucokinase regulatory GKRP protein which leads to increased hepatic glucokinase activity and thus, increased glycogen synthesis and storage (Beer *et al.*, 2009). The *MLXIPL* gene encodes the Carbohydrate response element binding protein (ChREBP), a transcription factor that is expressed in the liver and has a prominent function during consumption of high-carbohydrate diets (Agius, Chachra and Ford, 2020). The association of the *MLXIPL* rs7800944 T allele with lower coffee consumption, as reported by Cornelis *et al.* (2015), has not been replicated in other studies.

In the same study, an additional index SNP was the rs6265 in Brain-Derived Neurotrophic Factor (*BDNF*) gene. The *BDNF* gene belongs to the neurotrophins family and encodes BDNF protein. This protein modulates the activity of serotonin, dopamine and glutamate, which are involved in mood-related circuits and have significant role in memory and learning (Numakawa *et al.*, 2010). The *BDNF* T allele associated with lower coffee intake has been demonstrated to impair BDNF secretion. This impairment potentially attenuates the enjoyable effects of coffee and, consequently, the motivation to consume coffee (Egan *et al.*, 2003). An additional candidate SNP was rs9902453, which is near the Solute Carrier family 6-member 4 (*SLC6A4*) gene. *SLC6A4* gene encodes the serotonin transporter, which can influence a wide range of behaviours such as sensory processing and food intake through serotonergic neurotransmission (Canli and Lesch, 2007). Nevertheless, whether the effect A allele, which was associated with lower coffee intake in this study has any functional effects on the gene is unknown.

Finally, in a sample of 2,735 participants, individuals carrying the rs5751876 TT genotype in the Adenosine Receptor A2a (*ADORA2A*) gene were significantly more likely to consume less caffeine (i.e., <100 mg/day) than carriers of the C allele (Cornelis, El-Sohemy and Campos, 2007). Caffeine-induced anxiety may serve to illustrate this association. Caffeine's effects are largely mediated by the adenosine receptor system and adenosine is also thought to be involved in the regulation of anxiety (Alsense *et al.*, 2003). Interestingly, the adenosine A2a receptors are required for most of the synaptic actions of BDNF and regulate BDNF levels in the brain (Sebastião *et al.*, 2011).

1.3.2. Genetics of caffeine pharmacokinetics

Genetics and non-genetic factors together can explain 42% and 38% of *CYP1A2* variation at activity and protein level, respectively (Klein *et al.*, 2010). The heritability of CYP1A2 activity can be shown in twin studies (Rasmussen *et al.*, 2002; Matthaei *et al.*, 2016). After excluding smokers and users of hormonal contraceptives, 89% of caffeine pharmacokinetics variation was due to genetic effects and, in the entire group, 8% of caffeine pharmacokinetics variation could be explained by the rs2470893 *CYP1A1/CYP1A2* intergenic polymorphism (Matthaei *et al.*, 2016).

A functional study showed that an A-to-C substitution at position 163 (rs762551) in the *CYP1A2* gene decreases enzyme inducibility as reflected by plasma and urinary metabolite ratios after caffeine intake (Sachse *et al.*, 1999). Therefore, carriers of the C allele, 54% of the Caucasian population (AC and CC carriers) metabolise caffeine more slowly than individuals homozygous for the AA allele, who are considered fast caffeine metabolisers and represent 46% of the population (Sachse *et al.*, 1999; Cornelis, El-Sohemy and Campos, 2007). Moreover, in a sample from the UK Biobank, the rs2472297 T allele in the intergenic *CYP1A1/CYP1A2* region and the rs4410790 C allele near *AHR* gene previously associated with increased caffeine and coffee consumption (Cornelis *et al.*, 2011, 2015), were also associated with lower plasma caffeine levels, indicating faster caffeine metabolism (Cornelis *et al.*, 2016).

The same GWAS identified a polymorphism at the level of *CYP2A6*, which encodes the liver enzyme involved in the metabolism of paraxanthine to 1,7-dimethyluric acid. The *CYP2A6* rs56113850 minor T allele was associated with higher plasma paraxanthine/caffeine levels, indicating slow paraxanthine metabolism (Cornelis *et al.*, 2016). Moreover, the authors identified a SNP (rs62391270) near CD83 Molecule (*CD83*) gene, which was significantly associated with paraxanthine levels. CD83 is a member of the immunoglobulin superfamily (Li *et al.*, 2019) and the link with caffeine metabolism is unknown. On the contrary, variants in the *GCKR*, *ABCG2* and *POR* genes, previously associated with coffee consumption (Cornelis *et al.*, 2015), were only nominally associated with plasma caffeine and caffeine metabolites (Cornelis *et al.*, 2016).

These findings suggest that systemic caffeine levels play a crucial role in regulating daily caffeine consumption to achieve the desired stimulant and positive reinforcing effects of caffeine. The observation that certain SNPs previously linked to coffee or caffeine intake were not linked to with caffeine metabolites in plasma may suggest variations in the levels of caffeine in the CNS and warrants further study.

1.3.3. Genetics of caffeine pharmacodynamics

As established previously, the antagonism of adenosine is how caffeine exerts its effects on the brain (Sawynok and Yaksh, 1993; Carrillo and Benitez, 2000; Magkos and Kavouras, 2005), therefore genetic variants implicated in this mechanism are potential candidates to establish interindividual differences in caffeine effects. The *ADORA2A* gene encodes the A2a adenosine receptor, one of the most well studied G protein-coupled receptors and a major target of caffeine (Lee *et al.*, 2014).

The C-to-T substitution at nucleotide position 1976 (rs5751876) in the *ADORA2A* gene has been repeatedly linked with risk for elevated or pathological anxiety (Alsene *et al.*, 2003; Hohoff *et al.*, 2009, 2010). A previous study reported the rs5751876 T allele to be associated with increased brain A1a receptor availability in the brain of healthy participants as measured by positron emission tomography (PET), suggesting indirect effects on anxiety via A1a modulation (Hohoff *et al.*, 2014). This has also been suggested to affect caffeine-adenosine A2a receptor binding in a way that results in greater dopaminergic neurotransmission compared with the major allele, resulting in feelings of anxiety and insomnia (Alsene *et al.*, 2003; Childs *et al.*, 2008).

Therefore, the rs5751876 variant has been used by authors to categorise individuals as having a 'high' (TT genotype, approximately 15-20% of Caucasian population) or 'low' (CT/CC genotype) sensitivity to caffeine (Alsene *et al.*, 2003; Childs *et al.*, 2008; Carswell *et al.*, 2020). Other *ADORA2A* variants have also been studied for an association with anxiety, including rs2298383 and rs4822492 (Childs *et al.*, 2008; Hohoff *et al.*, 2010) and may be implicated in habitual caffeine intake to avoid side-effects such as caffeine-induced anxiety and insomnia.

In summary, *CYP1A1*, *CYP1A2* and *AHR* genes, implicated in caffeine pharmacokinetics and the *ADORA2A* gene, associated with caffeine pharmacodynamics are the most replicated genes in the literature with regard to caffeine-related outcomes such as caffeine metabolism, caffeine-induced anxiety and habitual caffeine intake. Importantly, the *CYP1A2* rs762551 variant has a demonstrated causal effect on the function of the gene product, i.e., on CYP1A2 enzyme activity (Sachse *et al.*, 1999), which provides a biologically plausible explanation for the gene-caffeine interaction, based on proposed guidelines (Grimaldi *et al.*, 2017).

1.4. Thesis aims

The elderly population is on the rise in the UK and worldwide, primarily due to increased life expectancy (Khavinson, Popovich and Mikhailova, 2020). By 2040, the global elderly population (> 60 years old) is projected to grow by 56% and the 'oldest old' (> 80 years old) is expected to triple by 2050 (United Nations Department of Economic and Social Affairs, 2021). In the UK, as of 2021, there were over 15.5 million elderly individuals, constituting 23% of the UK population. Additionally, the number of 'oldest old' individuals reached 3.2 million, with nearly 600,000 being aged > 90 years old (Office for National Statistics, 2021). This trend is expected to increase the prevalence of disease and disability, including the impairment of cognitive functions (Crimmins *et al.*, 2011). Cognitive function is particularly important because it influences the ability of individuals to maintain independence, engage in daily activities and enjoy a good quality of life (Shin, 2022).

Cognitive decline and dementia are important public health concerns. In 2020, the global prevalence of cognitive impairment was 19%, with incidence rate at 53.97 per 1000 person-years (Pais *et al.*, 2020). Moreover, there were 55 million documented cases of dementia and it is anticipated that this number will increase to nearly 80 million by 2030 (Shin, 2022). Cognitive impairment carries substantial social implications such as reduced autonomy, which leads to an increased demand for long-term care (Furuta *et al.*, 2013; Roberts and Knopman, 2013; Livingston *et al.*, 2020). Markedly, a recent study found that cognitive function accounted for 29% of the variance in quality of life in older adults with cognitive impairment (Song, Fan and Seo, 2023).

Cognitive decline and dementia have a diverse aetiology and effective treatment remains elusive (Petersen *et al.*, 2018). Consequently, there is a growing interest in exploring the potential benefits of various agents, including psychostimulants and nutrients, in preserving cognitive function across the lifespan and delaying the onset of cognitive impairment (de Jager *et al.*, 2014; Petersen *et al.*, 2018). This heightened interest has led to the emergence of the brain health supplements market, which has gained significant global popularity (Crawford *et al.*, 2020). Brain supplements claim to improve memory, concentration and overall cognitive performance and are targeted at diverse groups, including students, healthy adults and the elderly (Gestuvo and Hung, 2012; Crawford *et al.*, 2020).

Caffeine, a naturally occurring psychoactive substance, is among the most promising cognitive function enhancers (Cappelletti *et al.*, 2015). Due to caffeine being the most widely consumed psychostimulant globally (James, 1998; Ogawa and Ueki, 2007; Heckman, Weil and De Mejia, 2010), its impact on human health and cognitive function is substantial. However, as discussed in this chapter, research on caffeine and cognition has yielded mixed results due to a) the multifactorial nature of human cognition, b) the variable cognitive function assessment methods employed by researchers, c) the populations recruited in studies (caffeine consumers vs non-consumers), d) caffeine tolerance and withdrawal effects and e) caffeine anxiogenic properties. Moreover, caffeine pharmacokinetics and pharmacodynamics are well-studied and influenced by numerous factors, including genetics. Therefore, the magnitude of caffeine effects in cognition, or the lack of an effect when compared to placebo, can be partly explained by genetic variations.

The present research was perceived, designed and conducted to comprehensively investigate all the above-described variables aiming to explore:

1) Whether there are caffeine x gene interactions in brain-related outcomes (Chapter 2). Our literature review revealed that cognition has numerous effectors that may influence individual performance. Therefore, a systematic review was designed to investigate current evidence on genetics studies related to caffeine and the full spectrum of brain-related outcomes, including sleep, anxiety and cognitive performance. This study informed all future studies.

2) If there is an association between variations in genes implicated in caffeine pharmacokinetics and pharmacodynamics, habitual caffeine intake and cognitive performance (Chapter 3). The methodology of this study in terms of selection of cognitive domains and their assessment methods, assessment of habitual caffeine intake and variables such as sleep quality, as well as selection of SNPs for our research were informed from Chapters 1 and 2.

3) Whether variations in genes implicated in caffeine pharmacokinetics and pharmacodynamics impact the effect of acute caffeine intake on cognitive performance (Chapter 4). The study replicated the methodology from Chapter 3 in terms of cognitive assessment, assessment of habitual caffeine intake and health variables and selection of SNPs, while the trial protocol and the selection of caffeine supplementation dose were informed from Chapters 1 and 2.

Chapter 2. Systematic Review

This chapter presents the first study of this research, which is a systematic review. The systematic review investigated the existing body of research encompassing genetic studies related to caffeine and all brain related outcomes, including sleep, anxiety and cognitive performance. Within this systematic review, an exploration is undertaken to evaluate the evidence up to date and identify the gaps in literature of genetics studies of caffeine and cognition that this work aims to fill: a) which genes are associated with cognition and brain-related outcomes that may affect cognitive function; b) which cognitive domains have been frequently studied and which are underexplored; c) how cognitive functions are commonly assessed in this context and d) how habitual caffeine intake is measured and what doses of caffeine supplementation have been used. The findings of this study will be employed for the design and development of future studies in the present research.

2.1. Introduction

Caffeine is the most widely consumed psychostimulant, being used habitually by more than 80% of the world population (Ogawa and Ueki, 2007; Heckman, Weil and De Mejia, 2010; EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2015). On average, daily caffeine intake in adults worldwide equals to 227 mg, which is approximately two regular 125 ml cups of coffee (Nehlig, 2018; Reyes and Cornelis, 2018; Samoggia and Rezzaghi, 2021). Caffeine is readily available from a variety of foods and beverages, such as coffee, tea, chocolate and energy drinks, with coffee being the primary dietary caffeine source in Europe and the United States (Reyes and Cornelis, 2018; Erblang *et al.*, 2019; Samoggia and Rezzaghi, 2021).

The pharmacokinetics and pharmacodynamics of caffeine have been widely studied; more than 95% of caffeine biotransformation to its main metabolites paraxanthine, theophylline and theobromine, occurs in the liver via the CYP1A2 enzyme (Lelo *et al.*, 1986; Nehlig, 2018). At the cellular level, caffeine blocks A1 and A2a adenosine receptors in the brain, competitively antagonising their binding with adenosine, a neuromodulator that promotes sleep and suppresses arousal, thereby triggering dopaminergic neurotransmission and promoting wakefulness (Cappelletti *et al.*, 2015; Urry and Landolt, 2015; Nehlig, 2018).

Caffeine has notable enhancing properties in cognitive function and physical performance, which explain its popularity, especially in shift workers, students, athletes and anyone generally seeking to overcome fatigue or prolong their capacity to complete everyday activities (Cappelletti *et al.*, 2015; Renda *et al.*, 2015; Carswell *et al.*, 2020). Apart from exerting locomotor activity stimulation in the CNS, caffeine has also been reported to possess anxiogenic properties for some individuals (Alsene *et al.*, 2003; Rogers *et al.*, 2010).

Although the stimulant and anxiogenic properties of caffeine have been known for over a century and are widely accepted, research on its specific effects on the brain remains controversial (James, 2014; Cappelletti *et al.*, 2015). In fact, a review showed mixed results from intervention studies; while some report that caffeine improves simple cognitive functions in doses 32-300 mg, some others have failed to find significant effects (McLellan, Caldwell and Lieberman, 2016). Results from a systematic review of observational studies are also inconsistent, with only a few studies showing an association between caffeine and cognition and more recent studies detecting associations only among women or for specific exposures (Beydoun *et al.*, 2014).

Inconsistent findings may reflect methodological pitfalls commonly seen with dietary exposures (Smith, 2002). Observational studies may be biased by misclassification of caffeine exposure due to the use of self-reported data and measures of caffeine-containing foods and drinks (Smith, 2002; James, 2014). In clinical trials, stimulant properties of caffeine may reflect restoration of brain function impaired by caffeine withdrawal (Rogers, 2014). Indeed, participants in randomised studies are often asked to abstain from caffeine overnight, which may hinder cognitive performance in caffeine consumers (Rogers, 2014; Wilhelmus *et al.*, 2017). Additionally, nutrigenetics research has also found a considerable interindividual variability in the magnitude of caffeine effects, or in the lack of an effect when compared to placebo, suggesting that the inconsistencies in previous findings are, at least in part, due to genetic variations (Nehlig, 2018).

Growing evidence from genetic studies has associated the interindividual differences to caffeine response with variations in *CYP1A2* and *ADORA2A* genes (Cornelis *et al.*, 2011; Nehlig, 2018). The rs762551 SNP in the *CYP1A2* gene has been shown to affect CYP1A2 enzyme activity and has been used to identify

individuals as 'fast' or 'slow' caffeine metabolisers (Butler *et al.*, 1992; Sachse *et al.*, 1999). Further, it has been hypothesised that variations in the *ADORA2A* gene such as the rs5751876 may impact caffeine-adenosine A2a receptor binding and thus, downstream dopaminergic neurotransmission (Alsene *et al.*, 2003). This may lead to anxiogenic effects following caffeine consumption and individuals can be categorised as having a 'high' or 'low' sensitivity to caffeine (Alsene *et al.*, 2003; Nehlig, 2018).

With such widespread consumption of caffeine, the consequences of this stimulant on human health are of particular interest not only to the scientific community but also to the majority of adult population worldwide. To the authors' knowledge, there is currently no systematic review focusing on the associations between brain-related outcomes and SNPs related to physiological response to caffeine and its metabolism. A deeper understanding on this topic may provide a basis for further interdisciplinary approaches and personalised recommendations. Therefore, the purpose of the present systematic review was to identify, evaluate and discuss the current evidence on the associations between common genetic variations, caffeine and brain-related outcomes in humans, including indices of cognition, anxiety and insomnia.

2.2. Materials and methods

2.2.1. Search strategy

This systematic review was guided by the Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA) guidelines and was registered with PROSPERO (CRD42021257556). PubMed and Embase databases were independently searched for relevant reports by two investigators until 21 April 2021. The search strategy (Table 2.1) was decided based on consensus and the records identified from both databases were uploaded on Covidence software (Babineau, 2014). To identify possible eligible reports that were not identified by the initial search, a manual search of reference lists of included studies was also conducted.

Table 2. 1 Search strategy.

Search	Search Terms	PubMed	Embase
1	exp caffeine/ "caffeine"[MeSH Terms]	23,648	24,341
2	caffein*: ab,ti/ "caffein*"[Title/Abstract]	30,382	36,557
3	#1 OR #2 "caffeine"[MeSH Terms] OR "caffein*"[Title/Abstract]	35,352	54,007
4	gene*: ab,ti/ "gene*"[Title/Abstract]	5,314,513	6,540,751
5	genotype*: ab,ti/ "genotype*"[Title/Abstract]	279,282	372,639
6	polymorphism*: ab,ti/ "polymorphism*"[Title/Abstract]	278,762	341,559
7	varia*: ab,ti/ "varia*"[Title/Abstract]	2,187,945	2,810,360
8	#4 OR #5 OR #6 OR #7 "gene*"[Title/Abstract] OR "genotype"[Title/Abstract] OR "polymorphism"[Title/Abstract] OR "varia*"[Title/Abstract]	6,885,000	8,551,311
9	exp cognition/ "cognition"[MeSH Terms]	167,428	2,411,977
10	cogniti*: ab,ti/ "cogniti*"[Title/Abstract]	424,247	583,924
11	exp anxiety/ "anxiety"[MeSH Terms]	89,979	225,399
12	anxi*: ab,ti/ "anxi*"[Title/Abstract]	225,543	319,636
13	exp insomnia/ "sleep initiation and maintenance disorders"[MeSH Terms]	14,023	71,461
14	insomnia: ab,ti/ "insomnia*"[Title/Abstract]	22,860	38,641
15	exp sleep/ "sleep"[MeSH Terms]	82,223	246,492
16	sleep: ab,ti/ "sleep"[Title/Abstract]	176,002	265,948
17	exp mood/ "affect"[MeSH Terms]	34,318	45,501
18	mood: ab,ti/"mood"[Title/Abstract]	78,195	113,937
19	exp memory/ "memory"[MeSH Terms]	140,239	311,406
20	memory: ab,ti/ "memory"[Title/Abstract]	265,213	335,187
21	exp attention/ "attention"[MeSH Terms]	80,424	270,844
22	attention: ab,ti/ "attention"[Title/Abstract]	437,769	553,826
23	exp decision making/ "decision making"[MeSH Terms]	208,922	395,308
24	“decision making”: ab,ti/ "decision making"[Title/Abstract]	150,324	197,671
25	exp reaction time/ "reaction time"[MeSH Terms]	99,990	85,756
26	“reaction time”: ab,ti/ "reaction time"[Title/Abstract]	32,902	40,680
27	exp startle response/ "reflex, startle"[MeSH Terms]	6,569	7,780
28	“startle response”: ab,ti/ "startle response"[Title/Abstract]	2,914	3,432
29	alertness: ab,ti/ "alertness"[Title/Abstract]	6,786	9,640
30	reasoning: ab,ti/ "reasoning"[Title/Abstract]	24,031	28,332

31	#9 OR #10 OR #11 OR #12 OR #13 OR #14 OR #15 OR #16 OR #17 OR #18 OR #19 OR #20 OR #21 OR #22 OR #23 OR #24 OR #25 OR #26 OR #27 OR #28 OR #29 OR #30 "cognition"[MeSH Terms] OR "cogniti*"[Title/Abstract] OR "anxiety"[MeSH Terms] OR "anxi*"[Title/Abstract] OR "sleep initiation and maintenance disorders"[MeSH Terms] OR "insomnia"[Title/Abstract] OR "sleep"[MeSH Terms] OR "sleep"[Title/Abstract] OR "affect"[MeSH Terms] OR "mood"[Title/Abstract] OR "memory"[MeSH Terms] OR "memory"[Title/Abstract] OR "attention"[MeSH Terms] OR "attention"[Title/Abstract] OR "decision making"[MeSH Terms] OR "decision making"[Title/Abstract] OR "reaction time"[MeSH Terms] OR "reaction time"[Title/Abstract] OR "reflex, startle"[MeSH Terms] OR "startle response"[Title/Abstract] OR "alertness"[Title/Abstract] OR "reasoning"[Title/Abstract]	1,865,486	3,777,432
32	#3 AND #8 AND #31 ("caffeine"[MeSH Terms] OR "caffein*"[Title/Abstract]) AND ("gene*"[Title/Abstract] OR "genotype"[Title/Abstract] OR "polymorphism*"[Title/Abstract] OR "varia*"[Title/Abstract]) AND ("cognition"[MeSH Terms] OR "cogniti*"[Title/Abstract] OR "anxiety"[MeSH Terms] OR "anxi*"[Title/Abstract] OR "sleep initiation and maintenance disorders"[MeSH Terms] OR "insomnia"[Title/Abstract] OR "sleep"[MeSH Terms] OR "sleep"[Title/Abstract] OR "affect"[MeSH Terms] OR "mood"[Title/Abstract] OR "memory"[MeSH Terms] OR "memory"[Title/Abstract] OR "attention"[MeSH Terms] OR "attention"[Title/Abstract] OR "decision making"[MeSH Terms] OR "decision making"[Title/Abstract] OR "reaction time"[MeSH Terms] OR "reaction time"[Title/Abstract] OR "reflex, startle"[MeSH Terms] OR "startle response"[Title/Abstract] OR "alertness"[Title/Abstract] OR "reasoning"[Title/Abstract])	1,097	2,587
33	exp animals/ "animals"[MeSH Terms]	23,912,226	
34	exp humans/ "humans"[MeSH Terms]	19,109,594	
35	#33 NOT #34 "animals"[MeSH Terms] NOT "humans"[MeSH Terms]	4,802,632	
36	#32 NOT #35 (("caffeine"[MeSH Terms] OR "caffein*"[Title/Abstract]) AND ("gene*"[Title/Abstract] OR "genotype"[Title/Abstract] OR "polymorphism*"[Title/Abstract] OR "varia*"[Title/Abstract]) AND ("cognition"[MeSH Terms] OR "cogniti*"[Title/Abstract] OR "anxiety"[MeSH Terms] OR "anxi*"[Title/Abstract] OR "sleep initiation and maintenance disorders"[MeSH Terms] OR "insomnia"[Title/Abstract] OR "sleep"[MeSH Terms] OR "sleep"[Title/Abstract] OR "affect"[MeSH Terms] OR "mood"[Title/Abstract] OR "memory"[MeSH Terms] OR "memory"[Title/Abstract] OR "attention"[MeSH Terms] OR "attention"[Title/Abstract] OR "decision making"[MeSH Terms] OR "decision making"[Title/Abstract] OR "reaction time"[MeSH Terms] OR "reaction time"[Title/Abstract] OR "reflex, startle"[MeSH Terms] OR "startle response"[Title/Abstract] OR "alertness"[Title/Abstract] OR "reasoning"[Title/Abstract])) NOT ("animals"[MeSH Terms] NOT "humans"[MeSH Terms])	950	("limit 33 to humans") 2,065

2.2.2. Study selection

Two reviewers selected records for inclusion in the systematic review by independently a) screening records by title and abstract and b) reviewing full texts, according to predetermined inclusion and exclusion criteria. Records that met the population, intervention, comparison, outcome, and study design (PICOS) criteria were eligible for inclusion (Page *et al.*, 2021) and are shown in Table 2.2. The included populations involved healthy adult participants above 18 years of age. Interventions included a) habitual caffeine intake and caffeine supplementation, reviewed separately and b) genotyping for polymorphisms in genes associated with caffeine metabolism and effect reported as alleles, haplotypes or genetic scores. Comparators comprised a) different levels of habitual caffeine intake, different doses of caffeine supplementation and placebo and b) the variant allele compared with the ancestral allele, risk haplotypes and different genetic scores.

Table 2. 2 PICOS criteria for inclusion and exclusion of studies.

Parameter	Inclusion criteria
Population	Healthy adults above 18 years old.
Intervention	a) habitual caffeine intake and acute caffeine supplementation. All caffeine doses reported as grouped variables or continuous variables were considered. b) genotyping for polymorphisms in genes associated with caffeine metabolism and physiological effects in humans. All genetic data reported as alleles, haplotypes or genetic scores were considered.
Comparator	a) different levels of habitual caffeine intake, different doses of caffeine supplementation and placebo supplementation. b) the variant allele, risk haplotype and different genetic scores.
Outcome	a) mood and anxiety (subjective measures and startle response). b) sleep disturbance/insomnia (subjective measures of sleep quality and duration and polysomnography). c) cognitive performance (measures of attention, reaction time, memory, alertness, decision making, reasoning).
Study design	All observational and experimental studies.

The outcomes included brain-related effects such as mood and anxiety, insomnia and sleep deprivation, as well as measures of cognition such as RTs, alertness, attention, decision making and reasoning. All observational and experimental trials were included. Reviewers were blinded to each other's decisions

and disagreements between individual judgements were resolved based on consensus.

2.2.3. Data extraction

In the present systematic review, outcome data were extracted only from participants for whom both genetic and caffeine intake/supplementation information was available. Data extraction was performed independently by two investigators and conflicts were resolved by consensus. For all included studies, extracted information included the first author's name, year of publication, study design, participant characteristics (i.e., number, sex, age and intervention), the genetic variant(s) under study, as well as the main and secondary brain-related effects, including results from statistical analyses. Extracted data were grouped based on the study outcomes.

2.2.4. Risk of bias assessment

The risk of bias of included studies was assessed independently by two reviewers following the Cochrane Review guidelines and conflicts were resolved by consensus. The risk of bias in interventions was assessed using the revised tool for assessing risk of bias in randomised trials (RoB-2). The parallel group and crossover RoB-2 tools were used based on study design and reviewers rated each study on domain level and overall risk of bias as 'low', 'high', or 'some concerns' (Sterne *et al.*, 2019). For observational studies, the ROBINS-I (Risk Of Bias In Non-randomised Studies of interventions - NRSIs) risk of bias tool was used and reviewers rated each study on domain level and overall, as 'low', 'moderate', 'serious', 'critical', or 'unclear' risk of bias (Sterne *et al.*, 2016).

2.3. Results

2.3.1. Search procedure

The search yielded 3,021 records. After removing duplicates (n = 733), 2,228 records were screened by title and abstract. A total of 42 reports were assessed by full text for eligibility, with 22 reports of 19 independent studies meeting the inclusion criteria. A detailed mapping of the records identified, included

and excluded, as well as the reasons for exclusions is shown in the PRISMA flow diagram in Figure 2.1.

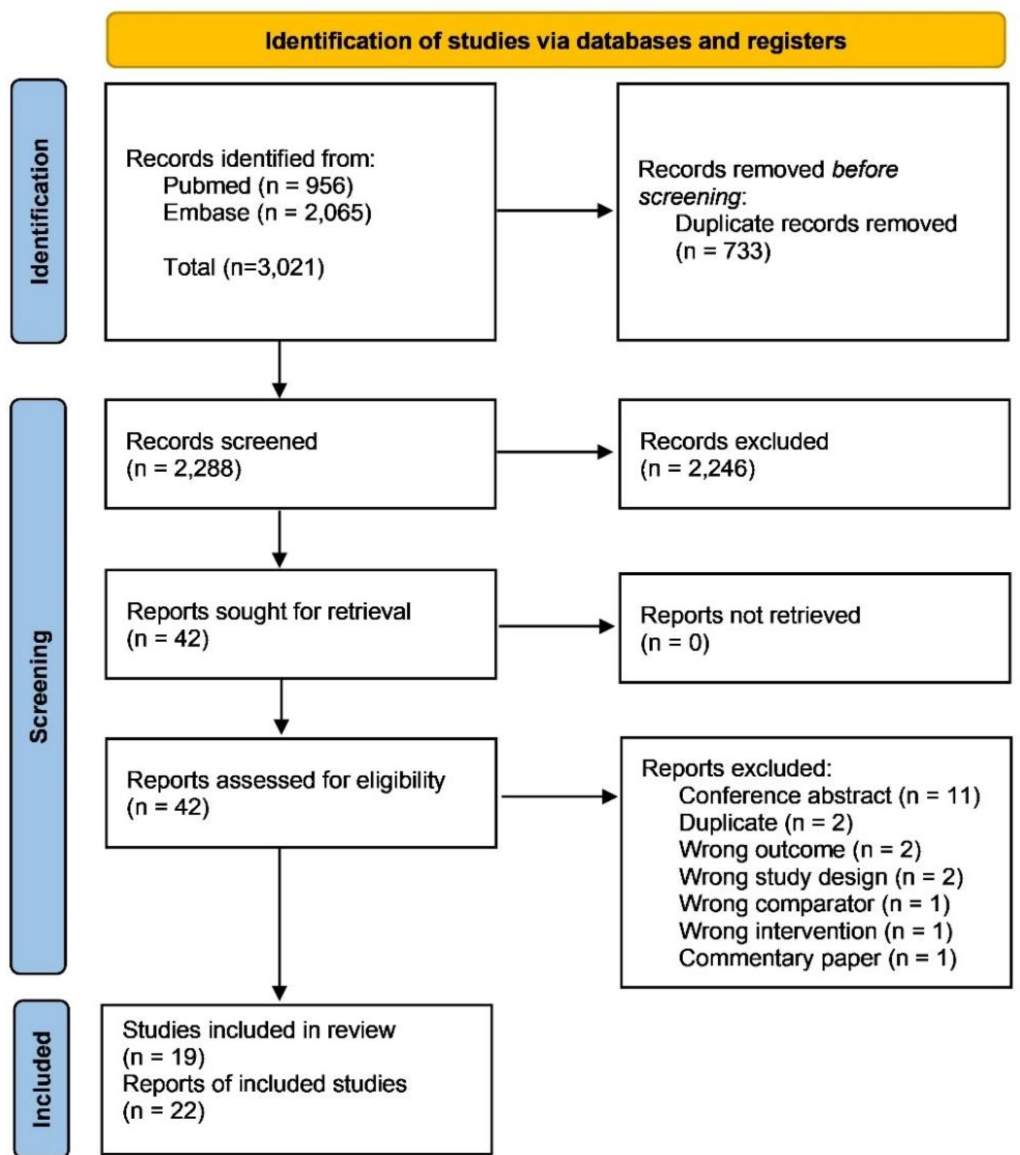

Figure 2. 1 The PRISMA flow diagram. Presentation of the procedure of literature search and selection with numbers of records at each stage.

2.3.2. Characteristics of included studies

The characteristics of the included studies are presented in Tables 2.3 – 2.5. Of the included 22 records, nine were crossover randomised controlled trials (RCTs)

(41%), six were parallel-group RCTs (27%), six were cross-sectional studies (27%) and one was a GWAS (5%). In these studies, 21 SNPs in 13 genes were identified, while two studies used a genetic score for caffeine metabolism based on two SNPs and one study assessed haplotypes including multiple variants instead of individual SNPs.

Table 2. 3 Summary of records identified from systematic review of genetic studies on caffeine and cognitive performance, cognitive performance during sleep deprivation and cognitive performance during and post-exercise.

Authors	Study design	No of participants - All* (M / F)	Age (years, range / mean ± SD)	Region, Ethnicity/ Nationality	Gene - SNP(s)	MAF	HWE met	Intervention / exposure	Outcome (measure)	Result
					cognitive performance					
Casiglia *et al.* (2017)	Cross-sectional	1,374 (601 / 773)	51.4 ± 15.3	Italy, Unknown	*CYP1A2* rs762551	0.33	yes	tertiles of habitual caffeine intake	Abstract reasoning (scores)	There was a significant genotype x caffeine interaction (*p* = 0.04). The CC homozygotes had significantly higher mean (SD) abstract reasoning in the 3d tertile of habitual caffeine intake (4.37 ± 0.24) compared to the 1st (3.39 ± 0.24; *p* < 0.005) and 2nd tertiles (3.49 ± 0.23; *p* < 0.01). Abstract reasoning in the A carriers was independent of caffeine intake (p > 0.05 for all tertiles of habitual caffeine intake).
Cornelis *et al.* (2020a)	Cross-sectional	295,492 (137,567 / 157,925)	37 – 73	UK, white British	rs6968554 (near *AHR*) rs2472297 (near *CYP1A2*) *CYP1A2* rs762551	NE NE NE	NA NA NA	caffeine vs no caffeine intake within the last h prior to tests	Prospective memory** (scores) Pairs matching (n errors) FI** (n correct) Vigilance/RT (ms)	No significant CMSG x recent caffeine drinking interactions on cognitive function were found. Recent caffeine drinking was associated with increasing RT performance (*β* = -9.02, CI: -14.15, -3.89, *p* < 0.0006) and with decreasing Pairs Matching performance (*β* = 0.05, CI: 0.01, 0.08, *p* < 0.004) with increasing CMSG score.
									Prospective memory** (scores)	Caffeine and tea intake were associated with decrements in FI performance among those with higher CMSG (*p* < 0.0003), but no

Authors	Study design	No of participants - All* (M / F)	Age (years, range / mean ± SD)	Region, Ethnicity/ Nationality	Gene - SNP(s)	MAF	HWE met	Intervention / exposure	Outcome (measure)	Result
Cornelis *et al.* (2020b)	Cross-sectional	320,333 (147,332 / 173,001)	37 – 73	UK, white British	rs2472297 (near *CYP1A2*) rs6968554 (near *AHR*) *CYP1A2* rs762551	NE NE NE	NA NA NA	0 vs < 1 vs 1 vs 2–3 vs 4–5 vs 6–7 vs ≥ 8 cups of habitual coffee or tea intake / day	Pairs matching (n errors) FI** (n correct) Vigilance/RT (ms) SDS (n correct) Trail Making Test A and B RT (ms)	significant CMSG × caffeine/tea interactions were observed. The AA genotype of the rs762551 presented with greater decrements in performance in pairs matching with higher coffee intake than those with CC or AC genotypes (p <0.0001); however, no significant rs762551 × coffee interaction was found.
Renda *et al.* (2015)	Crossover RCT	106 (106 / 0) low / moderate caffeine consumers	18-40	Italy, Unknown	*ADORA2A* rs5751876 *AMPD1* rs17602729 *ADRA1A* rs1048101 *ADRA2B* rs29000568 *ADRB1* rs1801252 *ADRB1* rs1801253 *ADRB2* rs1042713 *ADRB2* rs1042714 *ADRB2* rs1800888 *ADRB3* rs4994	NE NE NE NE NE NE NE NE NE NE	yes yes yes yes yes yes yes yes yes yes	3 mg/kg body mass of caffeine vs placebo	Alerting (ms) Orienting (ms) Verbal executive control (ms) Motor executive control (ms)	The CC homozygotes of the *ADORA2A* rs5751876 showed a significantly higher RT performance in orienting (ΔRT = 5.8 ms [CI: 0.5;11.0], p = 0.033), while the TT homozygotes showed higher RT performance in motor executive control (ΔRT = 19.2 ms [CI: 6.1; 29.4], p = 0.005) after caffeine compared to placebo. No other gene x caffeine interactions were identified.

Authors	Study design	No of participants - All* (M / F)	Age (years, range / mean ± SD)	Region, Ethnicity/ Nationality	Gene - SNP(s)	MAF	HWE met	Intervention / exposure	Outcome (measure)	Result
Salinero *et al.* (2017)	Crossover RCT	21 (14 / 7) light caffeine consumers	28.9 ± 7.3	Spain, Unknown	*CYP1A2* rs762551	NE	NA	3 mg/kg body mass of caffeine vs placebo	Vigilance: Mean RT (ms) Stage 1, 2, 3, 4 RT (ms)	There were no differences between genotype groups (AA homozygotes and C allele carriers) in any variable measured during the visual attention test.
cognitive performance & sleep deprivation										
Baur *et al.* (2021)	Parallel group RCT	26 (14 / 12)	20-40	Switzerland, Western Europeans	*ADORA2A* rs5751876 CC homozygous	NA	NA	400 + 200 g regular coffee containing 300 mg caffeine vs decaffeinated coffee	Vigilance: Speed (s-1) Lapses (n) Accuracy (%) LSNR (db) Visual search: Speed target present (s-1) Speed target absent (s-1) Accuracy (%)	Vigilance The impairment in speed, lapses, and accuracy on the PVT after sleep deprivation was attenuated in the regular coffee group when compared to the decaffeinated coffee group ('day' x 'group' interactions: speed: F (6, 672) = 7.72; lapses: F (6, 672) = 3.69; accuracy: F (6, 672) = 4.52; $p_{all} < 0.001$). The LSNR was higher than in the decaffeinated coffee group on restriction days 1 through 3 ('day' x 'group' interactions: F (6, 672) = 9.54, $p < 0.001$. Visual search The regular coffee group performed faster when the target was present ('day' x 'group' interaction: F (6, 2759) = 3.08, $p = 0.005$) on day 5 and when the target was absent ('day' x 'group' interaction: F (6, 2759) = 4.83, $p < 0.001$) on days 4 and 5 and more accurately throughout sleep restriction ('day' x 'group' interaction: F (6, 672) =

Authors	Study design	No of participants - All* (M / F)	Age (years, range / mean ± SD)	Region, Ethnicity/ Nationality	Gene - SNP(s)	MAF	HWE met	Intervention / exposure	Outcome (measure)	Result
									Working memory & executive control: 1, 2 and 3-back speed (s-1) 1, 2 and 3-back accuracy (%)	4.35, $p < 0.001$) compared to the decaffeinated coffee group. Visuo-spatial working memory & executive control The regular coffee group performed faster and more accurately than the decaffeinated coffee group on most days during sleep restriction ('day' x 'group' interaction: speed: F (6, 2062) = 9.52; accuracy: F (6, 2062) = 5.13; $p_{all} < 0.001$). Verbal working memory & executive control The regular coffee group performed faster and more accurately than the decaffeinated coffee group on all 3 workload levels on sleep restriction days 1 through 4 ('day' x 'group' interaction: speed: F (6, 2065) = 8.11; accuracy: F (6, 2062) = 4.23; $p_{all} < 0.001$).
Bodenmann *et al.* (2012)	Crossover RCT	45 (45 / 0)	20-30	Switzerland, Caucasian	HT4 and non-HT4 *ADORA2A* haplotypes including 8 variants: rs5751862 rs5760405 rs2298383 rs3761422	NE	yes	2 x 200 mg caffeine vs placebo	Vigilance: Speed (s-1) z scores	A significant haplotype x caffeine x session effect was found (F (26, 219) = 2.1, $p < 0.003$). Response speed scores were higher after caffeine compared with placebo in non-HT4 haplotype carriers of *ADORA2A* only after 15, 21, 24, 27 and 30h of wakefulness ($p_{all} < 0.05$).

Authors	Study design	No of participants - All* (M / F)	Age (years, range / mean ± SD)	Region, Ethnicity/ Nationality	Gene - SNP(s)	MAF	HWE met	Intervention / exposure	Outcome (measure)	Result
					rs2236624 rs5751876 rs35320474 rs4822492					
Skeiky *et al.*, (2020)	Crossover RCT	12 (6 / 6)	27.4 ± 6.9	US, Unknown	*TNFα* rs1800629	NE	yes	200 mg caffeine vs 300 mg caffeine vs placebo	Vigilance: LSNR (db)	A non-significant genotype x caffeine effect was observed $F(2, 20) = 0.21$, $p = 0.81$). No differences in performance between A allele carriers and GG homozygotes after 200 or 300 mg caffeine intake compared to placebo ($p_{all} > 0.05$) during 48 h of TSD.
cognitive performance & exercise										
Carswell *et al.* (2020)	Crossover RCT	18 (12 / 6) active individuals	24.0 ± 4.0	UK, Unknown	*ADORA2A* rs5751876 *CYP1A2* rs762551	NE NE	NA NA	3 mg/kg body mass of caffeine vs placebo	Vigilance: Δspeed (s-1) ΔRT (ms) Δlapses (n) Δslowest 10% response speed (s-1) Δfastest 10% RT (ms)	'Fast' metabolisers showed lower ΔRT scores ($p < 0.01$, Cohen's d = 1.6) and higher Δspeed and Δslowest 10% response speed ($p < 0.01$, Cohen's d = 1.5 and 1.9, respectively) during exercise and lower Δfastest 10% RT and Δlapses at rest ($p < 0.05$, Cohen's d = 1.1 and $p < 0.01$, Cohen's d = 1.7, respectively) after caffeine compared with 'slow' metabolisers. No differences emerged between *ADORA2A* genotypes during exercise or at rest ('high' vs 'low' sensitivity; $p_{all} > 0.05$).

M: male; F: female; SD: Standard deviation; SNPs: Single Nucleotide Polymorphisms; MAF: Minor Allele Frequency; HWE: Hardy Weinberg Equilibrium; NE: Not estimable based on data in published work: not distinct genotypes but genetic scores were used for analyses; NA: Not available; *individuals for whom both genetic data & data on caffeine intake were available; RT: Reaction Time; ** added part-way through the baseline assessment period; FI: Fluid Intelligence;

SDS: Symbol Digit Substitution; CI: Confidence Interval; CMSG: genetic caffeine metabolism score, derived by summing the number of single-nucleotide polymorphism alleles multiplied by their β-coefficients and recalibrated such that it ranged from 0 to 4, with higher scores predicting faster caffeine metabolism; ΔRT: difference in RT; PVT: Psychomotor Vigilance Task; LSNR: Log of the signal-to noise ratio; Δspeed: difference in speed, Δlapses: difference in number of lapses, Δslowest 10% response speed: difference in the slowest 10% response speed, Δfastest 10% RT: difference in fastest 10% RT; TSD: Total sleep deprivation.

Table 2. 4 Summary of records identified from systematic review of genetic studies on caffeine and anxiety.

Authors	Study design	No of participants - All* (M / F)	Age (years, range / mean ± SD)	Region, Ethnicity/ Nationality	Gene - SNP(s)	MAF	HWE met	Intervention / exposure	Outcome (measure)	Result
Alsene *et al.* (2003)	Crossover RCT	94 (51 / 43) no / low caffeine consumers	early 20s	US, Mixed - White (54), Black (15), Asian (20), Hispanic (5)	*ADORA2A* rs5751876 *ADORA2A* rs2298383	0.14 0.48	yes yes	150 mg caffeine vs placebo	POMS & VAS subjective anxiety (scores)	Only the TT group of the rs5751876 and rs2298383 polymorphism locus reported a significant increase in mean (SEM) anxiety after caffeine compared to placebo ($p < 0.05$) and this increase was significantly higher compared to the CC and CT genotype groups (POMS: 2.91 ± 0.59 vs -0.06 ± 0.59 vs 0.54 ± 0.41, respectively and VAS: 0.17 ± 0.04 vs -0.02 ± 0.05 vs 0.05 ± 0.05, respectively; $p_{all} < 0.05$).
Childs *et al.* (2008)	Crossover RCT	62 (32 / 30) no / low caffeine consumers	20.7 ± 0.3	US, subset of European-Americans	*ADORA2A* rs5751876 *ADORA2A* rs2298383 *ADORA2A* rs4822492 *DRD2* rs1110976	0.41 0.44 0.44 0.14	yes yes yes yes	150 mg caffeine vs placebo	VAS subjective anxiety (scores)	Individuals with the CC genotype at the rs2298383 and rs4822492 reported significantly higher mean (SEM) peak change in anxiety after caffeine compared with baseline than those with the TT genotype at the rs2298383 locus and GG at the rs4822492 locus (11.25 ± 5.09 vs -8.19 ± 3.51 and 11.32 ± 5.16 vs -8.19 ± 3.51, respectively: $p_{all} < 0.007$). Individuals with the G/− genotype of the *DRD2* rs1110976 reported higher mean (SEM) peak change in anxiety after caffeine compared with baseline than G/G individuals (4.34 ± 3.92 vs -2.78 ± 2.15; $p = 0.005$). No significant differences were observed among

Authors	Study design	No of participants - All* (M / F)	Age (years, range / mean ± SD)	Region, Ethnicity/ Nationality	Gene - SNP(s)	MAF	HWE met	Intervention / exposure	Outcome (measure)	Result
										the *ADORA2A* rs5751876 genotypes.
Domschke *et al.* (2012a)	Parallel group RCT	110 (56 / 54)	18-50	Germany, Caucasian	*ADORA2A* rs5751876	NE	NA	150 mg caffeine vs placebo	POMS & VAS subjective anxiety (scores) startle magnitude	Women but not men showed a significant genotype x caffeine interaction for unpleasant pictures F (1, 46) = 6.83, p = 0.01) with higher startle magnitudes for TT risk genotype in the caffeine condition and higher startle magnitudes in non-risk CC/CT genotypes in the placebo condition. There was a significant genotype x intervention interaction on POMS 'Depression – Anxiety' ratings: F (2, 212) = 5.25, p = 0.02), but no significant genotype x intervention on VAS ratings: all F (2, 212) = 1.28, p > 0.28.
Domschke *et al.* (2012b)	Parallel group RCT	116 (57 / 59)	18-50	Germany, Caucasian	*NPSR* rs324981	0.45	yes	150 mg caffeine vs placebo	startle magnitude	A significant interaction between picture valence, *NPSR* genotype, and challenge condition (F (2,216) = 3.61, p = 0.03) was identified. TT genotype had increased mean (SEM) startle magnitude in response to neutral stimuli (51.49 ± 0.43 vs 49.67 ± 0.53, $p \leq 0.05$) and a decrease in startle magnitude in response to unpleasant stimuli (49.81 ± 0.52 vs 51.78 ± 0.58, $p \leq 0.05$) in caffeine compared to

Authors	Study design	No of participants - All* (M / F)	Age (years, range / mean ± SD)	Region, Ethnicity/ Nationality	Gene - SNP(s)	MAF	HWE met	Intervention / exposure	Outcome (measure)	Result
										placebo condition, respectively. No change was observed in AA/AT genotypes.
Gajewska *et al.* (2013)	Parallel group RCT	114 (57 / 57) low / moderate caffeine consumers	26.6 ± 6.2	Germany, Caucasian	*ADORA2A* rs5751876	NE	NA	150 mg caffeine vs placebo	Prepulse modification (%)	A significant genotype × intervention × gender × SOA on the % startle inhibition was observed, *F* (4,424) = 4.48, *p* = 0.001. The TT genotype women reacted with a reduced % prepulse inhibition compared to TT genotype men in response to caffeine at 120 ms SOA: 32.85 % vs 66.41%, respectively; *t* 26 = 2.26, *p* = 0.03 and 240 ms SOA: -4.55 % vs 39.41 %, respectively; *t* 26 = 2.63, *p* = 0.01). No significant effects were observed between genotype groups.
Klauke *et al.* (2012)	Parallel group RCT	90 (45 / 45)	26.5 ± 6.2	Germany, European	*COMT* Val158Met	0.4	yes	150 mg caffeine vs placebo	startle magnitudes	No significant genotype x caffeine interaction was found and no differences in affect-modulated startle responses after caffeine based on the *COMT* Val158Met polymorphism.
Rogers *et al.* (2010)	Parallel group RCT	379 (180 / 199)	18-62		*ADORA2A* rs5751876	0.39	yes	100 + 150 mg caffeine vs placebo		A significant genotype x caffeine interaction was found, *F* (2, 365) = 6.57, *p* = 0.002**. The TT genotype significantly increased mean (SEM) anxiety after 100 mg

Authors	Study design	No of participants - All* (M / F)	Age (years, range / mean ± SD)	Region, Ethnicity/ Nationality	Gene - SNP(s)	MAF	HWE met	Intervention / exposure	Outcome (measure)	Result
				UK, Mixed - (95%) white Europeans					MAPSS subjective anxiety	caffeine compared to placebo (TT: 1.65 ± 0.15 vs CT/CC: 0.95 ± 0.17, $p < 0.01$). When considering habitual caffeine consumption, caffeine increased mean (SEM) anxiety only in non- and low consumers in all genotype groups (TT: 1.76 ± 0.18, $p < 0.05$ vs CT/CC: 1.1 ± 0.06, $p < 0.01$) after both caffeine sessions (100 + 150 mg).

M: male; F: female; SD: Standard deviation; SNPs: Single Nucleotide Polymorphisms; MAF: Minor Allele Frequency; HWE: Hardy Weinberg Equilibrium; NE: Not estimable based on data in published work; NA: Not available; *individuals for whom both genetic data & data on caffeine intake were available; SEM: Standard Error of the Mean; POMS: Profile of Mood States; VAS: Visual Analog Scale; Startle magnitudes: the difference between the highest peak 21–200ms after and the average during 50ms before startle probe presentation (anxiety-relevant, neutral, or pleasant picture); Prepulse modification (%): percent difference of the startle magnitude due to the preceding prepulse compared to control startle trials with positive values indicating prepulse inhibition of the startle response (PPI) and negative values indicating prepulse facilitation of the startle response (PPF); SOAs: Stimulus Onset Asynchronies; MAPSS: Mood, Alertness and Physical Sensations Scale; ** After 100 mg caffeine and baseline anxiety included as covariate.

Table 2. 5 Summary of records identified from systematic review of genetic studies on caffeine and sleep disturbance and insomnia.

Authors	Study design	No of participants- All* (M / F)	Age (years, range / mean ± SD)	Region, Ethnicity/ Nationality	Gene - SNP(s)	MAF	HWE met	Intervention / exposure	Outcome (measure)	Result
Byrne *et al.* (2012)	GWAS	2,110 (543 / 1,567)	NA	Australia, Caucasian	2,380,486 SNPs	NA	NA	drinking coffee in the evening	having ever experienced caffeine-induced insomnia vs having never experienced caffeine-induced insomnia	No SNPs reached the genome-wide significance level (7.2×10^{-8}) for caffeine-induced insomnia. Association analysis after adjusting for age, sex and insomnia factor score identified 8 loci related to caffeine-induced insomnia: rs521704 near the *GBP4* gene ($p = 1.9 \times 10^{-6}$, OR [95% CI] = 0.70 [0.62 – 0.78]); rs13172305 near the *RP11-772E11.1* gene ($p = 3.40 \times 10^{-6}$, OR [95% CI] = 1.76 [1.39 - 2.24]); rs11878836 near the *AC008556.1* gene ($p = 3.40 \times 10^{-6}$, OR [95% CI] = 1.37 [1.10 - 1.70]); rs561042 near the *GBP4* gene ($p = 6.20 \times 10^{-6}$, OR [95% CI] = 0.77 [0.66 - 0.91]); rs12725617 near the *LPHN2* gene ($p = 7.30 \times 10^{-6}$, OR [95% CI] = 0.74 [0.61 - 0.90]); rs12407812 near the *GBP1* gene ($p = 8.90 \times 10^{-6}$, OR [95% CI] = 1.41 [1.21 - 1.64]); rs9665295 near the *NEBL* gene ($p = 9.20 \times 10^{-6}$, OR [95% CI] = 2.55 [1.68 - 3.87]) and rs2103117 near the *RP1-21O18.1* gene ($p = 9.80 \times 10^{-6}$, OR [95% CI] = 0.61 [0.49 - 0.76]).

Authors	Study design	No of participants- All* (M / F)	Age (years, range / mean ± SD)	Region, Ethnicity/ Nationality	Gene - SNP(s)	MAF	HWE met	Intervention / exposure	Outcome (measure)	Result
Erblang *et al.* (2019)	Cross-sectional	1,023 (618 / 405)	32.5 ± 9.6	France, European ancestry	six *ADORA2A* SNPs: rs5751862 rs2298383 rs3761422 rs2236624 rs5751876 rs4822492	 0.46 0.44 0.38 0.22 0.41 0.44	 yes yes yes yes yes yes	low (0-50 mg) vs moderate (51-300 mg) vs high (≥300 mg) habitual caffeine intake / day from all sources	TST (h) Sleep complaints (n, %)	Significant genotype x caffeine interactions were found for rs2298383, rs3761422, rs5751876 and rs4822492 ($p_{all} < 0.04$) for TST. Mean (95% CI) TST was lower in the CT and TT compared to CC group of the rs5751876 (7.05 ± 0.32 vs 6.92 ± 0.48 vs 7.53 ± 0.30, respectively) and in the CT and CC compared to TT group of the rs3761422 (7.00 ± 0.32 vs 6.85 ± 0.45 vs 7.56 ± 0.28, respectively), while it was higher in the TT vs CC genotype groups of the rs2298383 (7.52 ± 0.32 vs 6.93 ± 0.44) and the GG and GC compared to CC group of the rs4822492 (7.52 ± 0.30 vs 7.26 ± 0.32 vs 6.93 ± 0.44, respectively) only for low caffeine consumers. The risk (OR, 95% CI) of sleep complaints was lower in the CT compared to CC genotype group for rs5751876 (0.6, 0.4–0.9 vs 1) but it was higher in TT compared to CC for rs2298383 (1.5, 1.1–2.8 vs 1) and in GG compared to CC genotype group for rs4822492 (1.8, 1.1–2.9 vs 1) in moderate caffeine consumers. No other differences were shown between genotypes in any of the outcomes.

Authors	Study design	No of participants- All* (M / F)	Age (years, range / mean ± SD)	Region, Ethnicity/ Nationality	Gene - SNP(s)	MAF	HWE met	Intervention / exposure	Outcome (measure)	Result
Holst *et al.* (2014)	Crossover RCT	16 (16 / 0)	18-35	Switzerland, Caucasian	*DAT1* VNTR	NE	NA	2 x 200 mg caffeine vs placebo	Wakefulness EEG power (%) NREM sleep EEG power (%)	A significant genotype x caffeine interaction was found on beta activity (21-24 Hz), F (1,14) ≥ 4.25; $p_{all} < 0.05$). Caffeine administered during sleep deprivation enhanced beta (21–24 Hz) EEG activity in wakefulness compared to placebo (151.6% ± 9.5 vs placebo: 109.3% ± 9.7; $p <$ 0.05) in 10R/10R homozygotes, yet not in 9R allele carriers. No genotype differences were observed for NREM sleep.
Mazzotti *et al.* (2011)	Cross-sectional	958 (421 / 537)	42.6 ± 14.4	Brazil, European ancestry	*ADA* rs73598374	0.05	yes	0 vs ≥ 1 cup of caffeine-containing drinks	Lights off time (h:m) Lights on time (h:m) Sleep latency (min) REM sleep latency (min) TST (min) Sleep efficiency (%) Stage 1, 2 and 3-4 sleep (%) REM sleep (%) Minutes awake Arousals / h	Among caffeine consumers, A allele carriers showed lower mean (SD) sleep latency (12.41 min ± 15.26 vs 17.40 min ± 22.51 for non-carriers; p = 0.03), higher % sleep efficiency (84.93% ± 12.12 vs 81.52% ± 12.45 for non-carriers; p = 0.01), higher % REM sleep (20.77% ± 6.37 vs 18.95% ± 6.41 for non-carriers; p = .02), and fewer minutes awake (51.04 min ± 43.85 vs 61.04 min ± 44.62 for non-carriers; p = 0.04). Among those who did not consume caffeine, no differences were found between genotypes in any of the sleep parameters ($p_{all} > 0.05$).

Authors	Study design	No of participants- All* (M / F)	Age (years, range / mean ± SD)	Region, Ethnicity/ Nationality	Gene - SNP(s)	MAF	HWE met	Intervention / exposure	Outcome (measure)	Result
Nunes *et al.* (2017)	Cross-sectional	926 (412 / 514)	42.8 ± 14.6	Brazil, European ancestry	*ADORA2A* rs5751876	0.46	yes	0.2 ± 0.3 caffeine load**	Sleep latency (min) REM sleep (%) Stage 1, 2 and 3-4 sleep (%)	Significant correlations between caffeine load and sleep latency (r = 0.12; p = 0.003; β = 0.174), % stage 3-4 sleep (r =0.09; p = 0.022; β = -0.077), and % REM sleep (r = 0.08; p = 0.04; β = -0.00004) only in T allele carriers. No differences were found among genotype groups in any of the outcomes (p_{all} > 0.05).
Retey *et al.* (2007)	Crossover RCT	19 (19 / 0)	NA	Switzerland, Unknown	*ADORA2A* rs5751876	0.41	NA	2 x 200 mg caffeine vs placebo	non-REM sleep EEG power density (%)	The CC genotype displayed a greater rise in the EEG power in the beta band (16.625–20.125 Hz) after caffeine compared to the CT and TT genotypes: mean (SEM): 115.45% ± 3.09 vs 106.91% ± 2.98 vs 100% ± 5.00, respectively; p < 0.03.

M: male; F: female; SD: Standard deviation; SNPs: Single Nucleotide Polymorphisms; MAF: Minor Allele Frequency; HWE: Hardy Weinberg Equilibrium; NE: Not estimable based on data in published work; NA: Not available; GWAS: Genome Wide Association Study; *individuals for whom both genetic data & data on caffeine intake were available; OR: Odds Ratio; TST: Total Sleep Time; EEG: Electroencephalogram; REM: Rapid-Eye Movement; NREM: Non-REM; ** caffeine load: total number of cups taken divided by the number of hours since the last caffeine-containing beverage was consumed on the day of polysomnography.

2.3.3. Risk of bias assessment

The overall and domain risk of bias assessment results, as well as summaries of the results are displayed in Figures 2.2 – 2.5.

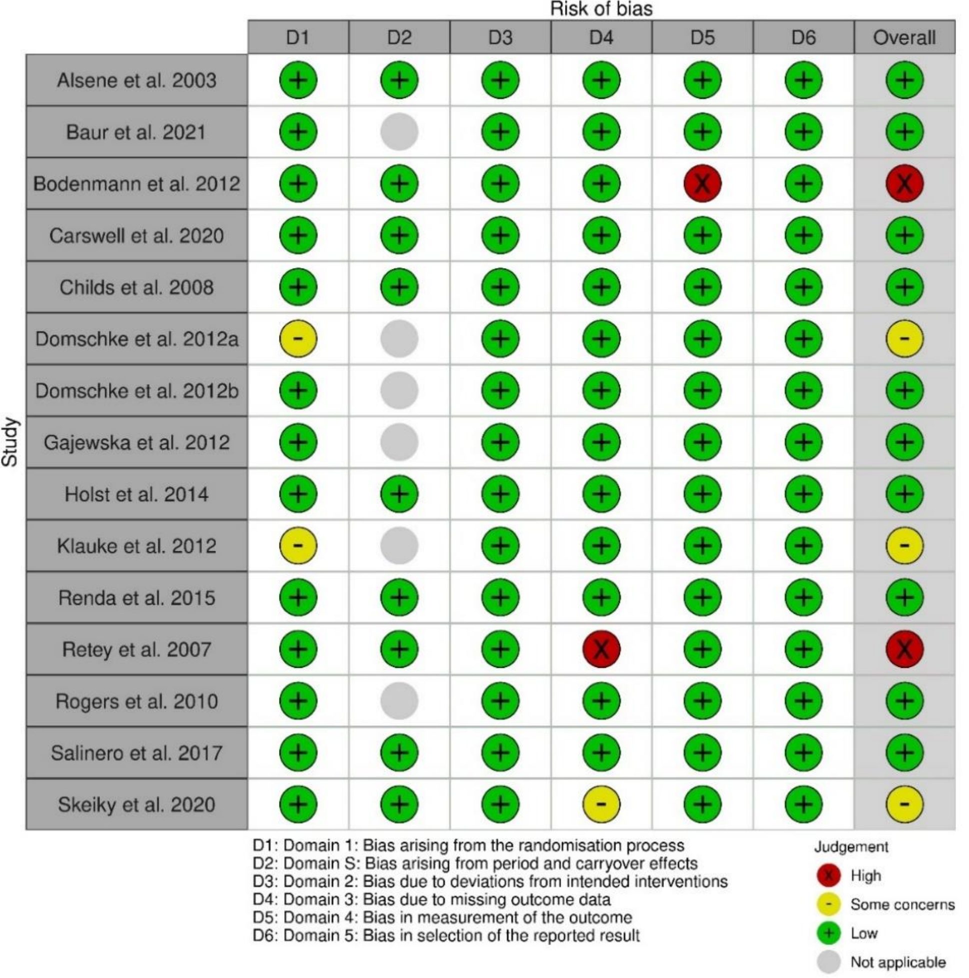

Figure 2. 2 Risk of bias assessment using the RoB-2 tool for crossover and parallel-group RCTs. The majority of RCTs (n =10, 67%) were of low overall risk of bias, while three studies (20%) were of unclear overall risk of bias and two studies (13%) were classified as high overall risk of bias. Some concerns were raised in random sequence generation (n = 2) and in bias due to missing outcome data (n = 1), while high risk of bias was demonstrated for bias due to missing outcome data (n = 1) and for bias in measurement of outcome (n = 1). From: McGuinness & Higgins, Risk-of-bias VISualization (robvis): An R package and Shiny web app for visualising risk-of-bias assessments, 2020.

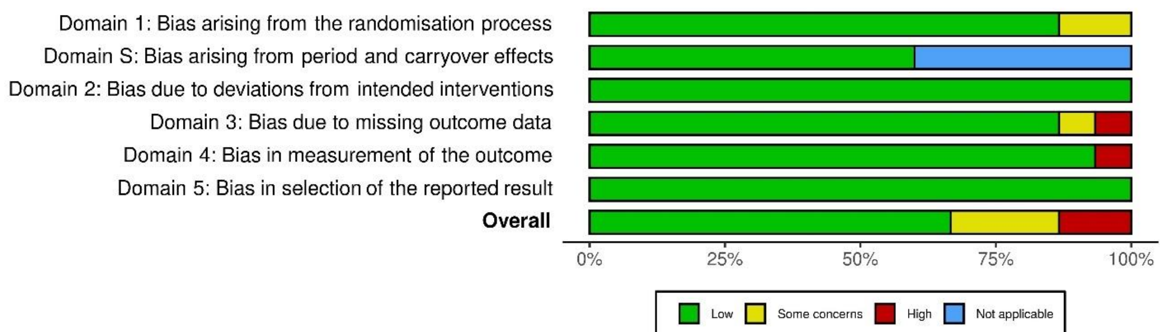

Figure 2. 3 Summary of risk of bias assessment for randomised controlled trials (crossover RCTs, n = 9 and parallel-group RCTs, n = 6, total n = 15). Bias arising from the randomisation process (low risk (n = 13, 86.7%), some concerns (n = 2, 13.3%); Bias arising from period and carryover effects (applicable only for crossover RCTs, low risk (n = 9, 100.0%); Bias due to deviations from intended intervention (low risk (n = 15, 100.0%); Bias due to missing outcome data (low risk (n = 13, 86.7%), some concerns (n = 1, 0.1%), high risk (n = 1, 0.1%); Bias in measurement of the outcome (low risk (n = 14, 93.3%), high risk (n = 1, 0.1%); Bias in selection of the reported result (low risk (n = 15, 100.0%); Overall risk of bias (low risk (n = 10, 66.7%), some concerns (n = 3, 20.0%); high risk (n = 2, 13.3%). From: McGuinness & Higgins, Risk-of-bias VISualization (robvis): An R package and Shiny web app for visualising risk-of-bias assessments, 2020.

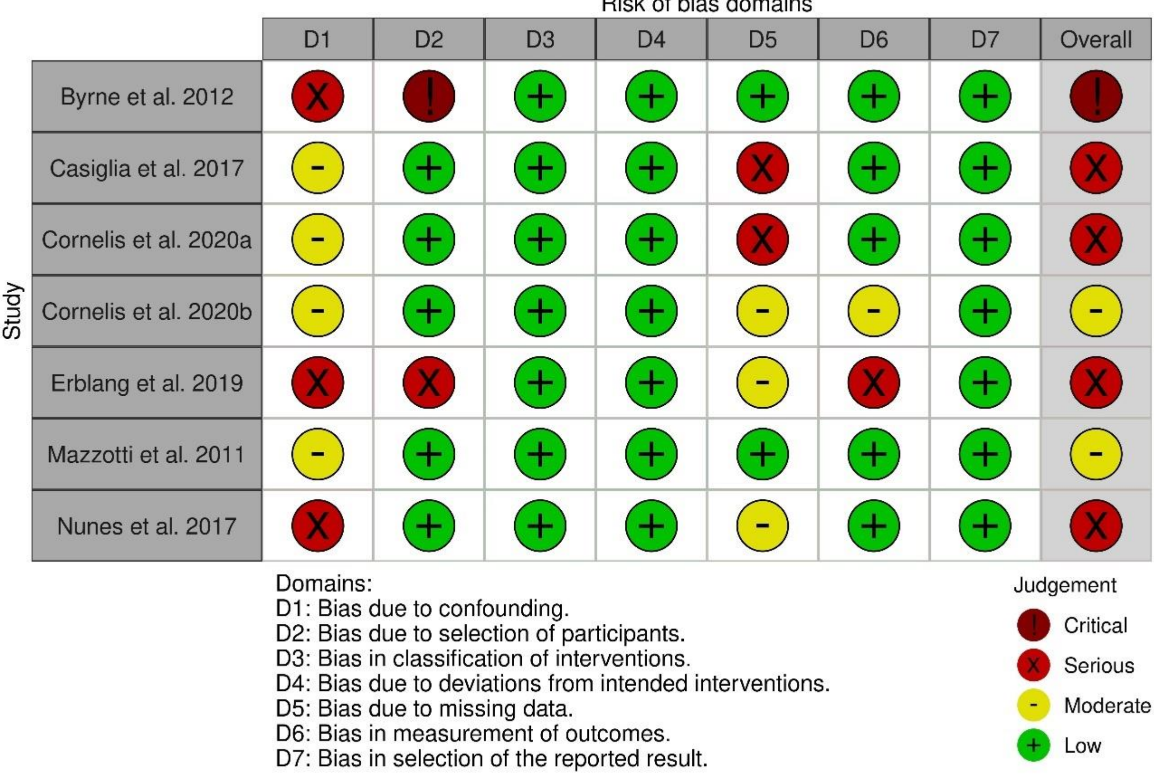

Figure 2. 4 Risk of bias assessment using the ROBINS-I for non-randomised trials of interventions. Overall, two studies (29%) demonstrated a moderate risk of bias, four studies (57%) demonstrated a serious risk of bias, while one study (14%) demonstrated a critical risk of bias. Bias due to confounding was the domain that demonstrated moderate (57%) and high risk of bias (43%) in all studies. The domains that demonstrated low risk of bias in all studies (100%) were bias due to classification of interventions, bias due to deviations from intended interventions and bias in selection of the reported result. A critical risk of bias was demonstrated for bias due to selection of participants in one study (14%). From: McGuinness & Higgins, Risk-of-bias VISualization (robvis): An R package and Shiny web app for visualising risk-of-bias assessments, 2020.

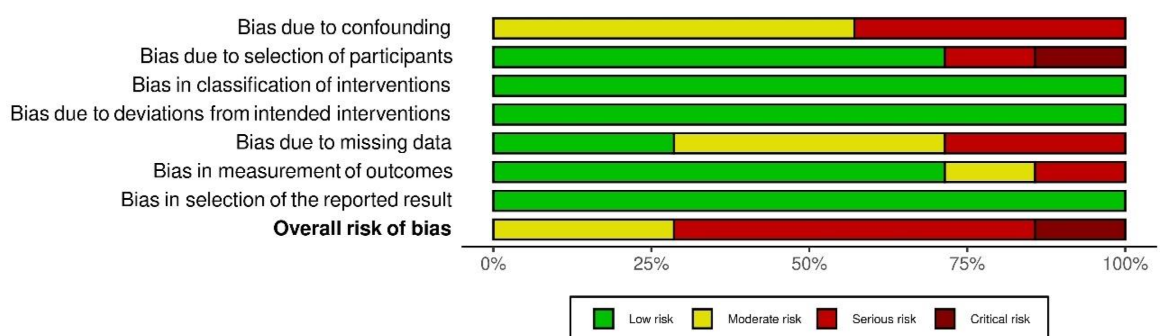

Figure 2. 5 Summary of risk of bias assessment for non-randomised trials of interventions (n = 7). Bias due to confounding (moderate risk (n = 4, 57.1%), serious risk (n = 3, 42.9%); Bias due to selection of participants (low risk (n = 5, 71.4%), serious risk (n = 1, 14.3%), critical risk (n = 1, 14.3%); Bias in classification of interventions (low risk (n = 7, 100.0%); Bias due to deviations from intended interventions (low risk (n = 7, 100.0%); Bias due to missing data (low risk (n = 2, 28.6%), moderate risk (n = 3, 42.9%), serious risk (n = 2, 28.6%); Bias in measurement of outcomes (low risk (n = 5, 71.4%), moderate risk (n = 1, 14.3%), serious risk (n = 1, 14.3%); Bias in selection of the reported result (low risk (n = 7, 100.0%); Overall risk of bias (moderate risk (n = 2, 28.6%), serious risk (n = 4, 57.1%); critical risk (n = 1, 14.3%). From: McGuinness & Higgins, Risk-of-bias VISualization (robvis): An R package and Shiny web app for visualising risk-of-bias assessments, 2020.

2.3.4. Reporting on the outcomes

Three major groups of outcomes were identified in the included studies: cognitive performance (n = 9), anxiety (n = 7) and sleep disturbance / insomnia (n = 6). Cognitive performance was assessed either alone (n = 5), during sleep deprivation (n = 3), or during and post-exercise (n = 1). Eight studies reported on deviations of study population from Hardy-Weinberg equilibrium (HWE), while 14 studies did not. HWE is an important tool in genetic studies primarily used to demonstrate whether the study population is representative of the general population (Namipashaki, Razaghi-

Moghadam and Ansari-Pour, 2015). Herein, all records that reported on HWE found that their study samples did not deviate from the HWE principle. Regarding Minor Allele Frequency (MAF), six studies either reported it or reported genotype frequencies that made it feasible to calculate it. For eight studies, genotype frequencies were reported only for total sample and not by caffeine intake groups, while no information on genotype frequencies was available for eight records. In terms of ethnicity, six studies were on unknown population, two studies were on mixed populations and 14 studies were on whites / Caucasian / Europeans. The findings of the included studies by outcome are shown in Tables 2.4 -2.6.

2.3.4.1. Cognitive performance

2.3.4.1.1 Cognitive performance without co-interventions

Five of the included studies reported genetic variation associated with cognitive performance (Renda *et al.*, 2015; Casiglia *et al.*, 2017; Salinero *et al.*, 2017; Cornelis, Weintraub and Morris, 2020a, 2020b). Indices of cognitive performance included abstract reasoning (Casiglia *et al.*, 2017), verbal-numerical reasoning, prospective memory, visual memory and search, processing speed, mental flexibility and executive function (Cornelis, Weintraub and Morris, 2020a, 2020b), alertness, orienting, executive motor control (Renda *et al.*, 2015) and visual attention (Salinero *et al.*, 2017).

Casiglia *et al.* (2017) demonstrated that caffeine in the highest tertile of caffeine intake was associated with significantly higher abstract reasoning in the CC homozygotes ('slow' metabolisers) compared to the lowest ($p < 0.005$) and middle tertiles ($p < 0.01$), while habitual caffeine intake was not associated with abstract reasoning in the A carriers ('fast' metabolisers) ($p > 0.05$ for all tertiles of habitual caffeine intake). On the contrary, Salinero *et al.* (2017), found no caffeine x *CYP1A2*

rs76255 genotype effects on visual attention after caffeine supplementation in a sample of active males and females.

Two analyses from the UK Biobank used two genome-wide significant SNPs (rs2472297 near *CYP1A2* and rs6968554 near *AHR*) of caffeine metabolites (Cornelis *et al.*, 2016) to formulate a weighted genetic score ranging from 0 to 4, with the highest score indicating faster caffeine metabolism. Focusing on a subset of Caucasian individuals, one of the studies investigated recent caffeine drinking (Cornelis, Weintraub and Morris, 2020a), defined as caffeine consumption through coffee or tea within the last hour prior to cognitive tests, while the other investigated habitual coffee, tea and caffeine intake (Cornelis, Weintraub and Morris, 2020b). Although recent caffeine drinking was associated with increasing performance in RT and with decreasing performance in pairs matching with increasing genetic caffeine metabolism score (CMSG), no significant CMSG x recent caffeine drinking interactions were found (Cornelis, Weintraub and Morris, 2020a). Moreover, a weak association between caffeine/tea and Fluid Intelligence (FI) among those with higher CMSG was found ($p < 0.0003$), while the 'fast' metabolisers (AA genotype of the rs762551) presented with greater decrements in performance in pairs matching with coffee intake than those with CC or AC genotypes ($p < 0.0001$). Nevertheless, no significant CMSG/genotype x coffee/tea/caffeine interaction was found for these tasks (Cornelis, Weintraub and Morris, 2020b).

Renda *et al.* (2015) demonstrated that the CC homozygotes ('low' caffeine sensitivity) of the *ADORA2A* rs5751876 showed a significantly higher RT in orienting ($p = 0.033$) in a sample of 106 males, while the TT homozygotes ('high' caffeine sensitivity) showed a higher RT in motor executive control ($p = 0.005$) after caffeine compared to placebo.

2.3.4.1.2 Cognitive performance and sleep deprivation

Three studies investigated the combined effects of specific genetic variants and caffeine intake on resilience to sleep deprivation by examining indices of cognition (Bodenmann *et al.*, 2012; Skeiky *et al.*, 2020; Baur *et al.*, 2021). Baur *et al.* (2021) reported that the regular coffee intervention group performed faster and more accurately than the decaffeinated coffee group on sleep restriction days 1 – 4 but not on day 5 in a sample of homozygous C-allele carriers of the *ADORA2A* rs5751876. A second study in 45 males investigated the effects of five *ADORA2A* haplotypes and caffeine on the sleep loss-induced impairment of attention. It was shown that caffeine improved psychomotor vigilance task (PVT) response speed during 40 h of sleep deprivation in non-HT4 (HT1, HT2, HT3, HT5 combined) haplotype carriers only ($p < 0.003$) (Bodenmann *et al.*, 2012). Another study found no differences between *TNFα* G308A genotype groups after caffeine intake compared to placebo ($p_{all} > 0.05$) on PVT performance after 48 h of sleep deprivation (Skeiky *et al.*, 2020).

2.3.4.1.3 Cognitive performance and exercise

Carswell *et al.* (2020) found that after caffeine supplementation, the 'fast' metabolisers (AA group of the *CYP1A2* rs762551) performed better than the 'slow' metabolisers (AC and CC group) at the PVT during exercise and at rest post-supplementation ($p_{all} < 0.05$). However, the study did not detect any differences in caffeine–placebo change scores in RT between 'high' and 'low' sensitivity genotypes of the *ADORA2A* gene during exercise or at rest post-supplementation ($p > 0.05$).

2.3.4.2. Anxiety

Seven of the included studies reported data on genetic variation and the anxiogenic effects of caffeine (Alsene *et al.*, 2003; Childs *et al.*, 2008; Rogers *et al.*, 2010; Domschke *et al.*, 2012a, 2012b; Gajewska *et al.*, 2012; Klauke *et al.*, 2012). Three of the included studies investigated the effects of polymorphisms on self-

reported anxiety following caffeine consumption (Alsene *et al.*, 2003; Childs *et al.*, 2008; Rogers *et al.*, 2010), while four studies investigated the effects of polymorphisms on startle responses to unpleasant optical or acoustic stimuli following caffeine intake (Domschke *et al.*, 2012a, 2012b; Gajewska *et al.*, 2012; Klauke *et al.*, 2012).

Alsene *et al.* (2003) demonstrated that only the TT groups of the rs5751876 and rs2298383 loci reported a significant increase in anxiety after caffeine compared to placebo and this increase was significantly higher compared to the CC and CT genotype groups ($p_{all} < 0.05$) in both SNPs. Rogers *et al.* (2010) demonstrated that *ADORA2A* rs5751876 TT genotype significantly increased self-rated anxiety after 100 mg caffeine compared to placebo ($p < 0.01$). However, when considering habitual caffeine consumption, 250 mg caffeine increased subjective anxiety only in non-to-low consumers in both TT and CT/CC genotype groups ($p < 0.05$ and $p < 0.01$, respectively). On the contrary, Childs *et al.* (2008) showed that genetic variations in the *ADORA2A* rs2298383 and rs4822492 and *DRD2* rs1110976 but not in the *ADORA2A* rs5751876 gene were associated with anxiety following 150 mg caffeine in no-to-moderate caffeine consumers.

Domschke *et al.* (2012a) reported that only females with the rs5751876 TT genotype demonstrated significantly higher startle magnitudes for unpleasant pictures in the caffeine condition ($p = 0.01$). Gajewska *et al.* (2013) showed that women with the rs5751876 TT genotype exhibited impaired prepulse inhibition compared to TT risk genotype men. Another study demonstrated that the Neuropeptide S receptor gene (*NPSR*) TT risk genotype had a decrease in startle magnitude in response to unpleasant stimuli in caffeine compared to placebo condition ($p \leq 0.05$) (Domschke *et al.*, 2012b). Lastly, a study on the effects of the *COMT* Val158Met variant on startle

response showed no influence of caffeine on startle responses dependent on the *COMT* Val158Met polymorphism (Klauke *et al.*, 2012).

2.3.4.3. Sleep disturbance and insomnia

Six of the included studies investigated the interaction between genetic variability and caffeine intake on sleep disturbance and insomnia (Rétey *et al.*, 2007; Mazzotti *et al.*, 2011; Byrne *et al.*, 2012; Holst *et al.*, 2014; Nunes *et al.*, 2017; Erblang *et al.*, 2019).

A GWAS on more than 2 million genetic loci identified eight SNPs that were associated with subjective caffeine-induced insomnia, although no SNPs passed the threshold of genome-wide significance level (7.2×10^{-8}) (Byrne *et al.*, 2012). Erblang *et al.* (2019) showed that total sleep time (TST) was lower in the T carriers compared to CC genotype of the *ADORA2A* rs5751876 and rs3761422, while it was higher in the TT vs CC group of the rs2298383 and the GG and GC compared to CC genotype of the rs4822492, but only in low caffeine consumers. The risk of sleep complaints was lower in the CT compared to CC genotype for rs5751876 and it was higher in TT compared to CC for rs2298383 and in GG compared to CC genotype for rs4822492 in moderate caffeine consumers.

Holst *et al.* (2014) revealed that 400 mg of caffeine was associated with increased vigilance to sleep deprivation in 10R/10R homozygotes of the *DAT* 1 gene when compared with 9R allele carriers ($p < 0.05$), as shown by EEG activity. Mazzotti *et al.* (2011) on the other hand, using polysomnography found that, among caffeine consumers, A allele carriers of *ADA* G22A compared to non-carriers showed lower sleep latency ($p = 0.03$), higher % sleep efficiency ($p = 0.01$), higher % Rapid-Eye Movement (REM) sleep ($p = 0.02$) and fewer minutes awake ($p = 0.04$). No difference

was found between genotypes for other sleep parameters, or for any of the sleep parameters among those who did not consume caffeine ($p > 0.05$).

Nunes *et al.* (2017) found no difference in sleep variables between the *ADORA2A* rs5751876 genotypes ($p_{all} > 0.05$). When stratified by genotype, significant, yet weak correlations were shown between caffeine load and sleep latency, % stage 3 sleep and % REM sleep only in T allele carriers. On the contrary, Retey *et al.* (2007) demonstrated that the CC genotype of the *ADORA2A* rs5751876 displayed a greater rise in the EEG power in the beta band after caffeine compared to the T carriers ($p < 0.03$), suggesting that the CC genotype exhibits acute insomnia following caffeine intake (Retey *et al.*, 2006).

2.4. Discussion

The purpose of the present systematic review was to identify the associations between common genetic variations, caffeine and brain-related outcomes in humans. The findings of this work are discussed below.

2.4.1. Cognitive performance

2.4.1.1. Cognitive performance without co-interventions

Caffeine is normally considered an enhancer of alertness and general cognitive performance (Nehlig, 2010; Cappelletti *et al.*, 2015). Cognitive performance is defined as the performance in functions that require mental effort (Lamport *et al.*, 2014). Cognitive functions are categorised as either ‘simple’ or ‘complex’; simple functions require very simple perceptual motor skills (e.g., RT, short-term memory), whereas complex functions require a greater effort (e.g., executive function, working memory) (Taylor *et al.*, 2016). Genetic studies on the association between caffeine and

specific functions of cognition are limited and are characterised by methodological heterogeneity.

Although caffeine intake has been shown to enhance simple cognitive functions such as RTs in a dose-dependent manner, the association between caffeine and complex cognitive functions is often argued (McLellan, Caldwell and Lieberman, 2016). The first study to show that high habitual caffeine intakes are associated with abstract reasoning only in 'slow' metabolisers (Casiglia *et al.*, 2017) may partly explain previous controversies in the literature regarding the association between habitual caffeine intake and complex cognitive abilities. Accordingly, the investigations from the UK Biobank found that the 'fast' metabolisers had lower performance in pairs matching with higher habitual coffee intake than those with AC or CC genotypes of the rs762551 (Cornelis, Weintraub and Morris, 2020b). After stratifying by CMSG, the results suggested that habitual caffeine and tea consumption were associated with decrements in fluid intelligence in 'fast' compared with 'slow' metabolisers (Cornelis, Weintraub and Morris, 2020b), while recent caffeine drinking was associated with improved cognition in simple cognitive functions with faster genetic caffeine metabolism (Cornelis, Weintraub and Morris, 2020a). Nevertheless, no significant genotype x coffee/tea/caffeine interactions were found for any of these tasks.

On the contrary, a study on light caffeine consumers found no differences in indices of cognition neither between trials nor between rs762551 genotype groups 1 h after supplementation with caffeine or placebo (Salinero *et al.*, 2017). Finally, in the only study on cognition and the *ADORA2A* gene*,* the rs5751876 genotypes performed faster in different cognitive indices - the CC genotype performed faster in orienting,

while the TT genotype performed faster in motor executive control after caffeine compared to placebo (Renda *et al.*, 2015). However, only male subjects were included in the study making the results non-generalisable.

A crucial factor that needs to be considered is that only the study from Casiglia and colleagues (2017) measured habitual caffeine from all sources, while in the UK Biobank investigations, habitual and recent caffeine drinking estimates were based solely on coffee and tea. In the UK, for example, the major caffeine source in the diet is tea, with coffee and cola drinks in second place and energy drinks in third place (Reyes and Cornelis, 2018). Moreover, the CMSG was derived using two SNPs (*CYP1A2* rs2472297 and *AHR* rs6968554) that have been presented with the largest effect sizes in a single GWAS of caffeine metabolites and may have not provided a valid measure of genetic caffeine metabolism because of the limited replication of data on these SNPs and because a known SNP associated with caffeine metabolism, *CYP1A2* rs762551 was not included in the scoring.

Moreover, it is important to note that the peak plasma caffeine concentration is shown to be reached in 30-60 min post ingestion and caffeine half-life in plasma is approximately 4-6 h in most adults and it is not yet known to what degree caffeine metabolism is altered between 'fast' and 'slow' metabolisers (Nehlig, 2018). Therefore, testing participants within 1 h post-caffeine ingestion would mostly measure caffeine absorption and not metabolism, which is determined by CYP1A2 enzyme. Moreover, it is unknown at what time point there would be a large enough difference in the circulating levels of caffeine between 'fast' and 'slow' metabolisers to have a significant impact on the stimulant effects of caffeine (Southward *et al.*, 2018).

In summary, based on genetic studies on caffeine and cognition, important parameters that should be considered are: a) habitual caffeine intake, since it is a known inducer of CYP1A2 enzymatic activity in a dose-dependent manner (Tantcheva-

Poór *et al.*, 1999), b) how caffeine intake is measured, c) the rate of acute caffeine metabolism based on *CYP1A2* rs2472297 and rs762551 and *AHR* rs6968554 polymorphisms and d) the nature of cognitive functions under study. More research is needed in both males and females stratified by *ADORA2A* rs5751876 genotype to investigate whether this SNP is implicated in the association between caffeine and cognition.

2.4.1.2. Cognitive performance and sleep deprivation

Sufficient sleep of 6–8 h and of good quality is essential for general health and optimal cognitive performance (Lo *et al.*, 2012). Whereas the neurobiological mechanisms are not yet fully understood, changes in the levels of adenosine in the brain appear to underly the sleep loss-induced reduction in cognitive functions such as working memory and sustained attention (Retey *et al.*, 2006). By blocking the binding of adenosine with the A2a receptors, caffeine countermeasures the detrimental effect of prolonged wakefulness by potentiating dopaminergic signalling, which leads to motor activation and subsequent alertness (Landolt, 2008). Hence, caffeine intake, particularly in the morning or early afternoon to enhance wakefulness in response to sleep restriction is very common (Martyn *et al.*, 2018). The present review identified limited data regarding the effect of genetics and caffeine on cognition in a sleep-deprived state.

The included studies found that caffeine attenuates the impairment in cognitive functions such as attention, orienting, memory and executive control caused by sleep deprivation in individuals who are C homozygous for *ADORA2A* rs5751876 (Baur *et al.*, 2021) and in non-HT4 haplotype carriers of *ADORA2A* compared with the HT4 haplotype (Bodenmann *et al.*, 2012). Although both studies tried to mimic real-life caffeine intakes which are very common in Europe (Reyes and Cornelis, 2018), only the CC genotype group of the *ADORA2A* rs5751876 genotype was included in one

study (Baur *et al.*, 2021) and the second studied only male subjects stratified by *ADORA2A* haplotypes instead of distinct genotypes (Bodenmann *et al.*, 2012).

The selective recruitment was based on the notion that these individuals are genetically sensitive to the effects of caffeine on cognition in rested (Renda *et al.*, 2015) and sleep-deprived states (Retey *et al.*, 2006), yet did not allow for comparisons between distinct genotype groups. Additionally, haplotypes are often ambiguous because of unknown linkage within the gene and, although haplotype frequencies are suitable for case-control studies (binary traits), they cannot provide a method of testing the statistical significance with a specific trait (Schaid *et al.*, 2002).

Further, although the A allele carriers of the *TNFα* rs1800629 polymorphism have been found to be relatively resilient to psychomotor vigilance impairment during sleep deprivation as compared to individuals homozygous for the G allele (Satterfield *et al.*, 2019), Skeiky *et al.* (2020) found no differences in RT between genotypes after caffeine intake.

Overall, genetic studies on cognition during sleep deprivation are limited. Further studies are needed to elucidate how distinct *ADORA2A* genotypes interact with different indices of cognition and the sleep–wake cycle and whether SNPs of other plausible genes in the dopaminergic system are implicated in these associations. For example, evidence suggests that the T carriers of the *ADORA2A* rs5751876 variant experience caffeine-induced anxiety (Alsene *et al.*, 2003) and that these individuals demonstrate low habitual caffeine intakes, most probably because of this anxiogenic effect (Cornelis, El-Sohemy and Campos, 2007; Cornelis *et al.*, 2015). These observations may provide a biological basis for habitual caffeine consumption that would drive the acute effects of caffeine in cognition after sleep deprivation and require further exploration.

2.4.1.3. Cognitive performance and exercise

Caffeine antagonises the effect of adenosine in the CNS, thereby decreasing feelings of tiredness and enhancing arousal, vigilance, and willingness to exert effort during exercise (Meeusen, Roelands and Spriet, 2013). In the only study up to date on the effects of caffeine and genetics on cognition and exercise, caffeine improved cognitive performance in RT in 'fast' compared with 'slow' metabolisers (based on *CYP1A2* genotype) both during and after exercise, but no differences were observed between individuals based on the *ADORA2A* genotypes. Nonetheless, it needs to be considered that only one heterozygous carrier of the *ADORA2A* C allele was included. Future studies with larger sample sizes are required to determine the influence of the *ADORA2A* gene on the cognitive effects of caffeine during exercise.

2.4.2. Anxiety

The most extensively researched SNP in association with the anxiogenic effects of caffeine is the *ADORA2A* rs5751876 silent polymorphism, because of its association with panic disorder and anxiety in Caucasians (Nehlig, 2018). There are two proposed explanations for the functional relevance of this polymorphism in anxiety: a) the variant can alter mRNA translation or stability and b) it is in LD with a functional variant in the *ADORA2A* gene such as the rs35320474 polymorphism (Alsene *et al.*, 2003; Domschke *et al.*, 2012a).

Three reports from mixed samples consisting of predominantly Caucasians support that the TT genotype of the specific variant is associated with increases in self-rated anxiety following caffeine consumption (Alsene *et al.*, 2003; Childs *et al.*, 2008;

Rogers *et al.*, 2010). However, when data only for European-American participants were considered, this effect was no longer significant in one of the studies (Childs *et al.*, 2008).

Interestingly, two studies on measured anxiety showed a possible sex-specific regulation of anxiety in response to caffeine, with female TT homozygous of the *ADORA2A* rs5751876 experiencing higher levels of anxiety (Domschke *et al.*, 2012a; Gajewska *et al.*, 2012). One possible explanation for these sex differences would reflect the hormonal differences between males and females and variations in levels of circulating oestrogens (Nehlig, 2010). Nevertheless, the studies assessed women using oral contraceptives and they were not tested during menstruation to control for such hormonal changes. On the other hand, previous data using fMRI show that caffeine effects may be also specific to different lateralisation in the dopaminergic response between genders (negative emotional stimuli activates the left hemisphere in women and the right hemisphere in men) and how males and females perceive and process anxiety (Seo *et al.*, 2017).

Two additional *ADORA2A* variants, rs2298383 and rs4822492, were also identified to be associated with self-rated caffeine-induced anxiety, however they lack replication and it remains unclear whether they have a functional role or are in LD with other functional polymorphisms (Alsene *et al.*, 2003; Childs *et al.*, 2008; Hohoff *et al.*, 2010). Additionally, the present review identified single reports on variants in genes that are biologically plausible modulators of caffeine effects on anxiety: the dopamine D2 receptor (*DRD2*) (Childs *et al.*, 2008), the *NPSR* (Domschke *et al.*, 2012b) and the *COMT* (Klauke *et al.*, 2012) genes. The *DRD2* and *COMT* genes are associated with the counteractive signalling between adenosine A2a and dopamine D2 receptors (Fuxe *et al.*, 2007) and the inactivation of dopamine and norepinephrine (Chen *et al.*, 2004),

while the *NPSR* rs324981 polymorphism has been found to be influencing emotion processing of anxiety-relevant stimuli (Dannlowski *et al.*, 2011).

Summarising the above, it is suggested that caffeine-sensitive individuals who habitually consume low-moderate caffeine doses are affected by caffeine in doses that can be consumed in one cup of coffee. It is also indicated that caffeine-naïve individuals may experience the anxiogenic consequences of caffeine regardless of genetic variations (Rogers *et al.*, 2010), or perhaps they do not habitually consume caffeine because it has an anxiogenic effect on them. Further investigations using similar measures of anxiety with higher caffeine doses and variants in genes implicated in neurotransmission are needed to reach to safe conclusions on the effect of habituality and gene x gene interactions in anxiety.

2.4.3. Sleep disturbance and insomnia

Most of the evidence for associations between genetics, caffeine intake and sleep effects relate to the *ADORA2A* gene. Retey *et al.* (2007) reported that caffeine can cause an insomnia-like EEG pattern only in CC homozygous individuals. Nunes *et al.* (2017) reported that caffeine was associated with shorter sleep duration only in T allele carriers. Erblang *et al.* (2019) reported that both alleles may be associated with different sleep parameters; T allele associated with shorter sleep duration in low habitual caffeine consumers and CC genotype associated with more sleep complaints in moderate habitual caffeine users. Differences in study design may account for the inconsistencies across studies. For example, Retey *et al.* (2007) supplemented participants with a measured caffeine dose after caffeine abstinence for two weeks, suggesting more accurate data on caffeine intake. Nunes *et al.* (2017) used an index (caffeine load) that incorporates the number of caffeine doses the individuals had taken before the polysomnography and the time since the last dose. Finally, Erblang *et al.* (2019) reported habitual caffeine intake from a self-administered questionnaire and

characterised caffeine intake as low, moderate and high. Additionally, both Retey *et al.* (2007) and Nunes *et al.* (2017) assessed sleep using polysomnography, while Erblang *et al.* (2019) used self-reported data.

In the only GWAS up to date on SNPs implicated in caffeine-induced insomnia, no SNPs reached the genome-wide significance level and, although association analyses revealed eight variants to be associated with insomnia (Byrne *et al.*, 2012), none of these loci has been replicated in genetic association studies. However, risk of insomnia was assessed through a dichotomised scale based on whether participants reported ever or never experiencing caffeine-induced insomnia, which may be a source of information bias. Moreover, the assignment of participants into two groups may have resulted in a loss of power in the study, as risk alleles related to more severe or minor caffeine-induced insomnia may have not been identified.

Single studies on genes related to neurotransmission were also identified (Mazzotti *et al.*, 2011; Holst *et al.*, 2014). ADA is an enzyme responsible for the clearance of extracellular adenosine and regulates sleep, while the *ADA* rs73598374 variant has been associated with better sleep duration and intensity in healthy adults (Landolt, 2008). Moreover, the 10R/10R genotype dopamine transporter 1 (*DAT1*) VNTR polymorphism has been associated with reduced DAT protein expression in the striatum when compared with 9R allele carriers (Costa *et al.*, 2011). Although both studies found genotype differences in sleep quality parameters with caffeine intake, results require replication.

2.4.4. Quality of evidence

The present systematic review used three different tools for risk of bias assessment: the RoB-2 tools for randomised parallel group and crossover trials and the ROBINS-I tool for observational studies. The included randomised trials displayed an

overall low risk of bias, while three studies raised some concerns and two studies were of high risk. The domains that raised concerns were selection and detection biases, indicating that the studies provided insufficient information on the sequence generation process and the blinding of allocated interventions by outcome assessors. A high risk of bias appeared in detection and attrition bias domains, suggesting that the outcome is likely to be influenced by lack of blinding and that there is missing outcome data that was not reported, respectively (Higgins *et al.*, 2022).

On the contrary, the non-randomised trials overall displayed a serious risk of bias, with two studies displaying moderate and one study displaying critical risk. Domains with serious or critical risk included bias due to missing data and detection biases, as well as bias due to confounding and selection of participants. Bias due to confounding in non-randomised trials is very common (Sterne *et al.*, 2016) and results from the use of self-reported measures, for example subjective sleep quality, which may have lower reliability than objective measures such as polysomnography. Moreover, the selective recruitment of participants based on specific characteristics suggests that the study population may not be representative of the target population (Sterne *et al.*, 2016).

Although the quality of the included observational studies seems to be low, it needs to be considered that the fundamental underlying principle of the ROBINS-I tool is that a non-randomised trial is compared against a target RCT (Sterne *et al.*, 2016; Sterne *et al.*, 2019). This means that, using this stringent tool, no observational study can be of low overall risk of bias and that a good quality observational study, which is comparable with a RCT, would be of moderate risk of bias. The present systematic review identified two observational studies that are of moderate risk of bias and it needs to be considered that the study that displayed an overall critical risk is a GWAS and it is uncertain whether the ROBINS-I tool is applicable to this study design. This

indicates that both the randomised and the non-randomised trials in this systematic review may provide fair quality evidence.

As no meta-analysis was conducted, it was not feasible to use the GRADE (Grading of Recommendations, Assessment, Development and Evaluations) framework for appraising quality of evidence by brain-related outcomes. Nonetheless, some issues need to be addressed regarding quality of evidence in this systematic review. The included studies were on three different brain-related outcomes of caffeine: cognition, anxiety and insomnia/sleep disturbance. Among the nine studies on cognition, five studied cognition alone, three studies explored cognitive performance during sleep deprivation and one study explored cognitive performance during and post exercise. Seven studies were investigating anxiety and six studies were on sleep disturbance and insomnia. Therefore, there is a variety of outcomes and the number of studies for some of them was limited. In addition, studies on the same outcome incorporated different outcome measures based on the study design (intervention vs observational) and the selection of different methods of assessing cognition (i.e., different cognitive tasks assessing executive control or memory), anxiety (i.e., subjective vs objective measures of anxiety) or sleep (subjective measures of sleep quality vs polysomnography).

Regarding the first comparator of this review, the genetic variability, most studies tested individual SNPs (21 SNPs in total), two studies formulated a genetic score based on more than one SNP and one study assessed haplotypes. With such diversity in genetic information, only a few SNPs are replicated in the literature. As far as the second comparator, caffeine, both habitual caffeine intake and caffeine interventions were considered. Still, studies compared: a) tertiles of habitual caffeine intake (lowest/middle/highest); b) different doses of caffeine supplementation vs placebo; c) recent acute caffeine intake vs no caffeine intake or d) different caffeine

loads based on quantity of recent caffeine drinking and the number of hours since the last caffeine-containing drink. Moreover, some studies estimated habitual caffeine intake of participants solely on coffee and tea and may have omitted important sources of caffeine (Reyes and Cornelis, 2018). Finally, four studies were in males and results may not be generalisable to females. On the other hand, two investigations studied no/low habitual caffeine consumers, who may not be representative of the general adult population worldwide (James, 2014).

Accordingly, based on the studies selected for the aim of the current systematic review, caution is recommended when forming conclusions regarding the impact of individual SNPs on the brain-related effects such as cognition, anxiety and sleep disturbance/insomnia of habitual or acute caffeine intake in humans.

2.4.5. Strengths and limitations

A strength of the present review is the inclusion of both experimental and observational study designs on the genetics of caffeine and brain-related outcomes. Indeed, if a review includes only randomised trials, it may omit other outcomes because of the importance of long-term effects of an exposure to human health or because only a small number of randomised trials is available on the topic (Higgins *et al.*, 2022).

The separation of randomised and observational studies was primarily a result of recognition that randomisation is the only way to fully protect against confounding and that confounding is always a concern in even the most rigorously conducted observational studies (Schünemann *et al.*, 2019). For this reason, three well-established tools, specific to different study designs were used to assess risk of bias of the included studies. Particularly, the use of a stringent tool, the ROBINS-I tool to compare the quality of observational studies against target RCTs ensured a high

quality approach for this review (Sterne *et al.*, 2016). Finally, no studies were excluded based on language.

A possible limitation of the current systematic review is that non-peer-reviewed studies were excluded. Using the grey literature is highly desirable in systematic reviews in order to reduce publication bias, through inclusion of research that is yet to be published or has received less exposure (Higgins *et al.*, 2022). Therefore, the present review might have not fully addressed publication bias and studies that report dramatic effects were more likely to be identified compared with studies that report smaller effect sizes (Garg, Hackam and Tonelli, 2008).

2.5. Conclusions

In conclusion, the present review has provided evidence that variability in the *CYP1A2* and the *ADORA2A* genes are associated with brain-related outcomes of caffeine. Nevertheless, it is not yet clear what specific genotypes are implicated in each brain outcome, which functions of cognition are particularly associated with caffeine (simple vs complex), whether there are gender differences in effects of caffeine on anxiety and how habitual caffeine intake may influence the acute effects of caffeine. The review also demonstrates that variability in additional genes may be involved in caffeine pharmacokinetics and brain neurotransmission collectively influence individual responses to caffeine; however, these studies lack replication.

Future studies in this area are recommended to use interdisciplinary approaches to investigate the complex interactions between genetic and environmental factors on brain function. Careful design to overcome the common methodological challenges of caffeine research is warranted. For example, the selection of caffeine-naïve or low caffeine consumers may not representative of the general population. Individuals who consume caffeine habitually may help investigate issues of caffeine tolerance, caffeine

withdrawal and withdrawal reversal. Moreover, there is need for studies that examine brain-related effects of caffeine not based solely on single sessions or a period of days, but also for weeks, months and possibly years.

Chapter 3. Habitual Caffeine Intake, Genetics and Cognitive Performance

This chapter presents the findings of the second study of this research. This study aimed to answer the second research question, i.e., whether there is an association between variations in genes implicated in caffeine pharmacokinetics and pharmacodynamics, habitual caffeine intake and cognitive performance. This chapter has four sections: firstly, an introduction to the topic; secondly, a detailed presentation of the materials and methods used for the study conduct; thirdly, the results of the study after statistical analysis and finally, the findings of the study are discussed and compared with previous research. Aiming to have comparable methods between our studies, the methodology from this study will inform Study 3, which is presented in Chapter 4 of this thesis.

3.1. Introduction

As discussed in detail in Chapter 1 of this thesis, cognitive function refers to the conscious mental efforts that enable humans to exert control over their environment (Lamport *et al.*, 2014; Taylor *et al.*, 2016). Cognitive functions are categorised as either 'simple' or 'complex'; simple functions include those which require very simple perceptual motor skills (e.g., attention), while complex functions require a greater effort, such as multiple/dual tasks (e.g., executive function) (Lezak, Howieson and Loring, 2004; Harvey, 2019). The six core components of neurocognitive function include perceptual-motor function, attention and processing speed, executive function, memory, language and social and emotional cognition (Harvey, 2019).

The interest in the effects of various agents on cognitive function has grown remarkably, driven by the desire to understand their impact on cognitive performance across the lifespan and improve cognitive function in various populations, including students, healthy adults and the elderly (Gestuvo and Hung, 2012; Anstey, 2014; Klimova, Dziuba and Cierniak-Emerych, 2020; Cohen Kadosh *et al.*, 2021). Caffeine is

the most widely consumed natural psychoactive substance and among the most promising cognitive function enhancers (Heckman, Weil and De Mejia, 2010; James, 2014; Cappelletti *et al.*, 2015).

Because chronic caffeine use causes an upregulation of adenosine receptors (Johansson *et al.*, 1993; Ralevic and Burnstock, 1998), caffeine habituation is of particular interest. Studies using fMRI have shown significant variability in regions of the brain related to vision between low and high habitual caffeine consumers after acute caffeine intake (Laurienti *et al.*, 2002) and in regions associated with somatosensory, motor and emotional processing in habitual caffeine consumers compared with non-consumers (Magalhães *et al.*, 2021). Such changes were replicated in non-caffeine consumers after a single coffee intake, suggesting possible causality between caffeine intake and altered patterns of neuronal networks (Magalhães *et al.*, 2021). Therefore, habitual caffeine consumption has been suggested to be essential when analysing the physiological effects of caffeine intake (Corti *et al.*, 2002).

Currently, there are data showing a positive relationship for habitual caffeine consumption with memory and executive function, but there is limited evidence for an association with simple cognitive functions (Jarvis, 1993; Hameleers *et al.*, 2000; Johnson-Kozlow *et al.*, 2002). Inconsistencies in caffeine research on cognitive performance are mainly due to the variable cognitive assessment methods employed by authors, participant selection (habitual consumers vs caffeine-naïve individuals) and misclassification of caffeine intake (Smith, 2002; Rogers *et al.*, 2013; James, 2014).

The past decades, part of the variability in study findings has also been attributed to common genetic variations, linked with caffeine metabolism and response (Nehlig, 2018). Nevertheless, the results from our systematic review (Chapter 2) demonstrated that genetic association studies on habitual caffeine intake and cognitive performance are limited and have also yielded mixed results. A study investigating the

association between caffeine intake and genetic caffeine metabolism based on *CYP1A2* rs762551 variant on abstract reasoning, demonstrated that caffeine in the highest tertile of intake was associated with significantly higher abstract reasoning compared with the lowest and middle tertiles, but only in 'slow' metabolisers (Casiglia *et al.*, 2017). Contrarily, the results from two investigations from the UK Biobank using the *CYP1A2* rs2472297 and *AHR* rs6968554 SNPs as proxies of caffeine metabolism, found no significant gene x caffeine interactions on memory, fluid intelligence and attention (Cornelis, Weintraub and Morris, 2020a, 2020b).

Notably, most genetic association studies on habitual caffeine intake and cognition are focusing on one variant, e.g., a variant associated with caffeine metabolism (Casiglia *et al.*, 2017), without considering genetic variants associated with factors affecting cognitive performance, such as sleep. Moreover, there are no genetics studies up to date investigating the associations between habitual caffeine intake and genetic caffeine sensitivity.

As shown in Chapter 1, everyday cognitive performance is influenced by various factors, such as environmental conditions (hot vs cold environment) (Morley *et al.*, 2012; Muller *et al.*, 2012), sounds/music (Perham and Vizard, 2011; Nemati *et al.*, 2019), diet (Lamport *et al.*, 2014) and sleep quality (Cunningham *et al.*, 2018). Therefore, research should contextualise daily cognition with extensive intrapersonal (e.g., subjective sleep quality, sleepiness) and contextual factors (e.g., location) (Verhagen *et al.*, 2019). Furthermore, past investigations have been restricted to one or two cognitive domains and, in fact, restricted to one assessment either in the laboratory or at home (Casiglia *et al.*, 2017; Cornelis, Weintraub and Morris, 2020a, 2020b). Repetitive online cognitive tasks in natural everyday environments that take multiple minutes to perform might provide more valuable information on daily

cognitive function compared with 'one-off' tasks in experimental settings (Bouvard *et al.*, 2018).

The aim of the present study was to investigate the interactions between genetics and habitual caffeine consumption on cognitive performance in all key domains of cognition that have been previously linked with caffeine, namely social and emotional cognition, memory, attention and psychomotor speed and executive function in healthy individuals in real-life conditions. The study methodology (SNP selection, cognitive domains and methods to assess cognitive performance, measurement of habitual caffeine intake etc.) was selected based on the evidence collected from Chapters 1 and 2 and is described below.

3.2. Materials & Methods

3.2.1. Participants

The study was approved by St Mary's University Ethics Committee and conducted in agreement with the Declaration of Helsinki (World Medical Association, 2013). A sample size of 131 was calculated using G*Power 3.1 for a medium effect size ($f^2 = 0.15$) at 80% power and an alpha level of 5% for 13 predictors representing potential enhancers or depressors of cognitive function, as discussed in the first chapter of this thesis: 1) age (Harada, Natelson Love and Triebel, 2013), 2) sex (Sherwin, 2003), 3) BMI (Michaud *et al.*, 2018), 4) habitual caffeine intake (McLellan, Caldwell and Lieberman, 2016), 5) genetic variants in genes involved in caffeine metabolism (*AHR* rs6968554 and *CYP1A2* rs2472297) and response (*ADORA2A* rs5751876), 6) physical activity (Erickson *et al.*, 2019), 7) level of education (Barulli & Stern, 2013), 8) subjective sleep quality (Zhongrong Wang *et al.*, 2022) and 9) subjective sleepiness prior to tasks (Alhola and Polo-Kantola, 2007), 10) tobacco (Campos, Serebrisky and Castaldelli-Maia, 2016), 11) alcohol use (Dry *et al.*, 2012),

12) *ADA* rs77819966 and 13) *APOE* ε4-/ε4+ genes. The *ADA* rs77819966 and *APOE* ε4+/ε4- were used as proxies of sleep quality and risk of cognitive impairment, respectively (Gharbi-Meliani *et al.*, 2021; Tartar *et al.*, 2021).

Participants were recruited via email, word of mouth and social media advertising between June 8th and August 31st, 2022. Data collection was completed remotely, while genotyping and statistical analyses were completed at St Mary's University Twickenham, London. Before enrolling, participants were asked to complete an online screening questionnaire directly on Jisc online platform. Data from ineligible participants were destroyed immediately.

Healthy adult males and females residing in the UK with no known neurocognitive disorder and uncorrected vision impairments who provided written informed consent were included in the study. Exclusion criteria included medication that may alter CYP1A2 enzyme activity, including oral contraceptives, exogenous hormones, SSRIs and quinolone antibiotics (Arnaud, 2011; Casiglia *et al.*, 2017; Grzegorzewski *et al.*, 2021), as well as pregnant or lactating women. Eligible participants' involvement lasted three days.

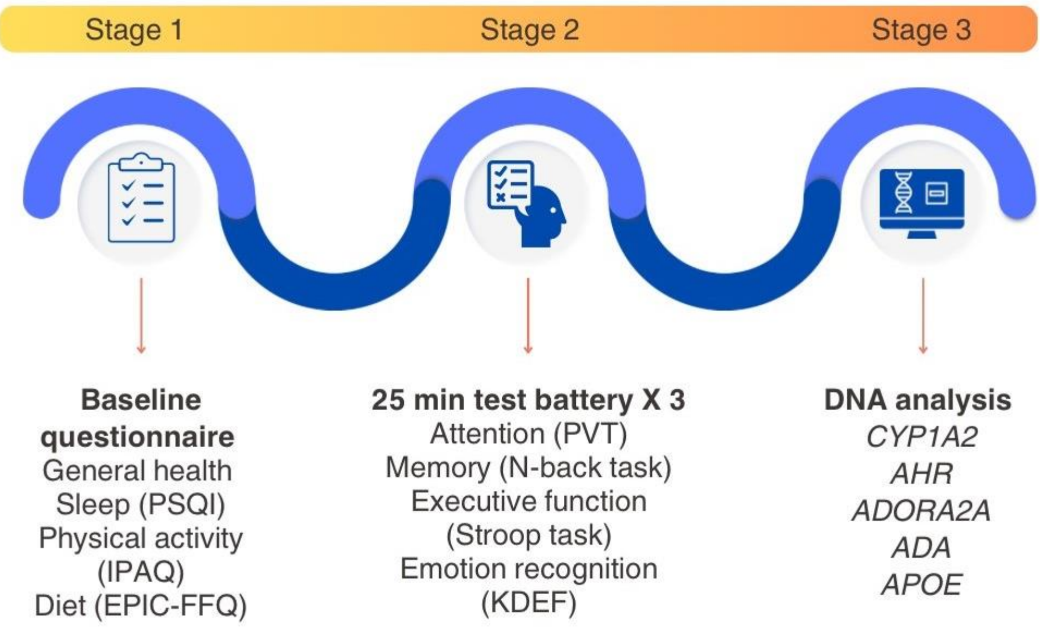

Figure 3. 1 Schematic representation of participant involvement across the three stages of the study. PSQI: Pittsburgh Sleep Quality Index; IPAQ: International Physical Activity Questionnaire; EPIC-FFQ: EPIC Food Frequency Questionnaire; PVT: Psychomotor Vigilance Task; KDEF: Karolinska Directed Emotional Faces.

3.2.2. Baseline Questionnaire

After providing written informed consent, participants were assigned a unique ID and were emailed with instructions of participation. The first part of the study included a baseline questionnaire consisting of sociodemographic, health and lifestyle questions (Figure 3.1). Some questions from the screening questionnaire such as medication and pregnancy were repeated in the health and lifestyle questionnaire, to account for changes in the period between the completion of the two questionnaires.

3.2.2.1. Sociodemographic and Health Data

Sociodemographic questions included sex, age, ethnic origin and level of education. For level of education, which was used in the model as an independent predictor of cognitive performance, a scoring of 1-7 was used, with higher scores representing higher academic qualification or years of schooling (Casiglia *et al.*, 2017). Moreover, participants were asked to provide information on medical history, medication, dietary supplement and tobacco use. For this part of the baseline questionnaire, participants also completed the short version of the International Physical Activity Questionnaire (IPAQ). The questionnaire includes seven open-ended questions surrounding individuals' last 7-day recall of physical activity. Based on the IPAQ scoring protocol, the Metabolic Equivalents of Task (METs) in min / week were used to calculate physical activity level (PAL), which was identified as 'Low', 'Moderate' or 'High' (Hallal and Victora, 2004).

3.2.2.2. Sleep Quality

Participant sleep quality was assessed using the Pittsburgh Sleep Quality Index (PSQI), the most frequently used validated questionnaire for measures of sleep (Buysse *et al.*, 1989; Fabbri *et al.*, 2021). The PSQI is a standardised self-rated 24-item questionnaire assessing past-month subjective sleep quality using seven "component" scores, ranging from 0 to 3 subscale scores: sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbance, use of sleeping medication and daytime dysfunction. The sum of scores for these components yields a global score of subjective sleep quality ranging from 0 to 21, with 0–4 indicating "good" sleep and 5–21 indicating "poor" sleep (Buysse *et al.*, 1989).

3.2.2.3. Caffeine and Alcohol Intake

Participants were asked to complete a Food Frequency Questionnaire (EPIC-Norfolk FFQ) to assess dietary intakes. To assess habitual caffeine intake, the EPIC FFQ software was modified to calculate caffeine intake from 11 typical foods and beverages that contain caffeine or cocoa: a) chocolate biscuits, b) buns and pastries, e.g., croissants, c) choc ices, d) chocolates, single or squares, e) chocolate snack bars, f) tea, g) coffee, instant or ground, h) coffee, decaffeinated, i) cocoa, hot chocolate, j) low-calorie fizzy soft drinks and k) fizzy soft drinks (Mulligan *et al.*, 2014). Cup volume and quantities of selected foods were used from the EPIC database and caffeine content was calculated based on published data (Fitt, Pell and Cole, 2013; Malczyk *et al.*, 2021). Where applicable, the average values of weak and strong caffeine / tea infusions were used, as well as the average caffeine content from milk and dark chocolate, or average from food labels of foods in the UK (Fitt, Pell and Cole, 2013; Malczyk *et al.*, 2021).

3.2.3. Cognitive Test Battery

For the second part of the study, participants completed a cognitive test battery on three separate days within a period of two weeks. The battery required a total of 22-25 min to complete, including administration time plus transition time between tasks. The Gorilla Experiment Builder was used to design and host the experiment (Anwyl-Irvine *et al.*, 2020). The test battery consisted of four tasks, one for each key domain of cognition (in order of appearance: social and emotional cognition, memory, attention and psychomotor speed and executive function). As discussed in Chapter 1, the domain of perceptual-motor skills is implicitly assessed in more complex functions, especially in timed computerised tests (Grissmer *et al.*, 2010; Lin *et al.*, 2017), while language assessment is commonly used in the diagnosis of developmental and

neurological disorders (Paulsen, 2011; Charidimou *et al.*, 2014). Hence, language was not included in our selected set of tasks.

Participants were advised to complete the tasks on the same device for all three assessments and at least 5 h after consuming any source of caffeine or alcohol, hypothesising that individuals would be neither under the acute effects of these substances nor under caffeine abstinence when the test battery described below was performed (George *et al.*, 1986; Paton, 2005; Casiglia *et al.*, 2017). They were also advised to complete the test battery in a quiet room, free of distractions.

3.2.3.1. Subjective Sleepiness

The Karolinska Sleepiness Scale (KSS) was used to assess subjective sleepiness before each test session (Åkerstedt and Gillberg, 1990). The KSS is a validated 9-point Likert scale to estimate self-reported momentary level of sleepiness and it ranges from '1' (extremely alert) to '9' (extremely sleepy, fighting sleep) (Kaida *et al.*, 2006).

3.2.3.2. Social and Emotional Cognition

Participants were asked to complete a facial Expression Recognition Task (ERT). Eight faces (half male and half female) representing six basic emotions (anger, fear, sadness, happiness, disgust and surprise) were selected from the Karolinska Directed Emotional Face System (KDEFS) (48 colour pictures in total) (Lundqvist, Flykt and Öhman, 1998). Images were displayed on the screen for 1 s to prevent residual processing of the image. Eight faces were selected for each emotional expression, with both male and female presenters included for each expression (half of the faces selected were male and half were female). Participants were asked to select the emotion displayed by each face from six options (sadness, happiness, fear, anger, disgust, or surprise). The interstimulus interval between faces was 3500 ms. The mean

RT (ms) for correctly identified emotions was used in analyses to calculate the three-day mean RT for emotion recognition of each participant.

3.2.3.3. Memory

Memory was assessed using the letter n-back task, a validated measure of working memory using a sequential-letter memory task with varied memory load (Owen *et al.*, 2005). For this investigation, the n-back task involved the 1-, 2- and 3-back letter tasks. In all conditions, a target is defined as any letter presented on the screen that is identical to the one presented one, two or three trials preceding it, respectively. Each of the three cognitive workload levels consisted of 20 targets and 40 non-targets. Sequences of single consonants were presented (3500 ms interstimulus interval) and participants responded to each stimulus by pressing the 'Yes' button if the stimulus on screen represented a target (33.3% of trials) and a 'No' if the stimulus on screen was a nontarget (66.6% of trials) (Perlstein *et al.*, 2003). Mean response latency for 1-, 2- and 3-back was computed for correct answers, excluding RTs < 100 ms (errors of commission) and the three-day mean memory RT (ms) was used in analyses.

3.2.3.4. Attention & Psychomotor Speed

The Psychomotor Vigilance Task (PVT) is a gold-standard measurement of sustained vigilant attention and psychomotor speed (Dinges and Powell, 1985). This test relies on measuring the RT to a digital time counter on a computer screen that starts to scroll randomly 100 times over a test duration of approximately 10 min. For this task, participants were instructed to focus on the screen and react to the time counter by pressing a response button as soon as a red dot (target) appeared in the centre of the screen. Pressing the response button stopped the counter. The inter-stimulus interval, defined as the period between the last response and the appearance

of the next stimulus, varied randomly from 2 to 10 s (Lim and Dinges, 2008). Responses with RTs < 100 ms (errors of commission) and > 500 ms (lapses) were both omitted from the analyses (Lim and Dinges, 2008). Three-day mean RTs (ms) were used in analyses for attention and psychomotor speed.

3.2.3.5. Executive Function

The Stroop colour and word task was used for selective cognitive flexibility and processing speed, and thus to assess executive function (Stroop, 1935). The administered task consisted of two subsets of stimuli according to Stroop’s first experiment, each composed by 100 items: a) the non-coloured words set, consisting of uppercase Italian words of 10 colours (red, yellow, pink, light blue, orange, black, grey, brown, green and purple) displayed in black colour (neutral stimuli) and b) the coloured words set, consisting of the Italian words of the same colours, with each word displayed in a colour different from that described (e.g., the word ‘RED’ written in blue ink, i.e., ink colour and word differ - incongruent stimuli). In the first part of the task, participants were asked to name the colour of the stimulus (word) on screen. In the second part, also known as the critical condition, participants were asked to name the colour in which the word was written. Participants were informed that there was no time limit to provide an answer for each item; they were requested, however, to complete all the items as fast as possible.

This task is based on the principle that reading words is a more automatic response than naming colours. Automatic processes do not require attention and happen faster than nonautomatic responses. Thus, the response to the word is available faster and interferes with the response to the colour (reduced automatisation), also known as Stroop asynchrony (Scarpina and Tagini, 2017). The three-day mean Stroop effect, i.e., the ability to inhibit a dominant response (i.e., reading) and attend a secondary response (i.e., colour naming) was calculated as the

difference between the mean RT in the coloured words set and the mean RT in the non-coloured words set (Chen, 1997). The three-day mean Stroop effect (Δms) was used in analyses.

3.2.3.6. Global Cognition Score

Considering the diversity of cognitive measures across domains of cognition, a composite global cognition score was computed. The term "global cognition" refers to overall or general mental performance (Llewellyn *et al.*, 2009; Zhou *et al.*, 2018). Measures of global cognitive function are often used for screening of individual and population cognitive status (de Jager et al., 2014; Arevalo-Rodriguez et al., 2015). Global cognition is usually assessed through domain-specific cognitive tasks which are combined in composite scores and aims at producing a more comprehensive representation of cognitive function, allowing comparability of different cognitive domains and different measures within each domain (Llewellyn *et al.*, 2009; Zhou *et al.*, 2018).

In the present study, composite scores were derived to capture global cognitive function. This involved constructing scores by calculating the mean of standardised scores (z scores) from four cognitive tests (n = 4). The utilisation of z scores standardises individual test results by expressing them in terms of SD from the mean, facilitating a meaningful comparison across diverse tests. These composite scores ranged from -3 to 3, with higher values indicating better cognitive function. Notably, individual cognitive test results were coded to have higher scores as indicative of higher cognitive performance. To achieve this, all task RTs were transformed into response speed (1/RT), where higher values correspond to faster performance. This coding and transformation aim to ensure a consistent interpretation across the cognitive measures employed in the study.

3.2.4. Genotyping

For the final part of the study, participants for whom complete data from baseline questionnaire and test battery were available provided a buccal swab (RapiDri™ Swab kit, Isohelix, Kent, UK) for DNA analysis. The swabs were sent at home via post together with a pre-paid return envelope and instructions on sampling.

All laboratory analyses were performed at St Mary's University, Twickenham following routine protocols that have been previously approved by St Mary's Ethics Committee. DNA extraction was performed using PSP® SalivaGene 17 DNA Kit 1011 (STRATEC Molecular, Berlin) following the standard manufacturer protocol. DNA quantification and quality control were assessed with spectroscopy (Nanodrop, ThermoFisher, Waltham, MA, USA). Genotyping of *AHR* rs6968554, *CYP1A2* rs2472297, *ADORA2A* rs5751876, *ADA* rs77819966, and *APOE* rs429358 and rs7412 was performed using TaqMan® SNP genotyping assays (ThermoFisher, Waltham, MA, USA) and a StepOnePlus thermocycler (Applied Biosystems, CA, USA). All samples were analysed in accordance with the manufacturer's protocol. Individual samples were accepted with a quality of > 98%.

Participants who completed the study were requested to provide consent for potential follow-up contact after completing the study to provide feedback on study results and for future studies.

3.2.5. Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 28.0. Data are shown as means ± standard deviation (SD) or medians ± interquartile range (IQR) for normally and non-normally distributed data, respectively. Participants were grouped based on level of habitual caffeine intake in low (0-50 mg/day), moderate (51-300

mg/day) and high (>300 mg/day) caffeine consumers according to previous research (Erblang *et al.*, 2019). Differences across levels of habitual caffeine intake were investigated using a one-way analysis of variance (ANOVA) for normally distributed data and Kruskal-Wallis H test for non-normally distributed data. Differences in categorical data were assessed using the chi square test of independence or Fisher's exact test, for expected cell counts > 5 and < 5, respectively.

Deviations from Hardy Weinberg Equilibrium (HWE) for each SNP were performed using the χ^2 goodness-of-fit test. For genetic caffeine metabolism, an unweighted score was computed for each participant by summing the number of alleles associated with faster caffeine metabolism in *CYP1A2* rs2472297 and *AHR* rs6968554 (0-4), with higher scores indicating faster metabolism. Using this scoring, a score of 0-1 indicated 'slow' and 2-4 indicated 'fast' caffeine metabolism. These two SNPs were selected because they have been presented with the largest effect sizes in GWAS of caffeine metabolites (Cornelis *et al.*, 2016). For genetic caffeine sensitivity, *ADORA2A* genotypes were grouped into caffeine-sensitive (TT group) and non-sensitive (C allele carriers) based on previous findings supporting that the TT genotype of the rs5751876 variant is associated with increases in self-rated anxiety following caffeine consumption (Alsene *et al.*, 2003; Childs *et al.*, 2008; Rogers *et al.*, 2010). Finally, an unweighted overall caffeine genetic score was constructed by summing the number of caffeine metabolism alleles in *CYP1A2* and *AHR* and caffeine sensitivity alleles in *ADORA2A* gene for each participant (0-6), with higher scores indicating faster caffeine metabolism and lower caffeine sensitivity. Using this scoring, a score of 0-2 indicated 'slow - sensitive' individuals and a score of 3-6 indicated 'fast - nonsensitive' individuals.

The *APO* ε4+/ε4- genotype was determined by the combinations of genotypes at rs429358 and rs7412, as shown in Table 3.1. The *ADA* rs77819966 and *APO* ε4+/ε4-

were used in the analyses separately, as proxies of sleep quality and risk of cognitive impairment, respectively.

Table 3. 1 *APOE* genotype determination.

***APOE* genotype**	**rs429358**	**rs7412**
APOε1/ε1	CC	TT
APOε1/ε2	CT	TT
APOε1/ε3	CT	CT
APOε1/ε4	CC	CT
APOε2/ε2	TT	TT
APOε2/ε3	TT	CT
APOε2/ε4	CT	CT
APOε3/ε3	TT	CC
APOε3/ε4	CT	CC
APOε4/ε4	CC	CC

Differences between genotype groups ('slow' vs 'fast' metabolisers, caffeine 'sensitive' vs 'non-sensitive' and 'slow - sensitive' vs 'fast - nonsensitive' individuals were investigated using independent samples t-test for normally distributed data and Mann-Whitney U test for non-normal data. The genetic caffeine score x caffeine, the genetic caffeine metabolism x caffeine and genetic caffeine sensitivity x caffeine interactions on indices of cognition were assessed using a two-way ANOVA with Bonferroni adjustments for multiple comparisons.

The relationship between caffeine, genetics and cognitive indices was investigated through multiple regression analysis, having included the potential enhancers or depressors of cognitive function discussed previously (age, sex, BMI, PAL, level of education, subjective sleep quality, subjective sleepiness prior to tasks, tobacco and alcohol use, *ADA* rs77819966 and *APOE* ε4-/ε4+ status). Statistical significance was assumed at the 5% level.

3.3. Results

3.3.1. Participant characteristics across levels of caffeine intake

Of the 247 eligible participants who signed up for the study, 181 completed the online questionnaire (72%) and 131 participants completed the full series of online cognitive tasks and provided a swab sample for analysis (53%). Descriptive characteristics of study participants are presented in Table 3.2.

The study sample consisted of participants aged between 23 and 64 years of mostly (94%) Caucasian/white descent (Table 3.1). Most of the participants (74%) had a moderate PAL and a normal BMI of 18.5 – 24.9 kg/m^2 (n= 74, 57%), while 5 participants (4%) were underweight (< 18.5 kg/m^2) and 50 participants (39%) overweight or obese (> 25 kg/m^2). All participants consumed alcohol within the safe levels, which is less than 10 standard drinks, or less than 100 g of alcohol per week (Conigrave *et al.*, 2021). Genotype frequencies for the *CYP1A2* rs2472297, *AHR* rs6968554, *ADORA2A* rs5751876, *ADA* rs77819966 and the *APOE* rs429358 and rs7412 did not deviate from HWE ($p_{\text{all}} > 0.05$).

Among the participants who completed the study, two were excluded from the attention analyses because their RTs to the optical stimulus were above 500 ms on all three days of the experiment. Data from 12 participants were also excluded from the executive function analyses because in the second part of the task they continued to indicate the written word, instead of the colour of the word on the screen. Consequently, 14 participants were not included in the analyses of global cognition because they had missing values either for executive function (n = 12) or attention (n = 2), resulting in 117 participants. Inspection of extracted data verified that all participants completed the tasks on the same device for all three assessments.

Using a box plot visualisation, two participants were defined as outliers for extremely high caffeine intake and were excluded. The median daily caffeine consumption was 184.5 ± 153.9 mg/day. Nineteen participants (15%) pertained to the low (0-50 mg/day) caffeine consumer group, 85 (66%) to the moderate (51-300 mg/day) and 25 (19%) to the high (>300 mg/day) caffeine consumers. Participant demographics did not differ across the levels of caffeine intake. What varied among the groups was three-day subjective sleepiness before tasks, habitual alcohol intake and caffeine intake ($p_{all} < 0.05$), as assessed by the Kruskal-Wallis test using Dunn's procedure with a Bonferroni correction for multiple comparisons. Post hoc analysis for alcohol intake revealed statistically significantly higher median alcohol intakes in moderate (21.8 ± 37.1 g/week) compared with high (5.6 ± 27.1 g/week) caffeine intake groups ($p = 0.026$).

Table 3. 2 Participant characteristics by total sample and level of habitual caffeine consumption.

	All (N = 129)	L (N = 19)	M (N = 85)	H (N = 25)	*P**
Age[b], years	35.0 ± 15.5	33.0 ± 9.0	34.0 ± 15.5	37.0 ± 13.5	0.261
Gender, F / M (%)	90 (69.8) / 39 (30.2)	14 (73.7) / 5 (26.3)	62 (72.9) / 23 (27.1)	14 (56.0) / 11 (44.0)	0.248
BMI[b] (kg/m^2)	23.9 ± 6.5	25.5 ± 9.7	23.7 ± 5.2	24.2 ± 9.1	0.373
Ethnicity, white / non-white (%)	121 (93.8) / 8 (6.2)	16 (84.2) / 3 (15.8)	80 (94.1) / 5 (5.9)	25 (100.0) / 0 (0.0)	0.106
Education level[b]	6.0 ± 1.0	5.0 ± 1.0	6.0 ± 1.0	5.0 ± 1.0	0.427
PAL, L / M / H (%)	12 (9.8) / 94 (73.8) / 23 (16.4)	1 (5.3) / 15 (78.9) / 3 (15.8)	7 (8.2) / 62 (72.9) / 16 (18.8)	4 (16.0) / 17 (68.0) / 4 (16.0)	0.789
Smoking status, No / Yes (%)	110 (85.3) / 19 (14.7)	19 (100.0) / 0 (0.0)	71 (83.5) / 14 (16.5)	20 (80.0) / 5 (20.0)	0.115
Sleep Quality, Poor / Good (%)	82 (63.6) / 47 (36.4)	11 (57.9) / 8 (42.1)	55 (64.7) / 30 (35.3)	16 (64.0) / 9 (36.0)	0.855
PSQI[b]	5.0 ± 3.0	5.0 ± 3.0	5.0 ± 4.0	6.0 ± 4.0	0.283
KSS[b]	5.0 ± 3.0	5.0 ± 4.0	5.0 ± 3.0	5.0 ± 1.0	0.046
Exam time, before 12 pm / after 12 pm (%)	19 (14.7) / 110 (85.3)	4 (21.1) / 15 (78.9)	11 (12.9) / 74 (87.1)	4 (16.0) / 21 (84.0)	0.576
Alcohol intake[b] (g/week)	14.5 ± 33.0	8.9 ± 12.2	21.8 ± 37.1	5.6 ± 27.1	0.017
Caffeine intake[b] (mg/day)	184.5 ± 153.9	11.6 ± 22.1	176.9 ± 120.8	382.8 ± 116.4	<0.001
AHR genotype (%)					
AA	17 (13.2)	2 (10.5)	13 (15.3)	2 (8.0)	
AG	70 (54.3)	11 (57.9)	44 (51.8)	15 (60.0)	0.927
GG	42 (32.6)	6 (31.6)	28 (32.9)	8 (32.0)	
CYP1A2 genotype (%)					
CC	102 (79.1)	17 (89.5)	66 (77.6)	19 (70.4)	
CT	23 (17.8)	2 (10.5)	15 (17.6)	8 (29.6)	0.641
TT	4 (3.1)	0 (0.0)	4 (4.8)	0 (0.0)	
ADORA2A genotype, sensitive / non-sensitive (%)	30 (23.3) / 99 (76.7)	5 (26.3) / 14 (73.7)	16 (18.8) / 69 (81.2)	9 (36.0) / 16 (64.0)	0.191
ADA genotype, GG / AG (%)	126 (97.7) / 3 (2.3)	19 (100.0) / 0 (0.0)	83 (97.6) / 2 (2.4)	24 (96.0) / 1 (4.0)	0.717
APOE genotype, ε4 carriers / ε4 noncarriers (%)	29 (22.5) / 100 (77.5)	6 (31.6) / 13 (68.4)	14 (16.5) / 71 (83.5)	9 (36.0) / 16 (64.0)	0.060

a Values represent means ± SD; b Values represent medians ± IQR; * P values represent differences across the three levels of caffeine intake, assessed by one-way ANOVA, Kruskal-Wallis H test or chi square test; L: Low; M: Moderate; H: High; PAL: Physical Activity Level; BMI: Body Mass Index; PSQI: Pittsburgh Sleep Quality Index; KSS: Karolinska Sleepiness Scale; Low, moderate, and high caffeine intake: 0-50, 51–300, and > 300 mg/day, respectively.

For subjective sleepiness before tasks, post hoc analysis revealed statistically significantly higher median subjective sleepiness scores in high (5.0 ± 1.0) compared with low (5.0 ± 4.0) (p = 0.046) caffeine intake groups. Finally, post hoc analysis for caffeine intake showed statistically significantly higher median caffeine intake in high (382.8 ± 116.4 mg/day) compared with moderate (176.9 ± 120.8 mg/day) and low (11.6 ± 22.1 mg/day) caffeine intake groups ($p_{all} < 0.001$).

3.3.2 Participant characteristics across genetic groups

In the present sample, MAF for *CYP1A2* rs2472297 (T allele) was 0.12, which is lower than the expected for a European population and 0.40 for *AHR* rs6968554 (A allele), which is in line with published data for a population of European descent. The MAF for *ADORA2A* rs5751876 (T allele) was 0.43, which is slightly higher than published data for Europeans (Cunningham *et al.*, 2022).

3.3.2.1. Genetic caffeine metabolism

Two SNPs previously associated with caffeine plasma metabolites (*CYP1A2* rs2472297 and *AHR* rs6968554) (Cornelis *et al.*, 2016) were used to group participants based on genetic caffeine metabolism score. Using this classification, 71 (55%) participants were identified as 'slow' caffeine metabolisers and 58 (45%) were identified as 'fast' metabolisers. Participant characteristics did not differ between groups, apart from the level of habitual caffeine consumption, as assessed by Mann-Whitney U test (Table 3.3). Habitual caffeine intake for 'fast' metabolisers was statistically significantly higher than in 'slow' metabolisers (212.3 ± 203.4 vs 124.0 ± 135.1), U = 2482.5, p = 0.045.

Table 3. 3 Participant characteristics by genetic caffeine metabolism.

	slow (N = 71)	fast (N = 58)	*P**
Age[b], years	36.0 ± 16.0	34.0 ± 12.0	0.264
Gender, F / M (%)	47 (66.2) / 24 (33.8)	43 (74.1) / 15 (25.9)	0.329
BMI[b] (kg/m^2)	27.2 ± 7.9	24.3 ± 4.4	0.549
Ethnicity, white / non-white (%)	65 (91.5) / 6 (8.5)	56 (96.6) / 2 (3.4)	0.294
Education Level[b]	5.0 ± 1.0	6.0 ± 1.0	0.142
PAL, L / M / H (%)	6 (8.5) / 49 (69.0) / 16 (22.5)	6 (10.3) / 45 (77.6) / 7 (12.1)	0.300
Smoking status, No / Yes (%)	61 (85.9) / 10 (14.1)	49 (84.5) / 9 (15.5)	0.819
Sleep Quality, Poor / Good (%)	41 (57.7) / 30 (42.3)	41 (70.7) / 17 (29.3)	0.129
PSQI[b]	5.0 ± 4.0	6.0 ± 3.3	0.088
KSS[b]	5.0 ± 3.0	5.0 ± 2.0	0.865
Exam time, before 12 pm / after 12 pm (%)	13 (18.3) / 58 (81.7)	13 (22.4) / 45 (77.6)	0.563
Alcohol intake[b] (g/week)	16.4 ± 34.1	12.8 ± 33.3	0.972
Caffeine intake[b] (mg/day)	124.0 ± 135.1	212.3 ± 203.4	0.045
ADORA2A genotype, sensitive / non-sensitive (%)	12 (16.9) / 59 (83.1)	18 (31.0) / 40 (69.0)	0.059
ADA genotype, GG / AG (%)	71 (100.0) / 0 (0.0)	55 (94.8) / 3 (5.2)	0.088
APOE genotype, ε4 carriers / ε4 noncarriers (%)	16 (22.5) / 55 (77.5)	13 (22.4) / 45 (77.6)	0.987

a Values represent means ± SD; b Values represent medians ± IQR; Participants are categorised according to AHR+CYP1A2 genotypes ('slow' or 'fast' metabolisers); * P values represent differences between genotype groups, assessed by independent samples t-test, Mann-Whitney U test or chi square test; BMI: Body Mass Index; PAL: Physical Activity Level; L: Low; M: Moderate; H: High; PSQI: Pittsburgh Sleep Quality Index; KSS: Karolinska Sleepiness Scale.

3.3.2.2. Genetic caffeine sensitivity

The *ADORA2A* SNP, previously associated with caffeine-induced anxiety (Alsene *et al.*, 2003; Childs *et al.*, 2008) was used to group participants in caffeine 'sensitive' and 'non-sensitive' individuals. Using this classification, 30 (24%) participants were classified as 'sensitive' and 99 (76%) were classified as 'non-sensitive' individuals. Participant characteristics were not different between groups, as assessed by Mann-Whitney U test (Table 3.4).

Table 3. 4 Participant characteristics by *ADORA2A* groups.

	sensitive (N = 30)	non-sensitive (N = 99)	*P**
Age[b], years	34.50 ± 15.3	35.0 ± 13.0	0.441
Gender, F / M (%)	21 (70.0) / 9 (30.0)	69 (69.7) / 30 (30.3)	0.975
BMI[b] (kg/m^2)	24.1 ± 6.4	23.9 ± 6.5	0.585
Ethnicity, white / non-white (%)	29 (96.7) / 1 (3.3)	92 (92.9) / 7 (7.1)	0.457
Education Level[b]	5.5 ± 1.0	6.0 ± 1.0	0.696
PAL, L / M / H (%)	3 (10.0) / 25 (83.3) / 2 (6.7)	9 (9.7) / 69 (69.7) / 21 (21.2)	0.162
Smoking status, No / Yes (%)	27 (90.0) / 3 (10.0)	83 (83.8) / 16 (16.2)	0.819
Sleep Quality, Poor / Good (%)	22 (73.3) / 8 (26.7)	60 (60.6) / 39 (39.4)	0.204
PSQI[b]	6.0 ± 3.0	5.0 ± 5.0	0.455
KSS[b]	5.0 ± 3.0	5.0 ± 2.0	0.844
Exam time, before 12 pm / after 12 pm (%)	5 (16.7) / 25 (83.3)	14 (14.1) / 85 (85.9)	0.732
Alcohol intake[b] (g/week)	16.3 ± 37.7	14.5 ± 32.3	0.682
Caffeine intake[b] (mg/day)	200.3 ± 216.2	177.6 ± 146.4	0.272
'Slow' / 'fast' metabolisers	12 (40.0) / 18 (60.0)	59 (59.6) / 40 (40.4)	0.059
ADA genotype, GG / AG (%)	30 (100.0) / 0 (0.0)	96 (97.0) / 3 (3.0)	0.584
APOE genotype, ε4 carriers / ε4 noncarriers (%)	7 (23.3) / 23 (76.7)	22 (22.2) / 77 (77.8)	1.000

a Values represent means ± SD; b Values represent medians ± IQR; Participants are categorised according to ADORA2A genotypes (caffeine 'sensitive' or' non-sensitive'); * P values represent differences between the ADORA2A groups, assessed by independent samples t-test, Mann-Whitney U test or chi square test; BMI: Body Mass Index; PAL: Physical Activity Level; L: Low; M: Moderate; H: High; PSQI: Pittsburgh Sleep Quality Index; KSS: Karolinska Sleepiness Scale.

3.3.2.3. Overall genetic caffeine score

To create an overall caffeine genetic score, participants were grouped based on both caffeine metabolism and caffeine sensitivity SNPs. Following this grouping, 63 (48%) participants were classified as 'slow - sensitive' and 66 (52%) were classified as 'fast - nonsensitive' individuals. Participant characteristics were not different between groups, as assessed by Mann-Whitney U test (Table 3.5).

Table 3. 5 Participant characteristics by overall genetic caffeine score.

	slow - sensitive (N = 63)	fast - nonsensitive (N = 66)	*P**
Age[b], years	35.0 ± 12.0	34.5 ± 13.5	0.858
Gender, F / M (%)	40 (63.5) / 23 (36.5)	50 (75.8) / 16 (24.2)	0.129
BMI[b] (kg/m^2)	24.1 ± 6.1	23.7 ± 6.9	0.296
Ethnicity, white / non-white (%)	58 (92.1) / 5 (7.9)	63 (95.5) / 3 (4.5)	0.425
Education Level[b]	5.0 ± 1.0	6.0 ± 1.0	0.153
PAL, L / M / H (%)	5 (7.9) / 45 (71.4) / 13 (20.6)	7 (10.6) / 49 (74.2) / 10 (15.2)	0.662
Smoking status, No / Yes (%)	54 (85.7) / 9 (14.3)	56 (84.8) / 10 (15.2)	0.890
Sleep Quality, Poor / Good (%)	41 (65.1) / 22 (34.9)	41 (62.1) / 25 (37.9)	0.727
PSQI[b]	5.0 ± 3.0	5.0 ± 4.0	0.818
KSS[b]	5.0 ± 3.0	5.0 ± 2.0	0.482
Exam time, before 12 pm / after 12 pm (%)	12 (19.0) / 51 (81.0)	7 (10.6) / 59 (89.4)	0.176
Alcohol intake[b] (g/week)	16.2 ± 32.7	12.8 ± 35.1	0.691
Caffeine intake[b] (mg/day)	177.6 ± 141.6	185.5 ± 154.0	0.925
ADA genotype, GG / AG (%)	63 (100.0) / 0 (0.0)	63 (95.5) / 3 (4.5)	0.087
APOE genotype, ε4 carriers / ε4 noncarriers (%)	19 (30.2) / 44 (69.8)	10 (15.2) / 56 (84.8)	0.057

a Values represent means ± SD; b Values represent medians ± IQR; Participants categorised according to genetic caffeine score derived from CYP1A2, AHR and ADORA2A genes ('slow-sensitive' or 'fast-nonsensitive); * P values represent differences between the genetic caffeine score groups, assessed by independent samples t-test, Mann-Whitney U test or chi square test; BMI: Body Mass Index; PAL: Physical Activity Level; L: Low; M: Moderate; H: High; PSQI: Pittsburgh Sleep Quality Index; KSS: Karolinska Sleepiness Scale.

3.3.3. Cognitive performance

3.3.3.1. Genetic caffeine metabolism

The possible interactions between genetic caffeine metabolism and levels of caffeine intake on indices of cognition were investigated and results are shown in Table 3.5 and Figures 3.2 – 3.4. Within 'slow' metabolisers, 12 participants were low caffeine consumers, 48 participants were moderate and 11 participants were high caffeine consumers. Within 'fast' metabolisers, seven participants were low, 37 participants were moderate and 14 participants were high caffeine consumers.

Two-way ANOVAs were conducted to examine the associations of habitual caffeine intake and genetic caffeine metabolism on cognitive performance. All pairwise comparisons were run for each simple main effect with reported 95% CIs. All p-values were Bonferroni-adjusted within each simple main effect and were accepted at the $p < 0.025$ level for two simple main effects and $p < 0.017$ for three simple main effects.

There was a statistically significant interaction between levels of habitual caffeine intake and genetic caffeine metabolism for emotion recognition, $F(2, 123) = 5.848$, $p = 0.004$ and executive function, $F(2, 109) = 3.690$, $p = 0.028$, but not for any other indices of cognition. A statistically significant difference was found in mean emotion recognition RTs between 'slow' and 'fast' metabolisers in high caffeine consumers, $F(1, 123) = 8.835$, $p = 0.004$. Within high caffeine consumers, mean emotion recognition RTs for 'fast' metabolisers were 246.3 (95% CI, 82.290 to 410.371) ms higher compared with 'slow' metabolisers (1604.5 ± 263.8 ms vs 1358.2 ± 204.5 ms, respectively, $p = 0.004$). No differences were observed between low and moderate caffeine consumers. There was also a statistically significant difference in mean emotion recognition RTs among levels of caffeine intake in 'fast' metabolisers, $F(2, 123) = 8.512$, $p < 0.001$. Within 'fast' metabolisers, mean emotion recognition RTs for high caffeine consumers were 276.9 (95% CI, 45.797 to 507.992) ms higher compared with low (1604.5 ± 263.8 ms vs 1327.6 ± 136.7 ms, respectively, $p = 0.013$) and 256.8 (95% CI, 100.139 to 413.431) ms higher compared with moderate (1604.5 ± 263.8 ms vs 1347.7 ± 187.6 ms, respectively, $p < 0.001$) caffeine consumers. No differences were observed within 'slow' metabolisers.

Table 3. 6 Genetic caffeine metabolism x caffeine interactions on social and emotional cognition, memory, attention, executive function and global cognition.

	slow			**fast**			*F*	*P**
	L (12)	M (48)	H (11)	L (7)	M (37)	H (14)		
Attention (ms)	406.0 ± 50.8	390.7 ± 45.9	383.5 ± 32.9	395.4 ± 50.8	386.6 ± 48.1	401.8 ± 41.4	0.668	0.515
Emotion recognition (ms)	1447.0 ± 221.6	1400.6 ± 204.3	1358.1 ± 204.5	1327.6 ± 136.7	1347.7 ± 187.6	1604.5 ± 263.8	5.848	0.004
Memory (ms)	999.6 ± 342.8	1083.3 ± 188.0	1103.5 ± 197.5	1038.6 ± 287.7	1076.5 ± 155.2	1253.1 ± 276.6	1.260	0.287
Stroop effect (Δms)	417.6 ± 217.6	549.3 ± 330.9	369.1 ± 285.3	453.5 ± 203.3	549.3 ± 330.9	501.3 ± 337.1	3.690	0.028
Global cognition score	-0.121 ± 0.844	-0.055 ± 0.569	0.035 ± 0.411	0.169 ± 0.698	0.166 ± 0.507	-0.345 ± 0.636	2.561	0.082

a Values represent means ± SD; P values represent the differences across the caffeine intake groups stratified by genetic caffeine metabolism groups, assessed by two-way ANOVA. L: Low habitual caffeine intake (0-50mg/day); M: Moderate habitual caffeine intake (51-300mg/day); H: High habitual caffeine intake (>300mg/day); Stroop effect is the measure of executive function; ms: milliseconds; Δms: difference in ms.

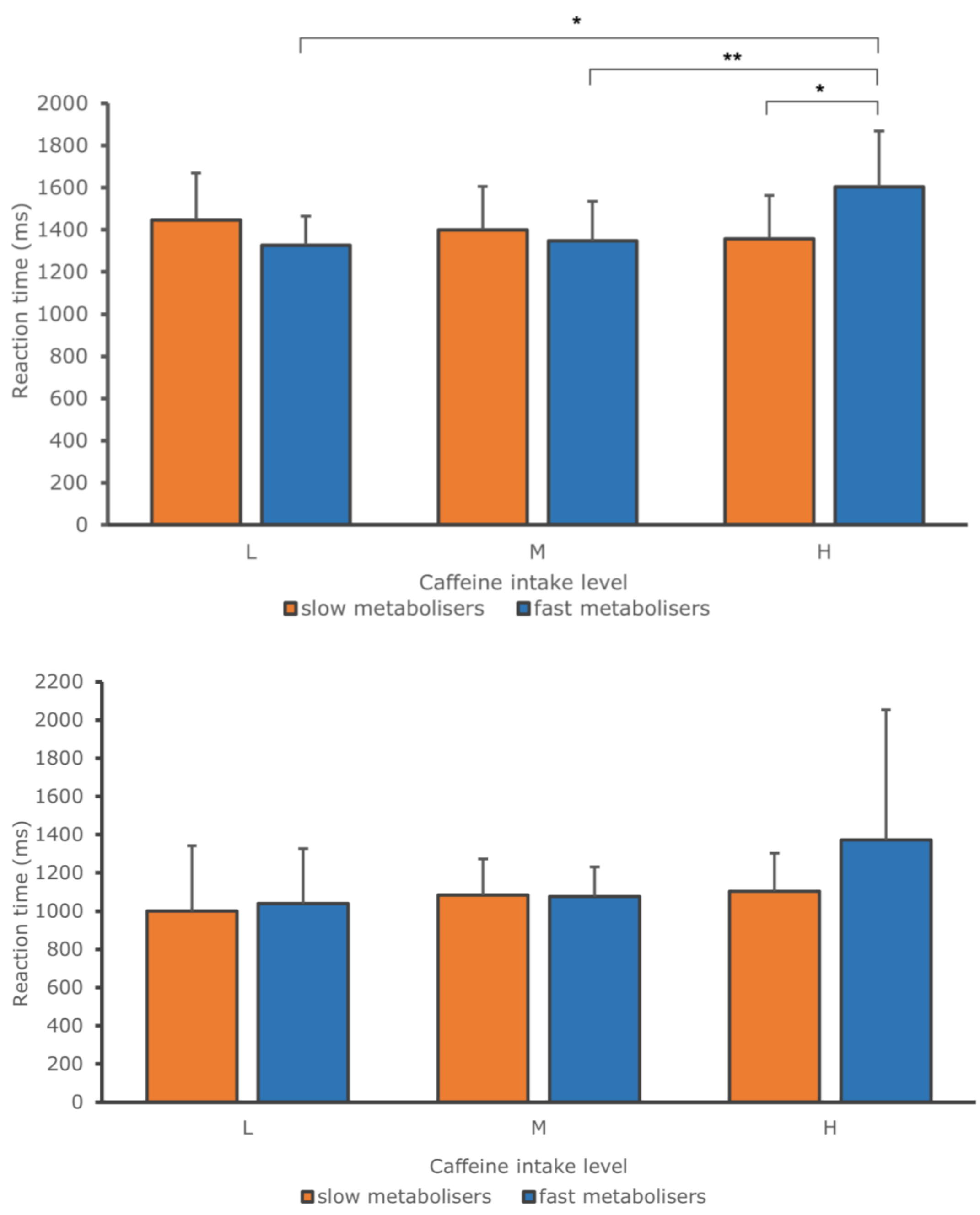

Figure 3. 2 Emotion recognition (above) and memory (below) mean RTs for 'slow' (orange bars) and 'fast' (blue bars) metabolisers for levels of caffeine intake (low vs moderate vs high). Error bars indicate standard deviations. * $p < 0.05$; ** $p < 0.01$.

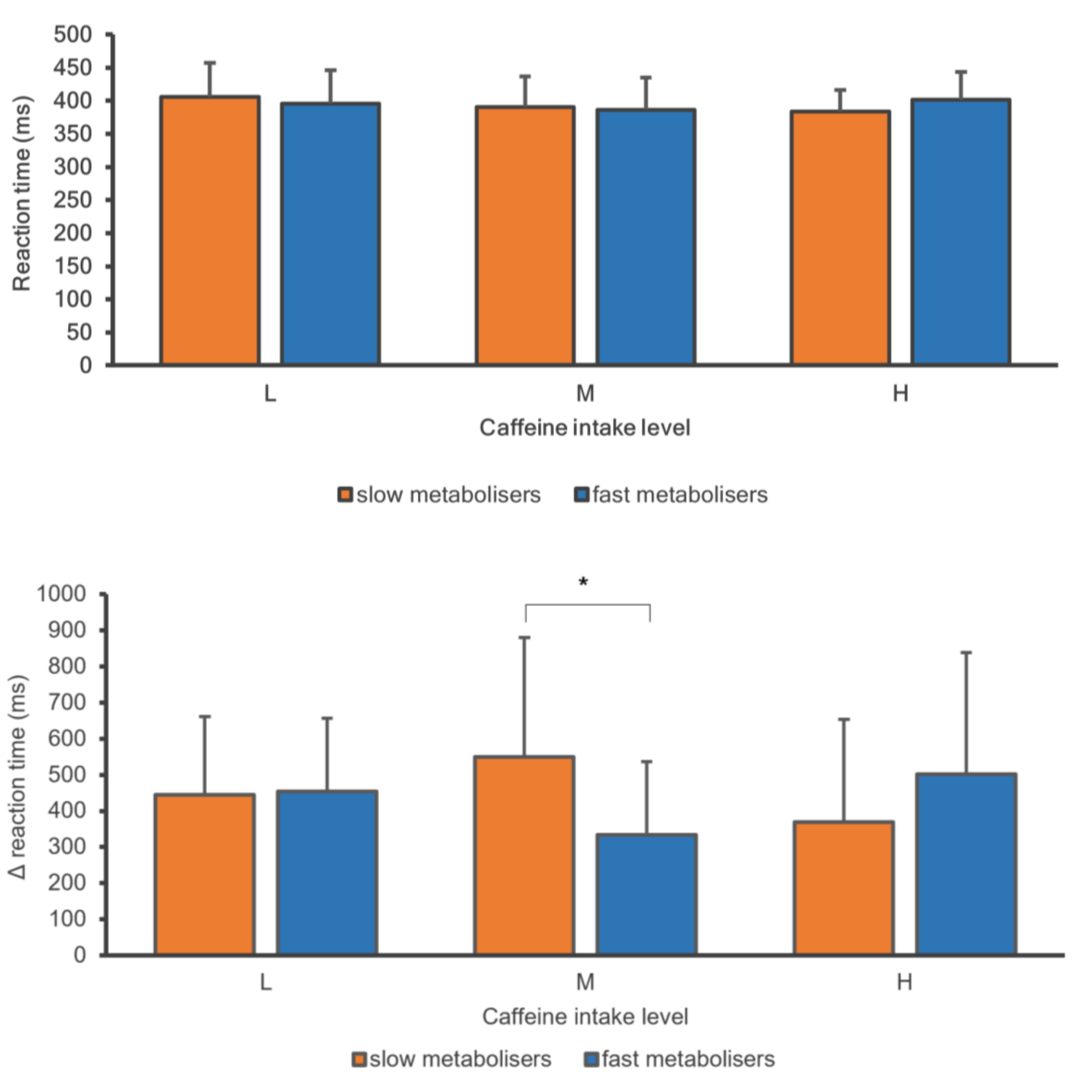

Figure 3. 3 Attention mean RTs (above) and Stroop task mean difference in RT between colour naming and word naming for 'slow' (orange bars) and 'fast' (blue bars) metabolisers for levels of caffeine intake (low vs moderate vs high). Error bars indicate standard deviations. * $p < 0.05$.

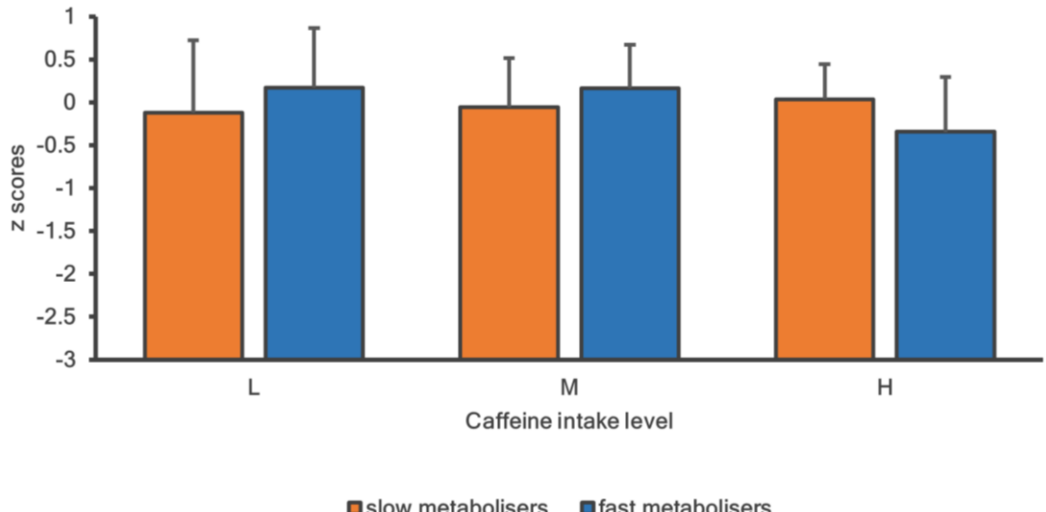

Figure 3. 4 Global cognitive performance scores for 'slow' (orange bars) and 'fast' (blue bars) metabolisers for levels of caffeine intake (low vs moderate vs high). Error bars indicate standard deviations.

There was a statistically significant difference in mean executive function ΔRTs between 'slow' and 'fast' metabolisers in moderate caffeine consumers, F (1, 109) = 10.583, p = 0.002. Within moderate caffeine consumers, the mean executive function ΔRTs for 'slow' metabolisers was 215.9 (95% CI, 84.374 to 347.479) ms higher compared with 'fast' metabolisers (549.3 ± 330.9 ms vs 333.4 ± 203.6 ms, respectively, p = 0.002). No differences were observed between low and high caffeine consumers.

3.3.3.2. Genetic caffeine sensitivity

No comparisons were performed between caffeine 'sensitive' and 'non-sensitive' individuals based on the level of habitual caffeine intake, since there were only three participants in the 'sensitive - low caffeine' intake group.

3.3.3.3. Genetic caffeine score

The possible interactions between the overall genetic caffeine score and levels of caffeine intake on indices of cognition was investigated and results are shown in Table 3.6. Within 'slow – sensitive' individuals, 11 participants were low caffeine consumers, 37 participants were moderate and 15 participants were high caffeine

consumers. Within 'fast – nonsensitive' individuals, 8 participants were low, 48 participants were moderate and 10 participants were high caffeine consumers.

Table 3. 7 Genetic caffeine score x caffeine interactions on social and emotional cognition, memory, attention, executive function and global cognition.

	slow - sensitive			**fast - nonsensitive**			*F*	*P**
	L (11)	M (37)	H (15)	L (8)	M (48)	H (10)		
Attention (ms)	412.7 ± 44.6	384.4 ± 50.1	379.6 ± 31.8	387.5 ± 55.5	392.5 ± 44.0	412.8 ± 39.2	2.158	0.120
Emotion recognition (ms)	1479.7 ± 195.3	1395.6 ± 205.9	1448.1 ± 256.1	1297.5 ± 160.4	1363.6 ± 192.3	1568.1 ± 277.3	2.734	0.069
Memory (ms)	1086.8 ± 327.5	1034.2 ± 158.9	1166.7 ± 281.4	913.7 ± 289.0	1116.0 ± 177.5	1218.2 ± 210.3	2.742	0.068
Stroop effect (Δms)	417.6 ± 211.3	456.0 ± 280.6	385.0 ± 304.4	522.4 ± 192.6	472.3 ± 342.9	552.1 ± 325.7	0.571	0.566
Global cognition score	-0.232 ± 0.721	0.035 ± 0.604	0.014 ± 0.341	0.269 ± 0.827	0.035 ± 0.508	-0.457 ± 0.547	3.171	0.046

a Values represent means ± SD; * P values represent the differences across the caffeine intake groups stratified by genetic caffeine score groups, assessed by two-way ANOVA. L: Low habitual caffeine intake (0-50mg/day); M: Moderate habitual caffeine intake (51-300mg/day); H: High habitual caffeine intake (>300mg/day); Stroop effect is the measure of executive function; ms: milliseconds; Δms: difference in ms.

Two-way ANOVAs were conducted to examine the association between habitual caffeine intake and genetic caffeine score on cognitive performance indices. Data are mean ± SD. Residual analysis was performed to test for the assumptions of the two-way ANOVA. Outliers were assessed by inspection of a boxplot, normality was assessed using Shapiro-Wilk's normality test for each cell of the design and homogeneity of variances was assessed by Levene's test. There were no outliers, residuals were normally distributed ($p > 0.05$) and there was homogeneity of variances ($p_{all} > 0.05$).

There was a statistically significant interaction between caffeine and genetic caffeine score on global cognition, $F(2, 107) = 3.171$, $p = 0.046$. Therefore, all simple main effects were performed with statistical significance receiving a Bonferroni adjustment and being accepted at the $p < 0.025$ level. After Bonferroni correction, no simple main effects were shown for global cognition, $p_{all} > 0.025$.

3.3.3.4. Predictors of cognitive performance

Multiple regressions (n = 5) were run to predict cognitive performance in all indices of cognition from age, sex, BMI, habitual caffeine intake, caffeine genetic

score, PAL, level of education, subjective sleep quality, subjective sleepiness prior to tasks, tobacco and alcohol use, genetic sleep quality and genetic risk for cognitive decline. Linearity of predictors against the predicted values was assessed by partial regression plots and variables with no linear relationship were excluded from the models. In all reported models, there was independence of residuals, as assessed by Durbin-Watson statistic. There was homoscedasticity, as assessed by visual inspection of a plot of studentised residuals versus unstandardised predicted values. There was no evidence of multicollinearity, as assessed by tolerance values greater than 0.1. There were no studentised deleted residuals greater than ± 3 SD, no leverage values greater than 0.2, and values for Cook's distance above 1. The assumption of normality was met, as assessed by Q-Q Plots. Results from multiple regressions for all cognitive indices are shown below.

3.3.3.4.1. Social and emotional cognition

Caffeine, age and subjective sleep quality score prior to tasks statistically significantly predicted emotion recognition RTs, F (3, 125) = 4.649, p = 0.004, accounting for 10% of the variation in emotion recognition performance with adjusted R^2 = 7.9%, indicating a trivial effect (Cohen, 1988). An additional year of age leads to an 8.4 (95% CI, 1.773 to 9.443, p = 0.004) ms increase in RT in emotion recognition. Regression coefficients and standard errors can be found in Table 3.8.

Table 3. 8 Multiple regression results for performance in emotion recognition (n=129).

		95% CI for B							
Model	*B*	*LL*	*UL*	*SE B*	*β*	*t*	*P*	R^2	ΔR^2
Constant	1114.383	952.835	1275.931	81.626		13.652	<0.001	0.100	0.079
Caffeine	0.204	-0.083	0.491	0.145	0.121	1.405	0.163		
PSQI	8.396	-4.316	21.108	6.423	0.111	1.307	0.194		
Age	5.608	1.773	9.443	1.938	0.249	2.894	0.004		

Model = "Enter" method; PSQI: Pittsburgh Sleep Quality Index; *B*: unstandardised regression coefficient; CI: Confidence Interval; *LL*: lower limit; *UL*: upper limit; *SE B*: standard error of the coefficient; *β*: standardised coefficient; R^2: coefficient of determination; ΔR^2: adjusted R^2.

3.3.3.4.2. Memory

Caffeine, genetic caffeine metabolism, age, BMI, subjective sleep quality score, level of education, alcohol intake and subjective sleepiness prior to tasks statistically significantly predicted memory RTs, F (8, 120) = 2.988, p = 0.004, accounting for 16.6% of the variation in memory performance with adjusted R^2 = 11.1%, indicating a trivial effect (Cohen, 1988). An additional year of age leads to a 7.5 (95% CI, 13.444

to 11.562, $p < 0.001$) ms increase in RT in memory. Regression coefficients and standard errors can be found in Table 3.9.

Table 3. 9 Multiple regression results for performance in memory (n=129).

Model	*B*	95% CI for B		*SE B*	*β*	*t*	*P*	R^2	ΔR^2
		LL	*UL*						
Constant	622.027	356.458	887.595	134.130		4.637	<0.001	0.166	0.111
Genetic caffeine score	8.702	-25.690	43.094	17.370	0.043	0.501	0.617		
Caffeine	0.232	-0.070	0.534	0.153	0.135	1.521	0.131		
BMI	-0.190	-6.588	6.209	3.232	-0.005	-0.059	0.953		
PSQI	2.351	-10.798	15.500	6.641	0.030	0.354	0.724		
Alcohol	2.742	-8.078	13.563	5.465	0.043	0.502	0.617		
Education score	11.726	-12.229	35.680	12.099	0.084	0.969	0.334		
Age	7.503	3.444	11.562	2.050	0.327	3.660	<0.001		
KSS	11.140	-12.398	34.678	11.888	0.084	0.937	0.351		

Model = "Enter" method; Genetic caffeine score derived from CYP1A2, AHR and ADORA2A genes, with higher scores indicating faster caffeine metabolism and low sensitivity; BMI: Body Mass Index; PSQI: Pittsburgh Sleep Quality Index; KSS: Karolinska Sleepiness Scale; *B*: unstandardised regression coefficient; CI: Confidence Interval; *LL*: lower limit; *UL*: upper limit; *SE B*: standard error of the coefficient; *β*: standardised coefficient; R^2: coefficient of determination; ΔR^2: adjusted R^2.

3.3.3.4.3. Attention

Age and subjective sleepiness prior to tasks statistically significantly predicted attention RTs, F (2, 124) = 3.477, $p = 0.034$, accounting for 5.3% of the variation in attention performance with adjusted R^2 = 3.8%, indicating a trivial effect (Cohen, 1988). An additional year of age leads to an 8.9 (95% CI, 0.061 to 1.715, $p = 0.036$) ms increase in RT in attention. Regression coefficients and standard errors can be found in Table 3.10.

Table 3. 10 Multiple regression results for performance in attention (n=127).

Model	*B*	95% CI for B		*SE B*	*β*	*t*	*P*	R^2	ΔR^2
		LL	*UL*						
Constant	340.105	300.536	379.674	19.992		17.012	<0.001	0.053	0.038
Age	0.888	0.061	1.715	0.418	0.186	2.125	0.036		
KSS	4.170	-0.546	8.886	2.383	0.154	1.750	0.083		

Model = "Enter" method; KSS: Karolinska Sleepiness Scale; *B*: unstandardised regression coefficient; CI: Confidence Interval; *LL*: lower limit; *UL*: upper limit; *SE B*: standard error of the coefficient; *β*: standardised coefficient; R^2: coefficient of determination; ΔR^2: adjusted R^2.

3.3.3.4.4. Executive function

Age, BMI and subjective sleepiness prior to tasks statistically significantly predicted executive function ΔRTs, F (3, 111) = 4.136, p = 0.008, accounting for 10.1% of the variation in executive function performance with adjusted R^2 = 7.6%, indicating a trivial effect (Cohen, 1988). An additional BMI unit leads to a 10.9 (95% CI, 1.958 to 20.006, p = 0.018) Δms increase in RT in executive function. Regression coefficients and standard errors can be found in Table 3.11.

Table 3. 11 Multiple regression results for executive function (n=115).

		95% CI for B							
Model	*B*	*LL*	*UL*	*SE B*	*β*	*t*	*P*	R^2	ΔR^2
Constant	174.543	- 140.515	489.601	158.994		1.098	0.275	0.101	0.076
Age	3.182	-2.374	8.739	2.804	0.107	1.135	0.259		
KSS	-23.480	-55.295	8.336	16.056	- 0.132	-1.462	0.146		
BMI	10.982	1.958	20.006	4.554	0.227	2.411	0.018		

Model = "Enter" method; KSS: Karolinska Sleepiness Scale; BMI: Body Mass Index; *B*: unstandardised regression coefficient; CI: Confidence Interval; *LL*: lower limit; *UL*: upper limit; *SE B*: standard error of the coefficient; *β*: standardised coefficient; R^2: coefficient of determination; ΔR^2: adjusted R^2.

3.3.3.4.5. Global cognition

Caffeine, genetic caffeine metabolism, age, BMI, subjective sleep quality score, level of education, alcohol intake and subjective sleepiness prior to tasks statistically significantly predicted performance in global cognition, F (8, 104) = 2.428, p = 0.019, accounting for 15.7% of the variation in global cognitive performance, with adjusted R^2 = 9.3%, indicating a trivial effect (Cohen, 1988). An additional year of age leads to a 0.21 (95% CI, 1.958 to 20.006, $p < 0.001$) unit decrease in global cognitive function score. Regression coefficients and standard errors can be found in Table 3.12.

Table 3. 12 Multiple regression results for global cognitive performance (n=113).

Model	B	95% CI for B LL	UL	$SE\ B$	β	t	P	R^2	ΔR^2
Constant	1.090	0.0326	1.854	0.385		2.830	0.006	0.157	0.093
Genetic caffeine score	-0.020	-0.116	0.076	0.048	-0.037	-0.405	0.686		
PSQI	-0.018	-0.058	0.023	0.020	-0.081	-0.864	0.389		
Caffeine	0.000	-0.001	0.001	0.000	-0.063	-0.660	0.511		
BMI	-0.003	-0.021	0.015	0.009	-0.032	-0.335	0.738		
Alcohol	-0.019	-0.050	0.012	0.016	-0.112	-1.213	0.228		
Education score	0.017	-0.052	0.087	0.035	0.046	0.497	0.620		
Age	-0.021	-0.033	-0.010	0.006	-0.361	-3.718	<0.001		
KSS	-0.016	-0.084	0.052	0.034	-0.045	-0.466	0.642		

Model = "Enter" method; Genetic caffeine score derived from CYP1A2, AHR and ADORA2A genes, with higher scores indicating faster caffeine metabolism and lower sensitivity; PSQI: Pittsburgh Sleep Quality Index; BMI: Body Mass Index; KSS: Karolinska Sleepiness Scale; *B*: unstandardised regression coefficient; CI: Confidence Interval; *LL*: lower limit; *UL*: upper limit; *SE B*: standard error of the coefficient; β: standardised coefficient; R^2: coefficient of determination; ΔR^2: adjusted R^2.

3.4. Discussion

The purpose of the present study was to investigate the interactions between genetics and habitual caffeine consumption on cognitive performance in four key domains of cognition, namely social and emotional cognition, memory, attention and psychomotor speed and executive function in healthy individuals in real-life conditions and on three separate occasions. The present study showed that habitual caffeine intake was higher in 'fast' compared with 'slow' metabolisers, while no other differences emerged between any of the genetic caffeine groups. Moreover, 'slow' metabolisers performed better than 'fast' metabolisers in emotion recognition task among high caffeine consumers. On the contrary, 'fast' metabolisers performed better than 'slow' metabolisers in the executive function domain, but only within moderate caffeine consumers. The findings are discussed in detail below.

3.4.1. Habitual caffeine intake

As discussed in Chapter 1 of this thesis, genetics are involved in individual variability in caffeine consumption, both at the pharmacodynamic and pharmacokinetic level (Laitala, Kaprio and Silventoinen, 2008; Yang, Palmer and de Wit, 2010). In the present sample, a significant difference in habitual caffeine consumption was found between genetic caffeine metabolism groups, but not between genetic sensitivity groups (Tables 3.2 – 3.4). Specifically, 'fast' metabolisers had a significantly higher

mean habitual caffeine intake compared with 'slow' metabolisers (p = 0.045), which is in line with previous findings (Cornelis *et al.*, 2011, 2015, 2016).

The SNPs employed in the current analysis as proxies for caffeine metabolism have been shown to be associated with habitual caffeine consumption (Cornelis *et al.*, 2011, 2015; Sulem *et al.*, 2011), supporting the hypothesis that individuals self-regulate their caffeine intake for optimal level of arousal (Zhou *et al.*, 2010). Caffeine metabolism can vary between individuals, largely owing to variations in CYP1A2 enzyme activity, the rate-limiting step for plasma caffeine clearance (Lelo *et al.*, 1986). Differential expression of *AHR* rs6968554 and *CYP1A2* rs2472297 has been linked with habitual caffeine intake, demonstrating the potential that high consumption is linked to faster caffeine metabolism (Cornelis *et al.*, 2011, 2015). Therefore, 'fast' caffeine metabolisers may need more caffeine to avoid symptoms of abstinence and withdrawal.

On the contrary, this study did not replicate previous findings of an association between the *ADORA2A* gene and habitual caffeine intake. It has been reported that carriers of the TT genotype in rs5751876 are more likely to consume less caffeine than carriers of the C allele (Cornelis, El-Sohemy and Campos, 2007). This association may be explained by the fact that the adenosine receptor system, the underlying mechanism of caffeine physiological effects, is also involved in the regulation of anxiety (Alsene *et al.*, 2003). However, it needs to be considered that the present study had a smaller sample size compared with the study by Cornelis *et al.* (2007) and that, despite the apparent plausibility of this association, this finding has not been replicated in GWAS of habitual caffeine consumption.

3.4.2. Cognitive performance

The current analysis of 129 participants in the UK provides support for a domain-specific association between habitual caffeine consumption and cognitive function. Furthermore, when it comes to predicting cognitive performance across various indices, only age and BMI were significant contributors in the models. Below, we discuss the results of the current analysis, examining cognitive performance within specific domains.

3.4.2.1. Social and emotional cognition

Social and emotional cognition refers to all mental processes underlying one's ability to understand the behaviours of others, as a requirement for social interactions

(Frith and Frith, 2007; Pinkham *et al.*, 2014). The emotion recognition task used in the present study assessed one of the key subdomains of social cognition – social understanding (Penn, Sanna and Roberts, 2008; Harvey and Penn, 2010) and, specifically, the ability to decode emotions in facial expressions (Lundqvist, Flykt and Öhman, 1998). Social cognitive skills are critical for successful communication and, consequently, mental health and wellbeing (Holt-Lunstad, Smith and Layton, 2010; Phillips *et al.*, 2010; Henry *et al.*, 2016; Jones *et al.*, 2017).

The present study showed a statistically significant difference in mean emotion recognition RTs between 'slow' and 'fast' metabolisers among high caffeine consumers. Specifically, within high caffeine consumers, mean emotion recognition RTs for 'fast' metabolisers were higher compared with 'slow' metabolisers (p = 0.004), while within 'fast' metabolisers, mean emotion recognition RTs for high caffeine consumers were higher compared with moderate (p < 0.001) and low (p = 0.013) caffeine consumers. This means that 'slow' metabolisers had a higher performance in emotion recognition compared with 'fast' metabolisers, but only among high caffeine consumers. Moreover, among 'fast' metabolisers, high caffeine consumers had higher RTs and thus, had a lower performance in emotion recognition compared with those consuming low and moderate levels of caffeine.

One for the reasons for the lower performance among 'fast' metabolisers - high caffeine consumers is due to withdrawal symptoms. For the purposes of this study, participants were asked to abstain from any source of caffeine for at least 5 h prior to taking the tests. As the half-life of caffeine is 4-6 h (Nehlig, 2018), habitual caffeine consumers would be neither under the acute effect of caffeine or in abstinence when cognitive tasks would be performed (Casiglia *et al.*, 2017). Moreover, it has been suggested that high caffeine consumers may fail to comply with abstinence requirements in research (James, 2005). Hence, abstaining from caffeine sources during the day for the purposes of the study would be challenging for this group.

A typical overnight caffeine abstinence results in substantial elimination of systemic caffeine by early morning; thus, upon awakening, caffeine consumers have entered the early stages of caffeine withdrawal (Shi *et al.*, 1993; Smit and Rogers, 2000; Juliano and Griffiths, 2004). Although there were no differences in three-day exam time between the caffeine groups, when the tests were performed, withdrawal symptoms may have been more profound in 'fast' metabolisers - high caffeine consumers, lowering their performance in cognitive tasks. It is therefore possible that high caffeine consumers who completed the tests in the morning, before their morning

coffee or tea to comply with the study requirements, may have been under more profound caffeine withdrawal symptoms.

Finally, it should be noted that high caffeine consumers had a higher subjective sleepiness before tasks compared with low caffeine consumers. This finding is in line with previous findings that heavier caffeine consumption, compared with no to low caffeine consumption is associated with higher daytime sleepiness (Chaudhary *et al.*, 2016). In the present investigation, the lower performance in high caffeine consumers may also be influenced by the subjective feelings of alertness/sleepiness, which is shown to exhibit performance impairments for several cognitive functions (Fortier-Brochu *et al.*, 2012).

The model for emotion recognition explained 10% of the variation in social and emotional cognition performance, with higher age being associated with lower performance in the domain. This aligns with findings from several studies, which have consistently shown that cognitive task performance tends to decrease with advancing age, especially in the domains of attention, executive function and memory (Salthouse, 2010, 2012; Harada, Natelson Love and Triebel, 2013; Tremblay *et al.*, 2016; Adólfsdóttir *et al.*, 2017).

To the authors' knowledge, this is the first study to investigate the associations between caffeine and the domain of social cognition. Despite documented effects of caffeine on emotional arousal (James and Gregg, 2004; Giles *et al.*, 2018), its association with emotion recognition is less understood and our findings warrant replication.

3.4.2.2. Memory

No significant gene x caffeine interactions were observed in the domain of memory and this is in line with the studies from the UK Biobank, employing the same genetic score for caffeine metabolism (Cornelis, Weintraub and Morris, 2020a, 2020b). In these studies, no significant CMSG x recent caffeine drinking, nor significant CMSG × caffeine/tea interactions were found on measures of memory. Nonetheless, it warrants highlighting that, despite the common methodology between our study and those from the UK Biobank in terms of genetic caffeine assessment SNPs, we used different methodologies for assessing memory and habitual caffeine intake. In terms of cognitive tasks, our study utilised the n-back task, a widely used test to assess working memory, as described in our methods (Owen *et al.*, 2005). The studies from

the UK Biobank however, utilised two memory tasks, one for prospective memory and a pairs matching task to assess episodic memory. Therefore, although the domain of memory was assessed in all three studies, we evaluated different subdomains. As mentioned in Chapter 1, this is very common in memory research, i.e., to use different tasks to assess the same broader process or the use of the same test for distinct subdomains, and this limits the internal consistency of memory research (Cheke and Clayton, 2013). In terms of habitual caffeine assessment, we measured habitual caffeine intake from all dietary sources, while the UK Biobank studies used a classification indicative of recent caffeine intake from coffee or tea within the hour preceding cognitive assessments, or habitual caffeine intake in cups of coffee or tea per day (Cornelis, Weintraub and Morris, 2020a, 2020b).

The findings from the regression model for memory accounted for 16.6% of the variation in memory performance, with higher age being associated with increased RTs and thus, lower performance in this domain. This is also in accordance with previous findings from several studies (Salthouse, 2010, 2012; Adólfsdóttir *et al.*, 2017).

3.4.2.3. Attention

Significant gene x caffeine interactions were not observed either for the domain of attention. This is also in line with the investigations from the UK Biobank, which failed to find any gene x caffeine interactions for the test of vigilance. Comparable to memory assessments, the studies used different cognitive tasks to assess vigilant attention: we used the PVT, the gold standard for assessing sustained attention (Lim and Dinges, 2008), while the studies by Cornelis *et al.* (2020a, 2020b) used a timed test of symbol matching. Therefore, the implications of the different methodologies between the studies, as mentioned for the domain of memory, need to be considered.

The model for attention was found to explain 5.3% of the variation in attention performance, with a similar trend to emotion recognition and memory: the higher the age, the lower performance in attention, as previously shown in research (Salthouse, 2010, 2012; Harada, Natelson Love and Triebel, 2013; Tremblay *et al.*, 2016; Adólfsdóttir *et al.*, 2017).

3.4.2.4. Executive function

A statistically significant difference was observed in executive function performance between 'slow' and 'fast' metabolisers among moderate caffeine consumers. Within moderate caffeine consumers, ΔRTs between the colour and word

parts of the Stroop task for ‘slow’ metabolisers was higher compared with ‘fast’ metabolisers ($p = 0.002$). Therefore, ‘fast’ metabolisers had a higher performance in executive function compared with ‘slow’ metabolisers among moderate caffeine consumers.

This finding may suggest a dose-specific association between caffeine intake and cognitive performance in executive function, a complex cognitive ability, where ‘fast’ metabolisers perform better than ‘slow’ metabolisers but only for moderate doses of habitual caffeine intake. It is possible that moderate caffeine intake in ‘fast’ metabolisers may strike a balance between providing cognitive benefits and avoiding potential negative effects. ‘Fast’ metabolisers may require higher doses of caffeine to achieve the same cognitive results for executive function, which can increase the likelihood of side effects such as anxiety, restlessness, or sleep disturbance (Alsene *et al.*, 2003; Childs *et al.*, 2008). Therefore, ‘fast’ metabolisers who consume moderate levels of caffeine may have a higher cognitive performance without experiencing these adverse effects. Similarly, it is possible that in ‘slow’ metabolisers, the steady-state plasma/brain caffeine concentration may have been sufficient to discourage caffeine intake (Cornelis *et al.*, 2015), but not enough to improve executive function.

Executive function has been assessed in three previous genetics studies of caffeine. The studies from the UK Biobank (Cornelis, Weintraub and Morris, 2020a, 2020b), using a test for verbal-numerical reasoning, did not find any significant associations. Another study on habitual caffeine intake and abstract reasoning showed that the higher the caffeine intake, the higher the abstraction score, but only for ‘slow’ metabolisers (Casiglia *et al.*, 2017). This contradicts our results, according to which ‘fast’ metabolisers performed better compared to ‘slow’ metabolisers among moderate caffeine consumers. Despite assessing the same domain, all studies used different tasks, making comparisons of findings challenging. Moreover, the mode of administration of tasks between studies may account for inconsistent results. The study from Casiglia *et al.* (2017) performed assessments in an experimental setting, our study was conducted using at-home testing, while the studies from the UK Biobank performed tests both in the laboratory and at home. Moreover, the study by Casiglia *et al.* (2017) used a single SNP in the *CYP1A2* gene as proxy of genetic caffeine metabolism. With regards to habitual caffeine measurement, Casiglia *et al.* (2017) used tertiles of caffeine intake, while we used previously defined (Erblang *et al.*, 2019) levels of caffeine intake. Lastly, it warrants highlighting that the study by Casiglia *et*

al. (2017) required participants' overnight caffeine abstinence, therefore their findings may be hampered by symptoms of withdrawal.

Considering the predictors of executive function, the model explained 10.1% of the variance in performance. Higher BMI was associated with lower performance in executive function, which is in line with the existing literature showing a negative association between overweight and obesity and cognitive performance in children and adults (Gunstad *et al.* 2007, Li *et al.* 2008, Lokken *et al.* 2009, Nilsson and Nilsson 2009).

3.4.2.5. Global cognition

This was the first genetics study on caffeine and cognition in an epidemiological setting to employ a global cognitive function score to assess overall cognition. Nonetheless, no significant associations were found for global cognitive performance. However, age was shown to predict global cognitive function, with the model explaining 15.7% of the variation in global cognitive performance and with every additional year of age being associated with lower overall cognition.

Considering the overall results on cognitive performance in the present sample, two questions may arise. The first question pertains to the differential results between emotion recognition and executive function. In executive function, 'fast' metabolisers - moderate consumers appeared with higher performance in executive function compared with 'slow' metabolisers-moderate consumers. In emotion recognition task, 'fast' metabolisers - high consumers had a lower performance compared with 'slow' metabolisers - high consumers, as well as compared with low and moderate caffeine consumers within the 'fast' metabolisers group. The second question refers to the lack of significant gene x caffeine interactions for the domains of attention, memory and for global cognition.

There are two possible explanations for these findings. First, caffeine is extensively metabolised to paraxanthine, which has as high potency at antagonising adenosine receptors and exerts several similar effects to caffeine, such as wake promotion and psychostimulation (Arnaud, 1987; Lin *et al.*, 2022). With long-term caffeine exposure, there is a substantial accumulation of paraxanthine, with average serum levels of paraxanthine reaching two thirds those of caffeine (Benowitz *et al.*, 1995). Caffeine and paraxanthine are shown to produce a similar magnitude of response at 4 mg/kg body mass; however, caffeine appears to produce greater

responses than paraxanthine at 2 mg/kg body mass (Benowitz *et al.*, 1995). Therefore, it is possible that the differential brain levels of caffeine and paraxanthine between moderate and high caffeine consumers are associated with differential effects of caffeine in emotion recognition and executive function.

Secondly, the order of task presentation within the cognitive test battery may have contributed to the differential outcomes observed between emotion recognition and executive function, or the lack of significant gene x caffeine interactions for the other domains. Specifically, the emotion recognition task was administered first, while the Stroop task was administered last. Although the current test battery employed validated tasks previously used in caffeine research related to cognition, these have been mostly administered separately, thus the order of task appearance within the test sequence could have influenced the validity of the tasks and subsequent results.

Moreover, it is essential to consider mental fatigue and task disengagement from tasks. Mental fatigue results in a vigilance decrement, or time-on-task (TOT) effect (Caldwell *et al.*, 2019), in which RTs and accuracy degrade over time. This degradation is thought to be due to high demands on neural resources (Boksem and Tops, 2008), as well as increased boredom and distractibility (Pattyn *et al.*, 2008), especially due to negative emotional stimuli (Borbély *et al.*, 2016). Although the duration of the battery in this study may not have been sufficiently long to induce cognitive fatigue based on previous findings (Dallaway, Lucas and Ring, 2022), mental fatigue and task disengagement are shown to deteriorate performance, especially in cognitively demanding tasks (Hopstaken *et al.*, 2015). As a result, as levels of fatigue rise, the value of exerting effort into a task declines, leading to reductions in performance (Müller and Apps, 2019). In our sample, this is especially important for high caffeine consumers, who presented higher levels of sleepiness prior to task completion, therefore their baseline alertness was already lower compared with the other caffeine intake groups. This may also be the source for a lack of associations for the domains of memory and attention.

In defense of the above statements, it should be noted that some participants were excluded from the final analysis because they failed to follow the task instructions or were too slow. Notably, no participants were excluded from the first two tasks (emotion recognition and n-back task), while two participants were excluded from the third task (PVT) and 12 from the final task, the Stroop task. The fact that 12 participants had to be excluded from the Stroop task could be explained by the Yerkes-Dodson law (Yerkes and Dodson, 1908), reflecting not only that it evaluates

the most complex cognitive function, i.e., it was the most difficult of all tasks, but also that it was last in order.

In summary, when employing a test battery, it is possible for all the above reasons, that only certain tasks remain sensitive enough to identify the association between caffeine and cognitive performance. In our study that has demonstrated domain-specific results, it may not necessarily mean that caffeine is associated only with specific functions. Instead, it could be that the cognitive battery lost the sensitivity to caffeine-related performance effects of individual tasks (Dye, Lluch and Blundell, 2000; Lieberman, 2003; Hoyland, Lawton and Dye, 2008). Consequently, null results for memory and attention could be due to the lack of sensitivity of the test battery, rather than the absence of an association. Provided that comparable cognitive tests between studies are currently scarce, caution should be applied when interpreting study findings, while replication of results is warranted.

3.4.3. Strengths and Limitations

This is the first genetic association study on caffeine assessing not only one domain, but all key four domains of cognition: a) social and emotional cognition b) memory, c) attention and psychomotor speed and d) executive function. This is also the first genetic association study in an epidemiological setting assessing cognitive performance remotely in real-life conditions on three separate instances to account for common day-to-day intra-individual variations in performance (von Stumm, 2016). It was hypothesised that this assessment would provide a more appropriate estimate of everyday cognition in real-time circumstances. Recent findings also support that cognitive tasks performed in naturalistic settings (e.g., at home or at work) provide measurements that are comparable in reliability to assessments made in controlled laboratory environments (Sliwinski *et al.*, 2018). Indeed, it has been suggested that, to improve the understanding of cognition in everyday life, assessments need to take place in natural daily environments (Dijk, Duffy and Czeisler, 1992).

Nevertheless, this study is not free of limitations. Firstly, because of participant data exclusion, the study was not appropriately powered and this may be one of the reasons no model could successfully identify genetics or caffeine as predictors of cognition. Moreover, the caffeine groups based on genetic caffeine metabolism were not balanced, with only seven participants in the low caffeine intake groups within the 'fast' metabolisers. This may impact the conclusions drawn from results presented and supports the need for further research. Furthermore, the modification of the EPIC-FFQ

software to assess habitual caffeine intake may have introduced misclassification of caffeine assessment. Finally, the remote nature of the study did not allow for a more personal contact between the research team and the participants to explain the tasks in more detail, leading to participant exclusion from the analyses because of poor understanding of the task instructions.

3.5. Conclusions

To summarise the above, we have confirmed previous findings that caffeine and cognition associations are domain-specific, with domains of social and emotional cognition and executive function being associated with habitual caffeine intake. We have also replicated that 'fast' caffeine metabolisers consume higher levels of caffeine, potentially to achieve the desired psychostimulant effects. Further, we demonstrated differential caffeine x cognitive function associations based on level of habitual caffeine intake, most probably via more profound caffeine withdrawal effects in 'fast' compared with 'slow' metabolisers. Finally, it is suggested that differential brain levels of caffeine and paraxanthine between high and moderate consumers may account for different associations in different domains, which may be further associated with mental fatigue and disengagement during prolonged tasks.

Our results are in part in line with other published literature; however, there is a substantial lack of comparable methods to assess cognitive function in research, therefore applicability should be approached with caution, specifically when dealing with diverse populations. More research in naturalistic environments using larger cohorts is needed to confirm these findings alongside investigating other discussed relevant influencers of human cognition to add to our understanding of how habitual caffeine may influence cognitive function based on individual genotype.

Chapter 4. Acute Caffeine Intake, Genetics and Cognitive Performance

This chapter presents the final study of this research project, aiming to answer the third research question, i.e., whether variations in genes implicated in caffeine pharmacokinetics and pharmacodynamics impact the effect of acute caffeine intake on cognitive performance. Having acquired a deeper understanding on the challenges in assessing the associations between habitual caffeine intake and genetics on human cognition (Study 2), a randomised double-blind cross-over trial aiming to investigate how acute caffeine intake affects cognition in all cognitive domains based on individual genotype was designed. This chapter has four sections: firstly, an introduction to the topic; secondly, a detailed presentation of the materials and methods used for the study conduct; thirdly, the results of the study after statistical analysis and finally, the findings of the study are discussed and compared with previous research.

4.1. Introduction

4.1.1. Caffeine – A cognitive function enhancer?

Caffeine, historically the most widely consumed psychoactive substance (James, 1997; James and Rogers, 2005; Fredholm, 2011), is perceived as a cognitive performance enhancer, in part explaining its popularity (Cappelletti *et al.*, 2015; Renda *et al.*, 2015; Carswell *et al.*, 2020). Caffeine possesses enhancing properties in cognitive performance in commonly consumed doses (32-300 mg) (Snel, Lorist and Tieges, 2004; Nehlig, 2010; McLellan, Caldwell and Lieberman, 2016). As discussed in detail in Chapter 1 of this thesis, among the indices of human performance that have been investigated in studies of caffeine, the most consistent results have been obtained in relation to simple functions (e.g., attention and processing speed), while with more complex cognitive functions (e.g., memory and executive function), findings have been less consistent (McLellan, Caldwell and Lieberman, 2016).

Overall, it appears that the performance-enhancing properties of caffeine are more likely to be noticeable when performance has already been degraded because of fatigue or lack of sleep, as indicated by previous reviews (Lieberman *et al.*, 2002; James, 2005; Cappelletti *et al.*, 2015; McLellan, Caldwell and Lieberman, 2016). Nevertheless, studies on caffeine and cognition up to date vary greatly in their methods, including for example, the tools used to measure habitual caffeine intake and to assess cognitive performance (Cappelletti *et al.*, 2015; McLellan, Caldwell and Lieberman, 2016). This variability also applies to the limited genetics studies on caffeine and cognition, as shown in Chapter 2.

In this chapter, the main challenge of caffeine research, which is the possible source of confounding and the subsequent inconsistent findings is discussed: caffeine withdrawal. This debate provided the basis for the third and final study of this programme of research, the results of which are presented herein.

4.1.2. Caffeine tolerance and withdrawal

Habitual use of caffeine has been linked with caffeine dependence in adults and adolescents, as indicated by various studies (Hughes *et al.*, 1998; Bernstein *et al.*, 2002; Oberstar, Bernstein and Thuras, 2002; Jones and Lejuez, 2005; Svikis *et al.*, 2005; Huntley and Juliano, 2012). The term caffeine dependence is used to describe the inability to control caffeine intake despite negative physical or psychological consequences associated with continued use (Meredith *et al.*, 2013). This dependency arises from a mechanism involving the upregulation of adenosine, leading to hypersensitivity and the development of tolerance and withdrawal symptoms (Juliano and Griffiths, 2004). In essence, caffeine dependence is characterised as a cluster of behavioural and cognitive symptoms that develop with repeated substance use (Meredith *et al.*, 2013).

Originally, drug tolerance was defined by behavioural plasticity, indicating a reduced response to repeated drug exposure (Kalant, 1998). Caffeine tolerance develops when the physiological, behavioural and subjective effects of caffeine diminish following repeated use (Meredith *et al.*, 2013). This means that with long-term use, the same dose of caffeine no longer yields positive or negative effects. Consequently, individuals gradually require higher doses of caffeine to achieve the desired psychostimulant effects (Meredith *et al.*, 2013; Nehlig, 2018). Caffeine tolerance has been reported to reach a plateau within 3-5 days of continuous use (Denaro *et al.*, 1990). A number of studies in humans have shown tolerance to the subjective effects of caffeine (Evans and Griffiths, 1992), as well as tolerance to other physiological effects such as diuresis, oxygen consumption, sleep disturbance and blood pressure (Bonnet and Arand, 1992; Griffiths and Mumford, 1996). At doses of 300 mg/day, caffeine is more likely to result in partial rather than complete tolerance (Juliano and Griffiths, 2004).

Caffeine tolerance is shown to result in withdrawal symptoms when caffeine intake is discontinued (Hughes *et al.*, 1993). Caffeine withdrawal has been well documented in humans (Juliano and Griffiths, 2004; Juliano, Evatt, *et al.*, 2012) and common symptoms include headache, fatigue, nervousness and dysphoric mood

(Griffiths, Bigelow and Liebson, 1989; Griffiths *et al.*, 1990; Juliano and Griffiths, 2004; Juliano, Huntley, *et al.*, 2012). The incidence or severity of symptoms is shown to increase with increases in daily dose; abstinence from doses as low as 100 mg/day can produce withdrawal symptoms (Juliano and Griffiths, 2004). Such symptoms appear after approximately 12–16 h, with effects peaking at around 24–48 h (Griffiths *et al.*, 1990; Hughes *et al.*, 1993; Smit and Rogers, 2000). However, it has been observed that these symptoms typically subside within 3–5 days of continuous abstinence (Griffiths, Bigelow and Liebson, 1986; Hughes *et al.*, 1993). A comprehensive review of the relevant literature reported that withdrawal consistently leads to symptoms of: 'headache', 'tiredness/fatigue', 'decreased energy/activeness', 'decreased contentedness/well-being', 'depressed mood', 'difficulty concentrating', 'irritability' and 'foggy/not clearheaded' (Juliano and Griffiths, 2004). The authors concluded that the available evidence 'overwhelmingly' supports that withdrawal effects are pharmacological in nature rather than expectancy-based, meaning that they do not constitute placebo effects.

Therefore, contrary to the widely accepted stimulating effects of caffeine (Cappelletti *et al.*, 2018), caffeine withdrawal refers to an acute syndrome that develops following discontinuation of chronic caffeine administration and can be summarised as a common CNS depression (Ammon, 1991). Importantly, withdrawal effects are shown to subside shortly after caffeine is reintroduced (Evans and Griffiths, 1999; James and Gregg, 2004; Juliano and Griffiths, 2004). In habitual caffeine consumers, the typical overnight abstinence period of 10-14 h aligns with the time course of caffeine withdrawal. This suggests that withdrawal effects are likely to have already started by the time the first caffeine dose of the day is ingested, whether self-administered or provided during experiments (Juliano and Griffiths, 2004; James and Rogers, 2005).

Considering the ubiquity of caffeine consumption, uncertainty over the possible psychostimulant effects of habitual caffeine use and their implications for cognitive performance are subjects of concern and ongoing debate among researchers (James, 2005, 2014; Nehlig, 2010, 2018; Einöther and Giesbrecht, 2013).

4.1.3. The debate in caffeine research

At the centre of the debate of caffeine research is the claim that most studies conducted to date contain a flaw derived from the widespread adoption of the typical drug-challenge protocol (James, 1994a, 2014; James and Rogers, 2005; Rogers *et al.*,

2013). In applying this protocol to investigate caffeine, researchers have failed to consider that most individuals consume caffeine daily, while placebo-controlled studies typically require participants to abstain from caffeine overnight prior to laboratory testing (James and Rogers, 2005; Gupta and Verma, 2013). This ensures a washout period after which all participants have comparable levels of the drug in their system at the time of testing (Gupta and Verma, 2013; Roberts, Dusetzina and Farley, 2015). Nevertheless, the typical drug-challenge protocol, although a gold standard in research, may not be a suitable design for revealing the effects of a drug that is both widely available and subject to development of tolerance and withdrawal (James, 2014).

Typically, most caffeine is consumed in the morning, with fewer doses ingested later in the day and very little in the evening before sleep, followed by overnight abstinence (Lieberman, Agarwal and Fulgoni, 2019). In placebo-controlled studies, when participants are asked to abstain from their usual morning caffeine intake before testing, they may experience early stages of caffeine withdrawal during the study (Juliano and Griffiths, 2004; James and Rogers, 2005). This withdrawal effect can then serve as a confounder and should be considered when interpreting the findings (Cohen and Recalt, 2020). Accordingly, while caffeine studies are framed as investigations of the cognitive effects of caffeine compared to placebo, they can also be viewed as studies examining the consequences of caffeine withdrawal compared to non-withdrawn conditions (James and Rogers, 2005).

Consequently, this perspective has raised the question of whether the perceived cognitive improvements (attributed to caffeine) reflect the net effects of the stimulant, or simply the restoration of processes degraded during periods of caffeine abstinence (either naturally-occurring or experimenter-prescribed), i.e., the reversal of withdrawal symptoms (Rogers *et al.*, 2003, 2013; James, 2005, 2014; James and Rogers, 2005; Rogers, 2014). This alternative explanation is known as the 'caffeine deprivation hypothesis' or the 'withdrawal reversal hypothesis' (Rogers *et al.*, 2003). In other words, do we consume coffee to enhance our speed and cognitive abilities, or simply to avoid a decline in cognitive performance due to omitting our usual dose?

The proposed approaches to control for caffeine withdrawal in caffeine research are discussed in the next section.

4.1.4. Proposed approaches to control for caffeine withdrawal reversal

Due to the limitations of the standard drug-challenge protocol in comprehending the effects of caffeine on human performance and mood, three alternative empirical paradigms have been proposed. These approaches employ different methodologies to tackle the issue of confounding caused by caffeine withdrawal. Below, these three methods are discussed, extending from the review of James and Rogers (2005) in the topic.

4.1.4.1. Recruitment of caffeine-naïve participants

One widely used approach involves the recruitment of caffeine-naïve individuals (i.e., non-consumers) to compare the effects of caffeine on human performance compared with placebo (Rogers *et al.*, 2003, 2013). Alternatively, recruitment of caffeine-naïve individuals has been employed to compare individuals who regularly consume caffeine with those who consume little or no caffeine, aiming to identify performance differences between the two groups (Haskell *et al.*, 2005; Childs and de Wit, 2006; Rogers *et al.*, 2013).

In this approach, caffeine is administered to caffeine-naïve individuals who have no recent history of caffeine use, reducing the likelihood of experiencing caffeine withdrawal. However, defining 'caffeine-naïve' participants has been challenging, with various criteria being used by researchers, such as 'no tea or coffee intake and less than 50 mg/day from other sources' (Haskell *et al.*, 2005), or 'less than 300 mg/week' (Childs and de Wit, 2006). Moreover, it has been suggested that relying solely on self-reported caffeine exposure may be unreliable due to participant varying perceptions (James *et al.*, 1988). Therefore, to improve the accuracy of selecting participants as caffeine-naïve, some researchers have combined self-reports with objective measurements, such as saliva caffeine concentration threshold of ≤0.2 µg/mL (Rogers *et al.*, 2013).

Apart from the challenges in defining caffeine-naïve participants, several issues arise from this approach. First, the prevalence of caffeine consumption in the general population is high, with over 80% of individuals consuming one or more caffeine beverages daily (Ogawa and Ueki, 2007; Heckman, Weil and De Mejia, 2010; EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2015). Secondly, finding participants who are truly caffeine-naïve can be challenging due to the widespread presence of caffeine in various sources (Fitt, Pell and Cole, 2013; Cappelletti *et al.*,

2015; European Food Safety Authority, 2015). Consequently, individuals with low or no caffeine consumption may represent a small, self-selected minority and the generalisability of the findings to regular consumers would be open to question (Rogers *et al.*, 2013).

The justifiable assumption that caffeine-naïve participants are non-tolerant to caffeine could suggest that the study of naïve participants avoids confounding due to withdrawal reversal (James and Rogers, 2005). In fact, as previously suggested, using caffeine-naïve participants in research can help mitigate the confounding effects from withdrawal reversal, but may introduce another source of confounding from variable responses to caffeine due to tolerance (James and Rogers, 2005). Indeed, naïve individuals may experience caffeine-induced anxiety and jitteriness (Bonnet and Arand, 1992; Griffiths and Mumford, 1996; Stafford, Rusted and Yeomans, 2006; Nehlig, 2010; Smith *et al.*, 2011). Caffeine-induced anxiety may influence performance following the Yerkes-Dodson law, as discussed in Chapter 1, which suggests that performance is an inverted U function of arousal, with a negative relationship between arousal and task difficulty (Yerkes and Dodson, 1908). Hence, cognitive performance improves with increased arousal up to a certain point, after which further increases in arousal can lead to a decline in performance (Stafford, Rusted and Yeomans, 2006; McLellan, Caldwell and Lieberman, 2016). Moreover, there is evidence that increased jitteriness from caffeine increased the speed of tapping (mouse clicks) in computerised tasks, thereby enhancing cognitive performance (Rogers *et al.*, 2003).

In conclusion, the strategy of recruiting caffeine-naïve individuals may introduce challenges to experimental internal and external validity, specifically in a) defining ‘caffeine-naïve’, b) ensuring representativeness of participants and c) addressing confounding due to caffeine tolerance and anxiety (James and Rogers, 2005).

4.1.4.2. Caffeine pre-treatment

Another method highlighted by James and Rogers (2005) in caffeine research involves the pre-treatment of participants with caffeine to eliminate caffeine withdrawal effects at the time of laboratory testing (James and Rogers, 2005). Participants in pre-treatment trials are administered caffeine so that they are not or are only mildly caffeine-deprived when examined for performance effects after a second dose of caffeine.

Nevertheless, the results of such studies have yielded inconsistent findings. Some have reported enhanced performance and mood after a subsequent caffeine dose following pre-treatment (Warburton, 1995; Warburton, Bersellini and Sweeney, 2001; Christopher, Sutherland and Smith, 2005; van Duinen, Lorist and Zijdewind, 2005), whereas others failed to observe any enhancement when caffeine was ingested within less than 6–8 h following pre-treatment (Robelin and Rogers, 1998; Yeomans *et al.*, 2002; Heatherley *et al.*, 2005). It is noteworthy that in studies reporting positive results, participants self-administered the pre-treatment caffeine dose while unsupervised. For example, in the study by van Duinen and colleagues (2005), participants were allowed to consume one cup of coffee before 10 a.m. on experimental days (van Duinen, Lorist and Zijdewind, 2005).

Due to the variability in caffeine levels in various coffee preparations (see Table 1.1) and the absence of verification regarding the timing and dose of consumption, there is a potential for participants to have provided inaccurate reports of their caffeine intake. This could involve underestimating their caffeine consumption or falsely stating they consumed caffeine when they did not. Therefore, the cognitive advantages attributed to caffeine intake in these studies could still be a result of withdrawal reversal. It has been suggested that enabling participants to be responsible for the pre-treatment dose is a less reliable way than delivering pre-treatment under laboratory supervision (Heatherley *et al.*, 2005). Evidently, studies in which pre-treatment was administered under supervision have reported no caffeine effects following a second dose, unless the interval between the two doses exceeded 6 h (Robelin and Rogers, 1998; Yeomans *et al.*, 2002; Heatherley *et al.*, 2005).

Nevertheless, such protocols may not be able to distinguish between withdrawal reversal and net caffeine effects. According to James & Rogers (2005), this is because it is not possible to determine whether the pre-treatment caffeine dose has fully removed and prevented the reappearance of withdrawal effects at the point when the subsequent dose of caffeine was administered. Provided the interindividual caffeine consumption patterns and rate of caffeine metabolism (Nehlig, 2018), it would be challenging to estimate the exact amount of pre-treatment caffeine needed to ensure uniform and complete removal of caffeine withdrawal effects among individuals (James and Rogers, 2005).

4.1.4.3. Long-term withdrawal studies

All the above led researchers to employ protocols entailing periods of long-term caffeine abstinence. Examples of such protocols in genetics studies of caffeine have been used in relation to exercise performance (Salinero *et al.*, 2017; Guest *et al.*, 2018) and cognitive performance in sleep-deprived states (Baur *et al.*, 2021). This approach allows investigators to assess the effects of caffeine in habitual caffeine consumers, however in non-withdrawn conditions. Nevertheless, these protocols may not reflect real-life conditions, since habitual caffeine consumers often ingest multiple caffeine doses daily, typically with higher amounts in the morning and fewer doses later in the day (Lieberman, Agarwal and Fulgoni, 2019).

Therefore, another approach for investigating the net effects of caffeine in habitual consumers, while avoiding withdrawal reversal was introduced in 1994, initially in the context of cardiovascular outcomes (James, 1994a, 1994b). Since caffeine tolerance has been reported to reach a plateau within 3-5 days of continuous use (Denaro *et al.*, 1990) and withdrawal effects are shown to subside within a similar timeframe (Griffiths, Bigelow and Liebson, 1986; Hughes *et al.*, 1993), James hypothesised that caffeine abstinence (washout) for a week would be enough to control for both tolerance and withdrawal reversal (James, 1994a, 1994b).

Based on this principle, the traditional drug-challenge paradigm was extended to include four consecutive 1-week periods, with participants following a strictly prescribed and biologically verified regimen of placebo/caffeine intake (James, 1994a, 1994b). During caffeine phases, participants ingested approximately one cup of coffee three times daily, simulating typical caffeine consumption patterns (Snel, Lorist and Tieges, 2004; Nehlig, 2010; McLellan, Caldwell and Lieberman, 2016). Using this alternating 1-week design, the study aimed to assess both the acute and chronic effects of caffeine, while also controlling for potential confounding due to tolerance and withdrawal effects associated with habitual consumption (James, 1994a, 1994b).

Four years later, the author used the same protocol to assess caffeine effects on mental processes (James, 1998). The study found that overnight caffeine abstinence negatively affected cognitive performance and these adverse effects were reversed when caffeine was reintroduced (withdrawal reversal). However, there was no evidence to support caffeine having any beneficial impact on performance under conditions of habitual caffeine use compared to sustained abstinence (caffeine-naïve conditions) (James, 1998). Interestingly, the results of studies employing this

approach have consistently shown no significant net benefits of caffeine on performance and mood (Silverman and Griffiths, 1992; Mumford *et al.*, 1994; Garrett and Griffiths, 1998; James, 1998; Tinley, Yeomans and Durlach, 2003; Rogers *et al.*, 2005).

Overall, findings from these better-controlled studies suggest that caffeine may have limited net effects on performance and rather reverses the withdrawal symptoms in habitual consumers.

4.1.5. Do genetics studies solve the debate?

While caffeine tolerance and withdrawal may explain the reported inconsistencies, or the lack of caffeine effects on cognition (Cappelletti *et al.*, 2015; McLellan, Caldwell and Lieberman, 2016), nutrigenetics research is promising in explaining at least part of the mixed results in caffeine research on cognition (Nehlig, 2018). Growing evidence from genetic studies has associated the interindividual differences in caffeine response with variations in genes implicated in caffeine pharmacokinetics (Sachse *et al.*, 1999; Cornelis *et al.*, 2011), pharmacodynamics (Hohoff *et al.*, 2014), as well as the behavioural aspects of habitual caffeine intake (Cornelis, El-Sohemy and Campos, 2007; Cornelis *et al.*, 2011; Sulem *et al.*, 2011; Amin *et al.*, 2012).

Nevertheless, as shown in our systematic review in Chapter 2, there are currently limited genetics studies on caffeine and cognitive performance and these have also yielded mixed results. For example, Carswell *et al.* (2020) found that 'fast' metabolisers (based on *CYP1A2* rs762551) had better performance in attention during exercise and post-exercise after 3 mg/kg body mass of caffeine compared with 'slow' metabolisers, while Salinero *et al.* (2017), using the same SNP, cognitive assessment task and caffeine dose, found no differences in attention performance between genotype groups for caffeine compared with placebo (Salinero *et al.*, 2017; Carswell *et al.*, 2020).

Further, Renda *et al.* (2015) reported a higher performance in executive control and orienting in caffeine sensitive and non-sensitive individuals, respectively (based on *ADORA2A* rs5751876) after 3 mg/kg body mass of caffeine compared with placebo. In sleep-deprived individuals, Baur *et al.* (2021) found that the impairments in cognition after sleep deprivation were attenuated with regular coffee (300 mg caffeine) when compared with decaffeinated coffee in non-sensitive individuals. In the study by

Bodenmann *et al.* (2012), performance was higher after 400 mg caffeine compared with placebo in non-HT4 vs HT4 haplotype carriers of *ADORA2A* after sustained wakefulness. Finally, Skeiky *et al.* (2020) found no differences in performance between genotypes of the *TNFa* rs1800629 after 200 mg or 300 mg caffeine supplementation compared with placebo after sleep deprivation.

Nevertheless, the methodologies employed by these genetics studies in participant selection and control for caffeine withdrawal are varied. For example, some researchers recruited low/moderate caffeine consumers (Renda *et al.*, 2015; Baur *et al.*, 2021), others recruited light caffeine consumers (Salinero *et al.*, 2017), while others did not report on habitual caffeine consumption of participants (Bodenmann *et al.*, 2012; Skeiky *et al.*, 2020). When it comes to control for withdrawal reversal, some studies requested participants to refrain from caffeine for a day (Renda *et al.*, 2015), two days (Carswell *et al.*, 2020), or a week (Salinero *et al.*, 2017; Baur *et al.*, 2021). Finally, two studies did not report on such requirements (Bodenmann *et al.*, 2012; Skeiky *et al.*, 2020).

In summary, the results from genetics studies on caffeine and cognition, although promising in unravelling the interindividual differences of caffeine effects, are not only limited in number, but also subject to methodological issues that are inherent to caffeine research. Therefore, genetics studies may not be yet capable of solving the debate in caffeine and cognition research. The present study was designed to address this gap by employing a robust methodology based on the protocol established by James in 1994 for the first time in genetics studies of caffeine.

4.1.6. Aims and objectives

The aim of the present study was to investigate the effect of caffeine on cognitive performance in all key domains of cognition (social and emotional cognition, memory, attention and psychomotor speed and executive function) and how genetics may impact this effect in healthy individuals.

Acknowledging the methodological issues of caffeine research, this study was designed to represent a better controlled study capable of delineating the complexities of the effects of caffeine on cognitive performance. Building upon the protocol introduced by James (1994a), the study did not follow the typical drug-placebo paradigm, but a 4-week protocol of long-term caffeine/placebo supplementation.

Therefore, we aimed to control for confounding for caffeine withdrawal reversal and investigate the net effects of caffeine on cognitive performance based on genetics.

4.2. Materials and methods

4.2.1. Study design

A double-blind, placebo-controlled, crossover trial was designed in which participants engaged in four consecutive 1-week experimental trials. In the present study, participants alternated between periods of caffeine intake and caffeine abstinence. This way, it was hypothesised that the acute effects of caffeine would be examined in conjunction with chronic caffeine intake.

4.2.1.1 Experimental trials

Each 1-week experimental trial consisted of two periods: the 'run-in' days of habitual consumption and the 'challenge' day of acute supplementation. Each 6-day period of habitual consumption was followed by a challenge day, comprising four distinct experimental conditions: a) 6 days of habitual placebo consumption followed by 1 day of placebo challenge (PP), b) 6 days of placebo followed by 1 day of caffeine (PC), c) 6 days of caffeine followed by 1 day of placebo (CP) and d) 6 days of caffeine followed by 1 day of caffeine (CC). The anticipated effects of each study arm are shown in Table 4.1. The order of the experimental trials was randomised and counterbalanced using Research Randomizer software version 4.0 and an alphanumeric code was assigned to each trial to blind participants and investigators to the substance.

Table 4. 1 Study design using a double-blind, placebo-controlled crossover protocol incorporating 'habitual' caffeine or placebo supplementation.

Week	**'Habitual' intake (Days 1-6)**	**Challenge (Day 7)**	**Effects revealed by Day 7**
1	Placebo	Placebo	Caffeine-naïve state or long-term abstinence
2	Placebo	Caffeine	Acute exposure
3	Caffeine	Placebo	Withdrawal effects
4	Caffeine	Caffeine	Habitual caffeine use

Available from: James, 1994a.

Throughout the 4 weeks of the study, participants were instructed not to drink coffee or tea and were provided with supplies of decaffeinated coffee and tea to facilitate adherence to this requirement.

4.2.1.2. Run-in days

Habitual intake was defined as the ingestion of caffeine or placebo (1.75 mg/kg body mass) for six consecutive days, three times daily at 9 a.m., 11 a.m. and 3 p.m., with water ad libitum. This level and rate selected by James (1994a) approximated the equivalent of 1-1.5 cups of coffee, producing steady-state plasma caffeine concentrations within 24 h (Pfeifer and Notari, 1988). Since previous studies have indicated that maximum caffeine tolerance is achieved within 3-5 days of the start of regular intake (Robertson *et al.*, 1978; Denaro *et al.*, 1990), it was assumed that 6 consecutive days of stable caffeine consumption would be adequate to allow the development of tolerance.

In preparation for the challenge days and to control for environmental factors which have been shown to affect inducibility of CYP1A2 enzyme (see Chapter 1), participants were asked to avoid strenuous exercise for 48 h (days 5 and 6) and abstain from alcohol and consumption of cruciferous vegetables (e.g., broccoli and Brussels sprouts) for 24 h (day 6) (Gunes and Dahl, 2008). Participant requirements are shown in Figure 4.1.

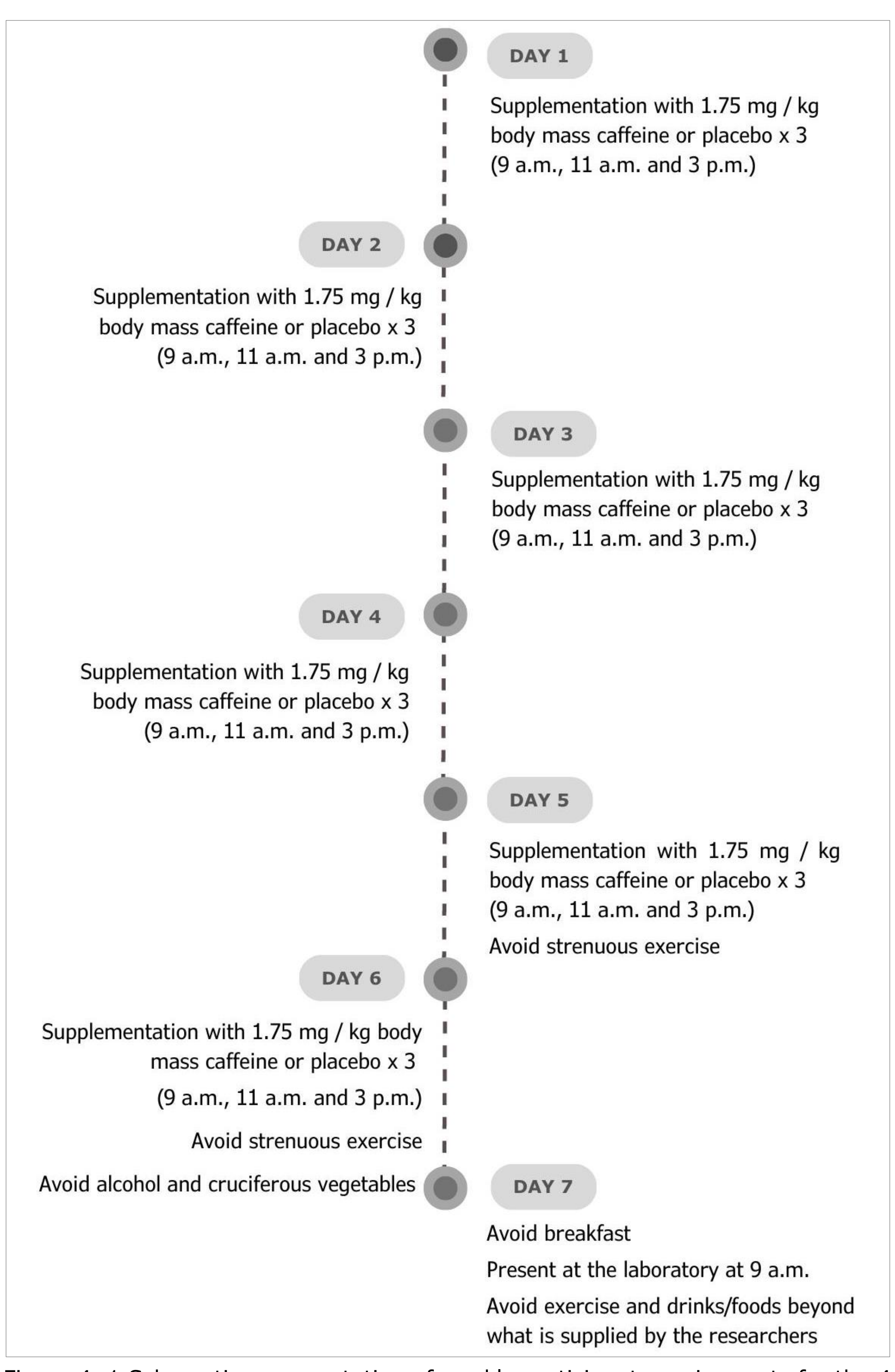

Figure 4. 1 Schematic representation of weekly participant requirements for the 4 weeks of the experimental protocol.

4.2.1.3. Challenge days

On the seventh (challenge) day of each week, participants arrived at the laboratory at 9 a.m., after an overnight fast and without having ingested any capsules. Laboratory sessions were timed to begin at 9:10 a.m., 10:30 a.m., 12.30 p.m. and 15:30 p.m., and participants attended at the same time for all four weekly sessions. Participants were seated in a sound-attenuated, temperature-controlled computer room at St Mary's University and the research team supervised the experiment. To avoid distractions during the experimental sessions, the members of the research team were sitting at the back of the room. At 9:30 a.m., participants completed the pre-challenge baseline cognitive test battery, after which they ingested placebo or caffeine supplementation. Based on previous research (Carswell *et al.*, 2020), the capsules were ingested with water proportional to participant body mass (3 ml/kg body mass). Figure 4.2 shows the experimental procedures during challenge days.

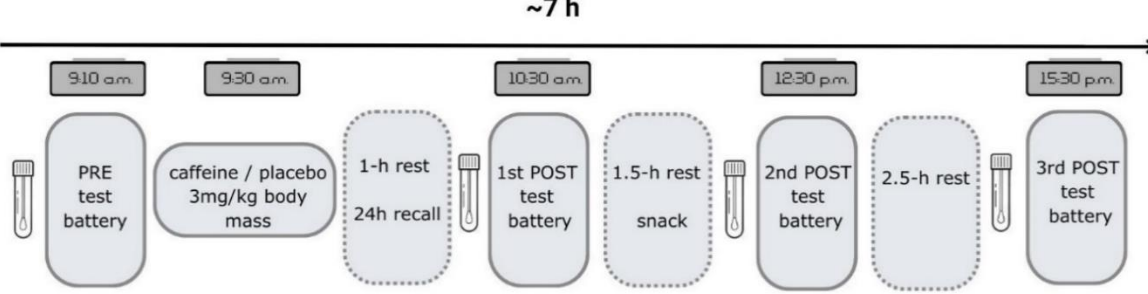

Figure 4. 2 Schematic representation of challenge day procedures. PRE: pre-supplementation; POST: post-supplementation; Swab icon represents saliva sample collected pre-supplementation and 1, 3 and 6h post-supplementation to assess caffeine metabolism.

Between the time of supplementation and the first post-challenge session, participants were provided with diet diaries to record all food and beverages consumed during the 24 h before the challenge day. They were also asked to report whether they engaged in any physical activity during the 48 h prior to their visit. This information was collected to assess compliance with research requirements. After the completion of the first post-supplementation session (1 h post-supplementation), at 11 a.m., participants were offered a light snack, consisting of fruit juice and a cheese sandwich. These were prepared by the research team following the information on participant

allergies and preferences collected at baseline. Caffeine is absorbed from the gastrointestinal tract within 60 min of oral intake (Blanchard and Sawers, 1983; Graham, 2001), while healthy adults absorbing 8 mg/kg caffeine are shown to reach peak plasma caffeine concentration in 75 min (Blanchard and Sawers, 1983; Kot and Daniel, 2008). Therefore, 80 min post-supplementation was deemed sufficient for the snack to not interfere with caffeine absorption.

During the experimental days, participants were asked to consume water ad libitum but avoid intake of drinks and foods other than what provided by the research team. They were also asked to avoid exercise between the trials. At the end of each challenge day, participants were provided their weekly supply of capsules.

4.2.2. Participants

The study was approved by St Mary's University Ethics Committee and conducted in agreement with the Declaration of Helsinki (World Medical Association, 2013). A sample size of 42 was calculated using G*Power 3.1.9.7 for a large effect size ($f^2 = 0.4$) at 80% power and an alpha level of 5%. Since the present study primarily focuses on the main effects based on genotype groups (n = 3), the repeated measures between factors mixed ANOVA was used for sample size calculations.

Participants were recruited via email, word of mouth and social media advertising. Additionally, individuals who had previously participated in Study 2, had granted permission to be contacted for future studies and were located in London, were also contacted to participate in this study. All data collection and statistical analyses were completed at St Mary's University, Twickenham, London. Before enrolling, participants received a link to the participant information sheet and the consent form and completed a screening questionnaire on Jisc online platform. If a participant was not deemed eligible to take part, their data were destroyed immediately.

Adult males and females residing in London with no known neurocognitive disorder and uncorrected vision impairments who provided written informed consent were included in the study. Participant exclusion criteria aligned with those outlined in Study 2 (Chapter 3) and included the additional exclusion of tobacco smoking and individuals who habitually consume alcohol above the publicly recommended upper safe limits (21 and 14 units per week for men and women, respectively). Eligible participant total involvement lasted four weeks.

4.2.3. Preliminary procedures and familiarisation

4.2.3.1. Capsule preparation and blinding

A member of St Mary's laboratory trained the research team to prepare the capsules for eligible participants. Calculations for capsule preparation were performed by the principal investigator based on self-reported weight provided by the participants before baseline. To account for differences between self-reported weight and measured weight higher than +/- 5 kg body mass, additional sets of capsules were prepared. This ensured that each participant would receive an appropriate dose of the supplementation based on their measured weight. Prepared capsule weights were recorded on the appropriate forms and signed by the principal investigator and the supervisors.

Identical vegan opaque capsules (Capsuline, Florida, US) containing placebo (microcrystalline cellulose, MCC Blackburn Distributions, UK) or caffeine (anhydrous powder, MCC Blackburn Distributions, UK) were prepared, containing 1.75 mg/kg body mass for run-in days and 3 mg/kg body mass for experimental days. Size 0 capsules were selected for participant convenience and because their capacity is up to 500 mg, which would help the research team account for calculation errors and prevent the administration of high caffeine doses. For each participant, the research team prepared the complete set of capsules (run-in days and challenge day). For the run-in days, the research team randomly prepared more than the required number (3 capsules / day x 6 days = 18 capsules) of capsules to account for losses and allow confirmation of participant compliance by capsule count.

Capsules were then blinded by a member of St Mary's laboratory. Participant weekly capsules were placed in white pots in random numbers, but always above what was needed for the run-in days. No members of the research team were aware of the number of capsules in each pot. To ascertain compliance to the protocol, participants were asked to bring the preceding week's pots on the experimental visits. The research team counted and recorded the remaining capsules for each participant for each week at the end of the study.

4.2.3.2. Baseline Visit

Eligible participants were contacted via email to schedule baseline visit at St Mary's University. Prior to baseline visit, the research team prepared the computer rooms by loading on desktops the appropriate links for the baseline questionnaire and

the cognitive test battery. During the first part of baseline visit, participants were informed in detail regarding study requirements and participant weight and height were measured by the research team following the Scottish Health Survey 2015 technical report instructions.

4.2.3.2.1. Baseline Questionnaire

Participants were then asked to complete the baseline questionnaire, which included questions on demographics, health, lifestyle, food intakes and sleep habits (described in detail in Chapter 3). The research team also collected information on participant allergies and preferences on coffee or tea, since they would be offered a light snack on experimental days and decaffeinated coffee and tea for the run-in days.

4.2.3.2.2. Familiarisation with experimental sessions

During the second part of the baseline visit, participants were familiarised with the test battery. The principal investigator showed explanatory videos on how to complete the tasks online and then participants completed the test battery once. At the end of baseline visit, which lasted approximately 2 h, participants were provided with week 1 capsules and with decaffeinated coffee and tea, based on their preferences.

4.2.4. Cognitive test battery

The cognitive test battery is described in detail in Chapter 3. The battery was completed on all experimental sessions during the laboratory visits and required a total of 22-25 min to complete, including administration time plus transition time between tasks.

4.2.5. Saliva caffeine

In this study, four different time points were selected to measure saliva caffeine levels and perform the cognitive assessments. Saliva sampling was preferred, since caffeine concentrations in blood and saliva correlate highly and it is less invasive compared to serum sampling (Alkaysi, Salem and El-Sayed, 1988; Tripathi *et al.*, 2015; Dobson *et al.*, 2016).

Following the evidence collected in Chapter 2, we hypothesised that studies investigating the effects of genotypes on caffeine metabolism and subsequent performance 1 h post-supplementation would reflect caffeine absorption (Graham, 2001) and not metabolism, which is catalysed by CYP1A2 enzyme. Therefore, activities

lasting longer than 1 h would provide more valid measures of potential interindividual differences in caffeine metabolism based on genotype. Moreover, it is unknown at what timepoint there would be a large enough difference in the circulating levels of caffeine between ‘fast’ and ‘slow’ metabolisers to have a significant impact on the performance-enhancing properties of caffeine (Southward *et al.*, 2018).

Therefore, in the present study, the first saliva sample was collected to verify adherence to the requirement of overnight fasting, thus verify caffeine abstinence regardless of the study arm, while subsequent samples were used to determine caffeine metabolism rate following caffeine treatment (Dodd *et al.*, 2015). The timepoint 1 h post-supplementation was selected as a measure of peak caffeine saliva concentration (Graham, 2001) and to permit comparisons with previous caffeine studies (Salinero *et al.*, 2017; Carswell *et al.*, 2020). The subsequent timepoints 3 h and 6 h post-supplementation were selected because they are within the average range of caffeine half-life (Nehlig, 2010). We hypothesised that ‘fast’ caffeine metabolisers would demonstrate lower saliva caffeine levels at 3 h and 6 h compared to ‘slow’ metabolisers (Cornelis *et al.*, 2016).

Samples were collected into pre-weighed test tubes (Salivettes®; Sarstedt, Nümbrecht, Germany) using a cotton swab. During sample collection, participants were seated and instructed to rinse their mouth with water. Participants were then requested to keep the swab in the mouth (e.g., in the cheek) for two minutes. The cotton swab was then placed back into the saliva collection tube. The saliva samples were collected at least 60 min after a meal (liquid/solid food intake) to avoid contamination of saliva. Since the onset of bacterial growth in saliva can be expected after a few hours in room temperature, the Salivettes® were sealed tightly after each sampling and centrifuged on collection of the final saliva sample, within 6 h of first sample collection. The tubes were centrifuged (Sanyo Centaur 2, MSE UK Ltd, London, UK) at 3,500 rpm for 2 min.

After being centrifuged, clear saliva samples were stored at −80 ° C until they were analysed using a spectrophotometric method, the Enzyme Multiplied Immunoassay Technique (EMIT), as described previously (Zysset, Wahlländer and Preisig, 1984; Tripathi *et al.*, 2015). The EMIT assay is an automated homogeneous enzyme immunoassay kit (SYVA, Siemens Healthineers Ltd, Surrey, UK) intended for use in the quantitative analysis of caffeine levels in human serum and saliva. The assay combines immunoassay and kinetic enzyme analysis. It is based on the photometric determination of the activity of an enzyme, which is chemically coupled to

the drug to be determined and that is inactivated by binding with the drug-specific antibody. In contrast to other immunoassays, no separation step is necessary, thus the method is simple and fast (Schobben and van der Kleijn, 1977). Caffeine saliva concentrations were provided by the instrument in μmol/L and were then converted to μg/L based on caffeine molecular weight (0.194 μg/mol) (National Center for Biotechnology Information, 2023) for comparability with previous studies.

4.2.6. Genotyping

All laboratory analyses were performed at St Mary's University, Twickenham as described in Chapter 3.

4.2.7. Drug guessing

In a double-blind study design, it should not be assumed that double-blind has been established or maintained just because placebo and active substance appear identical. Participants are expected to guess that they are taking placebo 50% of the time; otherwise, conclusions about the efficacy of the treatment may be misleading (Desbiens, 2002). Therefore, before the end of each laboratory visit, participants were asked to state whether they think they had ingested caffeine or placebo and provided two responses, i.e., one for the 6-day pre-challenge period and one for the challenge day, for each of the four conditions of the experiment (PP, PC, CP, CC).

4.2.8. Adherence to supplementation

To assess participant adherence to supplementation, a random oversupply of capsules was put in each pot during capsule blinding. On each visit, participants were asked to return the pot from the previous week. Two members of the research team collected the bottles and counted the remaining capsules for each participant per experimental trial. The number of capsules initially included in each pot was revealed to the research team after the completion of data collection. Adherence rates were calculated as the ratio of missing capsules to the number initially included in the pot for each week and for the four weeks collectively. Adherence was then assessed by dichotomising the continuous measure of adherence and classifying participants above the threshold of 80% as adherent and those below it as non-adherent (Karve *et al.*, 2009; Warren *et al.*, 2013).

4.2.9. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 28.0 for Windows. Data are shown as means ± SD or median ± IQR and were tested for normality with Shapiro-Wilk test. Categorical variables are presented as frequencies. Differences in participant characteristics based on genotype groups ('slow' vs 'fast' metabolisers and 'sensitive' vs 'non-sensitive' individuals) were tested with an independent samples t-test (with Levene's test for equality of variance), Mann–Whitney U or Fisher's Exact test, where appropriate. One-sample t-test was used to compare the mean accuracy of correct drug guesses (correctly reporting that caffeine or placebo had been ingested) with chance alone (50-50%).

Grouping of participants based on level of habitual caffeine intake and analyses of cognitive performance were in line with Study 2 (Chapter 3). Deviations from HWE were performed using the χ^2 goodness-of-fit test. Genetic caffeine metabolism from *CYP1A2* rs2472297 and *AHR* rs6968554, caffeine sensitivity from *ADORA2A* rs5751876 and APO ε4-/ε4+ genotype were calculated as in Study 2 (Chapter 3).

The model used was the three-way mixed ANOVA consisting of one between-subjects factor for genetics and two within-subjects factors for experimental conditions and timepoints for subjective sleepiness, measures of cognitive function, global cognition and saliva caffeine concentrations. The experimental condition factor incorporated the four dosing conditions (PP x PC x CP x CC) and the timepoint factor incorporated the baseline and 1, 3 and 6 h post challenge laboratory measurements. Two-way repeated measures ANOVAs were also performed for the above measures using the two within-subjects factors (condition x timepoints). All tests were 2-tailed, with significance assumed at the 5% level.

4.3. Results

4.3.1. Participant characteristics

Fifteen eligible participants signed up for the study. Of those, three did not consent to proceed to the experimental protocol. All twelve participants who agreed to enrol completed the 4-week protocol. No participants were excluded from the study because of lack of compliance to study requirements. Participant characteristics are shown in Table 4.2.

The study sample consisted of healthy adults (9 (75%) females and 3 (25%) males) aged between 21 and 55 years of mostly (75%) Caucasian/white descent. Eight participants (67%) had a normal BMI (18.5-24.9 kg/m^2), while four participants (33%) were classified as overweight (25-29.9 kg/m^2). The sample consisted of moderate caffeine consumers (130.6 ± 114.2 mg/day), while the mean alcohol consumption was 22.4 ± 30.7 g/week, which corresponds to less than two drinks/week and is within the safe levels of consumption (Conigrave *et al.*, 2021).

The *CYP1A2* rs2472297, *AHR* rs6968554, *ADORA2A* rs5751876, *ADA* rs77819966 and the *APOE* rs429358 and rs7412 did not deviate from HWE (p_{all} > 0.05). Allele frequencies for *CYP1A2* rs2472297 were C (88%) and T (12%), for *AHR* rs6968554 were A (83%) and G (17%), for *ADORA2A* rs5751876 were C (67%) and T (33%), for *ADA* rs77819966 were A (96%) and G (4%), for *APOE* rs429358 were T (79%) and C (21%) and for *APOE* rs7412 were T (4%) and C (96%). All allele frequencies were in line with publicly available data on allele frequencies, apart from rs6968554 and rs77819966 SNPs (Cunningham *et al.*, 2022).

Table 4. 2 Participant characteristics.

	All (N = 12)
Age[b], years	31.6 ± 11.2
Gender, F / M (%)	9 (75.0) / 3 (25.0)
BMI[a] (kg/m^2)	22.9 ± 3.1
Ethnicity, white/non-white (%)	9 (75.0) / 3 (25.0)
Education level[b]	5.0 ± 4.5
PAL, L / M / H (%)	1 (8.3) / 8 (66.7) / 3 (25.0)
Sleep Quality, Poor / Good (%)	8 (66.7) / 4 (33.3)
PSQI[b]	5.5 ± 1.0
Alcohol intake[b] (g/week)	22.4 ± 30.7
Caffeine intake[a] (mg/day) [L / M / H]	130.6 ± 114.2 [4 / 7 / 1]
ADA genotype, GG/AG (%)	11 (91.7) / 1 (8.3)
APOE genotype, ε4 carriers / ε4 noncarriers (%)	5 (41.7) / 7 (58.3)

[a] Values represent means ± SD; [b] Values represent medians ± IQR; BMI: Body Mass Index; PAL: Physical Activity Level; L: Low; M: Moderate; H: High; PSQI: Pittsburgh Sleep Quality Index; Low, moderate, and high caffeine intake: 0–50, 51–300, and > 300 mg/day, respectively.

4.3.2. Participant characteristics across genetic groups

For the rs5751876 SNP in the *ADORA2A* gene, five participants were homozygous for the C allele (CC, i.e., non-sensitive), while one was homozygous T allele (TT) and six were heterozygous (TC). Therefore, we grouped T allele carriers

together (TT and TC, n = 7) and classified them as caffeine-sensitive. For the rs2472297 SNP in the *CYP1A2* gene, 10 participants were homozygous for the C allele (CC), one was heterozygous carrier of the C allele (CT) and one participant was homozygous for the T allele (TT). For the rs6968554 SNP in the *AHR* gene, eight participants were homozygous for the A allele (AA) and four were heterozygous carriers of the A allele (AG). Using an unweighted score for genetic caffeine metabolism (Cornelis *et al.*, 2016), six participants had a score of 0 and six participants had a score of 1-2, while no participants had a score of 3-4. Therefore, participants with score 0 were classified as 'slow' metabolisers and participants with score 1-2 were classified as 'fast' metabolisers. Participant characteristics were not different between genotype groups ($p_{\text{all}} > 0.05$, Table 4.3); except gender proportions and education level between sensitive and non-sensitive individuals ($p < 0.05$).

Table 4. 3 Participant characteristics by genotype groups.

	ADORA2A			***AHR + CYP1A2***		
	sensitive n = 7	non-sensitive n = 5	*P*	slow n = 6	fast n = 6	*P*
Age, years	31.0 ± 10.1	32.4 ± 13.7	0.842	34.2 ± 10.7	29.0 ± 11.9	0.065
Gender, F / M (%)	7 (100) / 0 (0)	2 (40) / 3 (60)	0.045	5 (83) / 1 (17)	4 (67) / 2 (33)	1.000
BMI (kg/m^2)	21.2 ± 2.1	25.3 ± 2.6	0.012	23.1 ± 2.9	22.8 ± 3.4	0.884
Ethnicity, white / non-white (%)	5 (71) / 2 (29)	4 (80) / 1 (20)	1.000	3 (50) / 3 (50)	6 (100) / 0 (0)	0.182
Education level	6.0 ± 1.0	5.0 ± 2.0	0.030	5.5 ± 1.0	5.0 ± 2.3	0.180
PAL, L / M / H (%)	1 (14) / 4 (57) / 2 (29)	0 (0) / 4 (80) / 1 (20)	1.000	1 (17) / 5 (83) / 0 (0)	0 (0.0) / 3 (50) / 3 (50)	0.182
Sleep Quality, Poor / Good (%)	5 (71) / 2 (29)	3 (60) / 2 (40)	1.000	4 (67) / 2 (33)	4 (67) / 2 (33)	1.000
PSQI	7.0 ± 4.0	5.0 ± 4.5	0.149	6.5 ± 4.5	5.0 ± 4.0	0.394
Alcohol intake (g/week)	35.5 ± 34.9	4.0 ± 6.0	0.078	9.4 ± 18.9	35.3 ± 36.3	0.152
Caffeine intake (mg/day) [L / M / H]	162.4 ± 127.6 [2 / 4 / 1]	86.1 ± 84.9 [2 / 3 / 0]	0.274	112.2 ± 126.7 [3 / 2 / 1]	149.0 ± 108.7 [1 / 5 / 0]	0.601
ADA genotype, GG/AG (%)	7 (100) / 0 (0)	4 (80) / 1 (20)	0.417	6 (100) / 0 (0)	5 (83) / 1 (17)	1.000
APOE genotype, ε4 carriers / ε4 noncarriers (%)	3 (43) / 4 (57)	2 (40) / 3 (60)	1.000	2 (33) / 4 (67)	3 (50) / 3 (50)	1.000

Participants are categorised according to *ADORA2A* ('sensitive' or 'non-sensitive') and *AHR+CYP1A2* genotypes ('slow' or 'fast' metabolisers). BMI: Body Mass Index; PAL: Physical Activity Level; L: Low; M: Moderate; H: High; PSQI: Pittsburgh Sleep Quality Index; Low, moderate, and high caffeine intake: 0–50, 51–300, and > 300 mg/day, respectively.

4.3.3. Cognitive performance

4.3.3.1 Condition x timepoints

To compare subjective alertness before tasks and cognitive performance between conditions x timepoints, two-way repeated measures ANOVAs were performed for subjective sleepiness, for each domain of cognitive function and for global cognition scores using the four conditions and four timepoints as within-subjects factors (Table 4.4).

Table 4. 4 Summary of two-way repeated-measures ANOVAs of subjective sleepiness scores, emotion recognition, memory, attention, executive function and global cognition (n=12).

	condition x timepoints	
	F (9, 99)	*P*
KSS	0.643	0.758
Emotion recognition (ms)	1.326	0.233
Mean memory (ms)	1.835	0.071
Attention (ms)	2.190	0.029
Executive function (Δms)	0.865	0.473
Global cognition score	1.022	0.428

Values represent the results from two-way repeated-measures ANOVA using two within-subjects factors (4 conditions x 4 timepoints). KSS: Karolinska Subjective Sleepiness; ms: milliseconds; Δms: difference in ms.

As shown in Table 4.4, a statistically significant condition x timepoints interaction was found for the domain of attention. After Bonferroni correction, pairwise comparisons indicated that pre-supplementation and 1 h post-supplementation performance in attention were statistically significantly different in the PC condition (F (3, 33) = 3.524, p = 0.025). Compared with baseline, 1 h post-supplementation RTs were 15.8 ms lower (390.0 ± 31.9 ms vs 374.2 ± 28.6 ms, respectively; CI 95%, -29.078 to -2.437, p = 0.018). No other significant differences were observed.

4.3.3.2. Genetics x condition x timepoints

To investigate whether genetics may impact the effects of caffeine on tasks and cognitive performance between conditions x timepoints, three-way mixed repeated-measures ANOVAs were performed using the conditions and timepoints as within-subjects factors and a) caffeine metabolism ('fast' vs 'slow') and b) caffeine sensitivity ('sensitive' vs 'non-sensitive') as between-subjects factors (Table 4.5). The Greenhouse-Geisser correction was used when the Mauchly's test of sphericity indicated that the assumption of sphericity had been violated.

Table 4. 5 Summary of three-way repeated-measures ANOVAs of subjective sleepiness scores, emotion recognition, memory, attention, executive function and global cognition (n=12).

	ADORA2A sensitive vs non-sensitive		*AHR+CYP1A2* slow vs fast	
	F (9, 90)	*P*	*F* (9, 90)	*P*
KSS score	1.035	0.402	1.312	0.242
Emotion recognition (ms)	1.553	0.142	0.414	0.925
Memory (ms)	1.139	0.344	2.237	0.026
Attention (ms)	0.560	0.826	1.292	0.252
Executive function (Δms)	2.247	0.026	1.445	0.248
Global cognition score	0.599	0.684	0.577	0.717

Values represent the results from three-way repeated-measures ANOVA using two within-subjects factors (4 conditions x 4 timepoints) and one between-subjects factor ('sensitive' vs 'non-sensitive' and 'slow' vs 'fast'). KSS: Karolinska Subjective Sleepiness; ms: milliseconds; Δms: difference in ms.

There was a statistically significant three-way interaction between genotype ('fast' vs 'slow'), conditions and timepoints for memory RTs and between genotype ('sensitive' vs 'non-sensitive'), conditions and timepoints for Stroop effect ΔRTs.

Simple two-way interactions were performed to assess whether performance in memory and executive function is affected by a condition x timepoint effect at one or both levels of the third factor, genetics. Data are mean ± standard deviation. There was a statistically significant simple two-way interaction between condition and timepoints for 'fast' metabolisers, F (9, 45) = 3.483, p = 0.02, but not for 'slow' metabolisers, F (9, 45) = 0.744, p = 0.667 for memory performance. However, there was no statistically significant simple two-way interaction between condition and timepoints for caffeine sensitive individuals, F (9, 54) = 1.880, p = 0.075 or non-sensitive individuals, F (9, 36) = 1.304, p = 0.269 in executive function. The reason why there is a significant three-way interaction but no two-way interaction between condition and timepoints in executive function may be due to small sample size, a high number of factor levels, or both (Weisberg, 2014).

For 'fast' and 'slow' metabolisers, all simple simple pairwise comparisons were run between the different timepoints in each condition. Investigations for simple simple main effects after Bonferroni corrections showed that there was a statistically significant simple simple main effect in timepoint 1 (pre-supplementation) for 'fast' metabolisers performance in memory, F (3, 15) = 5.840, p = 0.008, but not for 'slow' metabolisers, F (3, 15) = 3.490, p = 0.042. 'Fast' metabolisers had 207.5 (95% CI, -386.6 to -28.4) ms lower RTs in the CC condition compared with the CP condition, p = 0.027 (825.1 ± 74.4 ms vs 1032.6 ± 142.0 ms, respectively). No other simple simple main effects were revealed (Figures 4.3 and 4.4).

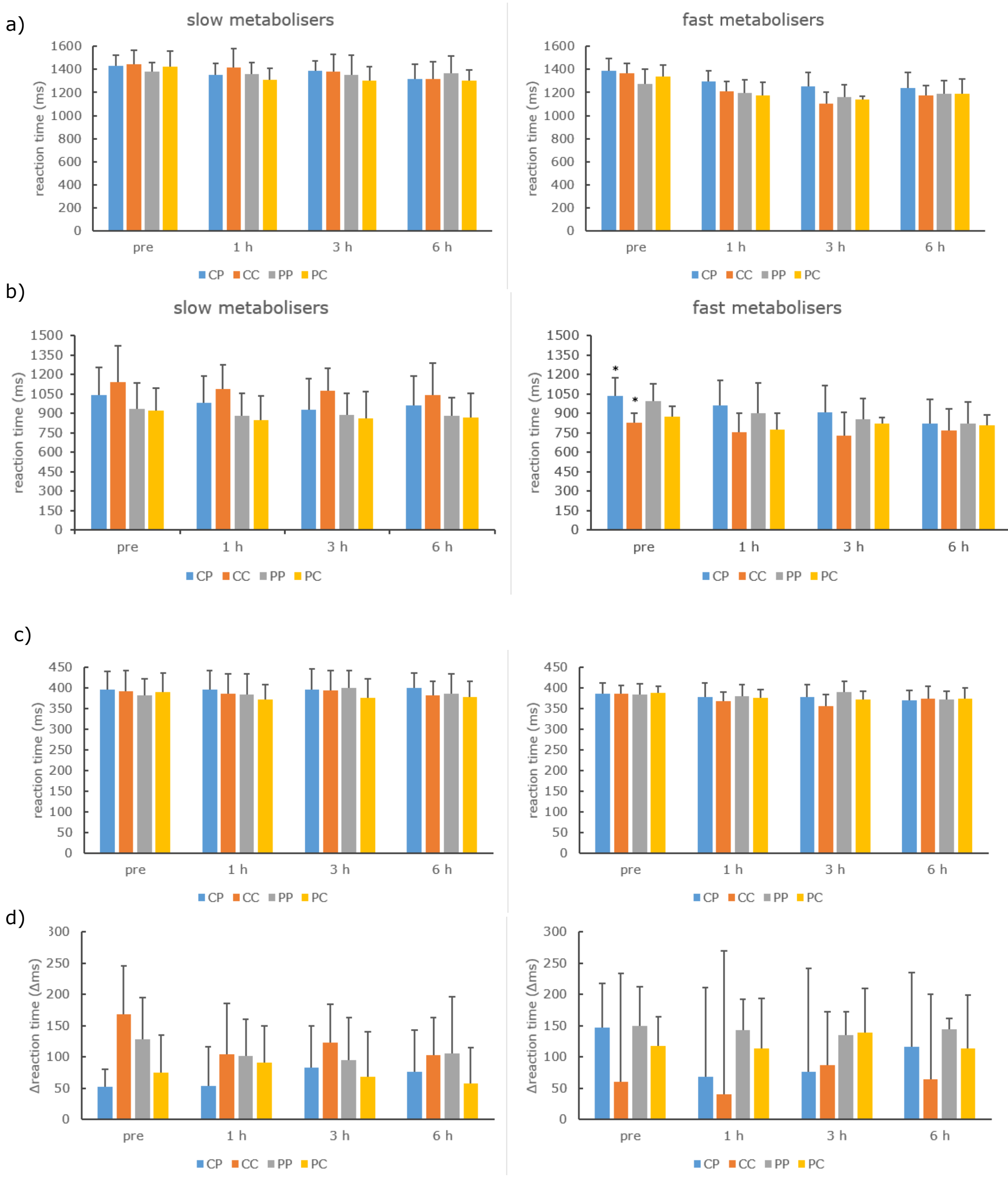

Figure 4. 3 Performance in the domains of a) emotion recognition, b) memory, c) attention and d) executive function pre-, 1 h, 3 h and 6 h post-supplementation. Participants are categorised based on genetic caffeine metabolism: 'slow' (left) or 'fast' caffeine metabolisers (right). Data are mean ± SD. **P* < 0.05 between CP and CC conditions.

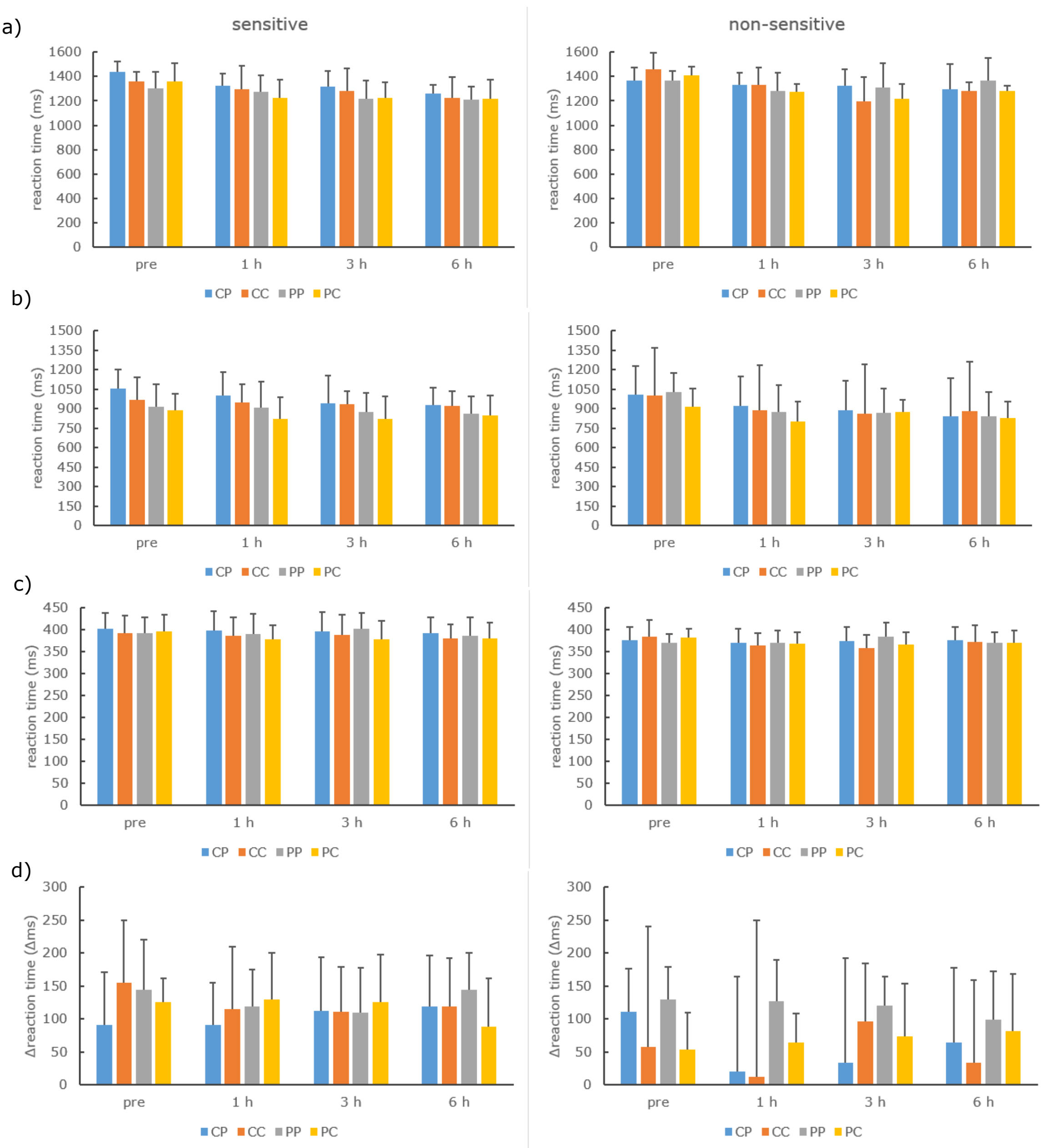

Figure 4. 4 RTs in the domains of a) emotion recognition, b) memory, c) attention and d) executive function pre-, 1 h, 3 h and 6 h post-supplementation. Participants are categorised based on genetic caffeine sensitivity: 'sensitive' (left) or 'non-sensitive' caffeine metabolisers (right). Data are mean ± SD.

4.3.4. Saliva caffeine

It has been previously reported that mean saliva caffeine concentrations below 1 µg/ml correspond to overnight caffeine abstinence (Evans and Griffiths, 1999). In

the present sample, mean baseline caffeine values were 0.86 ± 0.57 µg/ml for the CP condition, 0.89 ± 0.67 µg/ml for the CC condition, 0.07 ± 0.06 µg/ml for the PP condition and 0.09 ± 0.11 µg/ml for the PC condition.

A two-way repeated measures ANOVA was performed to compare saliva caffeine levels for the different timepoints in the two conditions involving caffeine supplementation on challenge day. After Greenhouse-Geisser correction, there was a statistically significant two-way interaction between condition and timepoints, F (3, 33) = 34.872, $p < 0.001$. In pairwise comparisons, there was a statistically significant difference between conditions ($p = 0.01$) and between timepoints (pre-supplementation with all post-supplementation and between timepoints 3 with 4). Conditions were different for timepoint 1 ($p = 0.002$) and timepoint 2 ($p = 0.005$), but not for timepoints 3 and 4. Within the CC condition, timepoint 1 was statistically significantly different from all timepoints ($p_{all} < 0.001$), while there was a statistically significant difference between timepoints 2 and 4 ($p = 0.030$). Within the PC condition, timepoint 1 was statistically significantly different with all timepoints ($p_{all} < 0.007$), while there was a statistically significant difference between timepoints 3 and 4 ($p < 0.001$) (Figure 4.5).

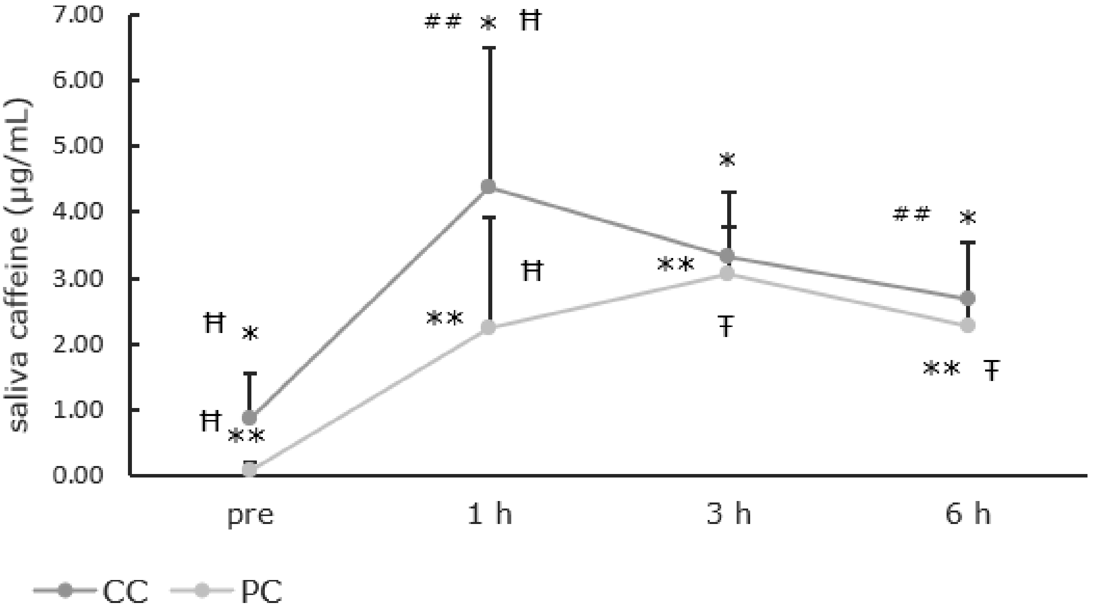

Figure 4. 5 Saliva caffeine for CC and PC conditions measured pre-supplementation and 1, 3 and 6 h post-supplementation. Data are mean ± SD. *P < 0.05 for pre- vs 1 h vs 3 h vs 6 h post-supplementation; **P < 0.05 for pre- vs 1 h vs 3 h vs 6 h post-supplementation; ##P < 0.05 for 1 h vs 6 h post-supplementation; ŦP < 0.05 for 3 h vs 6 h post-supplementation; ĦP < 0.05 for CC vs PC conditions pre- and 1 h post-supplementation.

To assess whether saliva caffeine levels were different based on genetically predicted caffeine metabolism, caffeine levels were compared between 'fast' and 'slow' metabolisers on the two conditions which involved caffeine supplementation on challenge day using a three-way mixed ANOVA. There was no statistically significant three-way interaction between genotype ('fast' vs 'slow' metabolisers), condition (CC vs PC) and timepoints (pre- and 1, 3 and 6 h post-supplementation), F (3, 30) = 0.973, p = 0.415. Table 4.6 shows the mean caffeine saliva levels for all conditions and timepoints based on genotype.

Table 4. 6 Saliva caffeine 1, 3 and 6 h post-supplementation based on condition and genetic caffeine metabolism.

	Baseline		**1 h**		**3 h**		**6 h**	
	slow	**fast**	**slow**	**fast**	**slow**	**fast**	**slow**	**fast**
CC saliva caffeine (μg/ml)	1.19 ± 0.78	0.58 ± 0.38	5.32 ± 1.40	3.41 ± 2.42	3.65 ± 1.24	2.99 ± 0.57	2.92 ± 1.11	2.42 ± 0.49
PC saliva caffeine (μg/ml)	0.06 ± 0.09	0.13 ± 0.14	2.72 ± 1.84	1.74 ± 1.55	3.33 ± 0.69	2.79 ± 0.66	2.39 ± 0.38	2.12 ± 0.47
PP saliva caffeine (μg/ml)	0.05 ± 0.04	0.08 ± 0.07	0.04 ± 0.03	0.06 ± 0.04	0.0 ± 0.03	0.05± 0.04	0.03 ± 0.02	0.04 ± 0.03
CP saliva caffeine (μg/ml)	0.88 ± 0.59	0.84 ± 0.60	0.91 ±0.85	0.96 ± 0.90	0.79 ± 0.70	0.73 ± 0.63	0.55 ± 0.59	0.49 ± 0.39

Participants are categorised according to genetic caffeine metabolism (*CYP1A2* + *AHR*, 'fast' or 'slow' metabolisers) and condition (CC vs PC vs PP vs CP). Data are mean ± SD.

4.3.5. Adherence to supplementation

By capsule count, the overall mean adherence rate of the group was 85.1 ± 16.1%. Adherence rate was also above the threshold of 80% for each week: 88.0 ± 15.4% for week 1, 82.4 ± 21.8% for week 2, 85.7 ± 15.5% for week 3 and 84.3 ± 11.5% for week 4. Based on the overall pot count (4 weeks x 12 participants), 31.3% (n=15) were considered non-adherent, while 68.8% (n=33) were considered adherent.

4.3.6. Drug guessing

The mean drug guessing accuracy (0.63 ± 0.19), representing the number of correct guesses of supplementation divided by the total guesses (four guesses for run-in supplementation and four guesses for challenge days per participant) was not statistically significantly different than guessing by chance alone, as assessed by a one-sample t-test, t (11) = 2.171, p = 0.056.

4.4. Discussion

The present study is the first trial to investigate the influence of three SNPs (*ADORA2A* rs5751876 for caffeine sensitivity and *AHR* rs6968554 and *CYP1A2* rs2472297 combined for caffeine metabolism) on the effects of caffeine on cognitive performance. Genotype was not found to affect caffeine levels in saliva, nor performance in any cognitive domains. The results of the present findings are discussed in detail in the following sections.

4.4.1. Cognitive performance

4.4.1.1. Condition x timepoints

This study differed from most previous research on the effects of caffeine on cognitive performance in two important aspects. First, caffeine effects were examined in relation to controlled exposure over several weeks, consisting of periods of repeated ingestion of the drug versus abstinence. Second, the study used a schedule of caffeine consumption that is broadly representative of population consumption patterns. As such, the findings may have greater generalisability than the results of many previous studies in this field.

The manipulations of caffeine intake, which were in the core of this experiment, did not produce a clear pattern of effects on performance. First, at baseline, performance was not impaired in the CP and CC conditions (overnight abstinence – withdrawal symptoms) relative to the PP and PC conditions (no withdrawal symptoms). Moreover, performance was not impaired post-supplementation when caffeine was withdrawn following habitual use (CP condition) compared to the other conditions. Therefore, these findings do not support the argument by James *et al.* (2005) that previous reports of significant caffeine-induced improvements in performance may have been methodologically flawed due to failure to control for abstinence effects in caffeine trials involving habitual consumers.

Caffeine withdrawal refers to a time-limited syndrome that develops following cessation of chronic caffeine administration and can be summarised as a common CNS depression (Ammon, 1991). Caffeine withdrawal in humans includes symptoms of irritability, headache, fatigue, nervousness, difficulty concentrating, loss of energy and dysphoric mood (Griffiths, Bigelow and Liebson, 1989; Griffiths *et al.*, 1990; Juliano and Griffiths, 2004; Juliano, Huntley, *et al.*, 2012). Herein, we investigated the

subjective sleepiness (and consequently, alertness) of participants prior to tasks; however, no differences between conditions were observed. Hence, the present findings suggest that impaired cognitive performance and reduced subjective alertness, at least as measured in this study, may not be added to the list of effects known to be associated with caffeine withdrawal.

Secondly, 3 mg/kg caffeine was shown to improve performance in attention 1 h post-supplementation compared to baseline in the PC condition, i.e., after acute caffeine intake. This supports previous findings that caffeine, in amounts commonly consumed by the majority of the population, has beneficial effects in attention performance (Ruxton, 2008; Tieges *et al.*, 2009; Brunyé, Mahoney, Lieberman and Taylor, 2010; Serra-Grabulosa *et al.*, 2010; Foxe *et al.*, 2012). Interestingly, it has been noted that the beneficial effects of caffeine in attentional processes are evident even with small doses (~0.5 mg/kg), regardless of level of alertness of individuals (Smith, 2002; Nehlig, 2010). Nevertheless, there was an absence of evidence of caffeine-induced enhanced performance in other domains of cognition or in other timepoints, either in the context of acute or habitual use, perhaps adding to the argument that caffeine only makes us faster but does not enhance other, more complex cognitive processes (Rogers *et al.*, 2013).

4.4.1.2. Genetics x condition x timepoints

4.4.1.1.1. Genetic caffeine metabolism

The present findings suggest that genetically predicted caffeine metabolism did not modify the effect of caffeine on cognitive performance. Specifically, the CP condition exhibited a lower memory performance when compared to the CC condition at baseline within the group of 'fast' metabolisers, while no genotype differences were observed.

There are two genetics studies to date assessing genetic caffeine metabolism x caffeine interactions on cognitive performance (Salinero *et al.*, 2017; Carswell *et al.*, 2020). In the study by Carswell *et al.* (2020), caffeine enhanced cognitive performance of 'fast' metabolisers in the domain of attention more than 'slow' metabolisers during exercise and at rest 120-min post-supplementation. Conversely, in the study by Salinero *et al.* (2017), no effect of *CYP1A2* genotype on attention RTs was observed 1 h post-supplementation (Salinero *et al.*, 2017).

Comparable to the present work, the studies assessed the domain of attention using the PVT after 3 mg/kg body mass caffeine compared to placebo. However, both studies used the *CYP1A2* rs762551 SNP as proxy of caffeine metabolism, contrary to our study. The rs762551 variant has been found to exhibit modest LD with other *CYP1A2* variants (R^2 = 0.05–0.29), however it did not reach genome-wide significance in a GWAS of caffeine metabolites (Cornelis *et al.*, 2016). Although functional data show that this variant affects CYP1A2 enzyme inducibility after caffeine intake (Sachse *et al.*, 1999), perhaps these effects are due to LD of this SNP with the other *CYP1A2* variants. In the present study, we selected the two variants (one of which is a *CYP1A2* SNP in LD with rs762551) with the largest effect sizes in a GWAS of caffeine metabolites (Cornelis *et al.*, 2016). Nevertheless, it needs to be considered that because of the small sample size, in the present analysis there were no participants who were indeed 'fast' caffeine metabolisers, and comparisons were made between 'slow' and what could have been classified as 'intermediate' caffeine metabolisers.

Additional elements of the study methodologies need to be considered. Notably, while our study included mostly females, both investigations by Carswell *et al.* (2020) and Salinero *et al.* (2017) predominantly featured males who were physically active and of a younger mean age compared to our sample. Furthermore, we need to consider the different assessment conditions. For instance, cognitive assessments in the study by Carswell *et al.* (2020) were performed during a 20-min cycling trial (being completed 50-min post-supplementation) and post-exercise, 120min post-supplementation. Therefore, the observed enhancements in cognitive performance may not represent a net effect of caffeine but rather be influenced by the effects of aerobic exercise. As discussed in Chapter 1 of this thesis, exercise interventions have demonstrated improvements in motor function, response speed and attention (Angevaren *et al.*, 2008). Moreover, the cycling trial was concluded 50-min post-supplementation, which is probably not enough time to reflect differences between 'fast' and 'slow' metabolisers. As such, we argue that there is a biological basis to attribute differences in cognitive performance to genotype groups.

Salinero *et al.* (2017) also evaluated cognitive performance 1 h post-supplementation, potentially reflecting caffeine absorption rather than metabolism. Finally, a notable difference between the studies pertains to the washout period between trials. Similar to our study, Salinero *et al.* (2017) utilised a 1-week washout period, while Carswell *et al.* (2020) employed a 2-day washout period, potentially subjecting habitual caffeine consumers to caffeine withdrawal effects (Griffiths,

Bigelow and Liebson, 1986; Hughes *et al.*, 1993). Therefore, the beneficial effects of caffeine on attention in this study may represent the reversal of withdrawal symptoms.

4.4.1.1.2. Genetic caffeine sensitivity

Our investigation suggests that genetically predicted caffeine sensitivity (*ADORA2A* gene) did not either modify the effect of caffeine on cognitive performance. Apart from stimulatory effects, caffeine has been reported to possess anxiogenic properties, which can subsequently affect cognitive performance (Rogers *et al.*, 2010; Shields, Sazma and Yonelinas, 2016). The *ADORA2A* rs5751876 SNP has been linked with risk for elevated or pathological anxiety (Alsene *et al.*, 2003; Hohoff *et al.*, 2009, 2010), a finding that has been confirmed by functional studies (Hohoff *et al.*, 2014). This has also been suggested to affect dopaminergic neurotransmission, resulting in feelings of anxiety and insomnia following caffeine ingestion (Alsene *et al.*, 2003; Childs *et al.*, 2008).

Although previously used by authors to categorise individuals as having a 'high' (TT genotype) or 'low' (CT/CC genotype) sensitivity to caffeine (Alsene *et al.*, 2003; Childs *et al.*, 2008; Carswell *et al.*, 2020), whether this SNP may impact cognitive performance remains to be elucidated. It is important to note that in the only study to date showing a differential effect of *ADORA2A* genotypes (TT vs CC) on cognition (Renda *et al.*, 2015), only male participants were recruited. This is particularly important, since previous studies on anxiety measures showed possible gender-specific caffeine effects, with females TT homozygous for *ADORA2A* rs5751876 experiencing higher levels of anxiety (Domschke *et al.*, 2012a; Gajewska *et al.*, 2012). These differences, probably reflecting variations in levels of circulating oestrogens (Nehlig, 2010) limit the generalisability of the findings.

It is possible that genetic caffeine sensitivity may modify the effects of caffeine on cognitive performance only in sleep-deprived states, as shown in the studies by Baur *et al.* (2021) and Bodenmann *et al.* (2012). The results from these studies suggest that caffeine attenuates the impairments of insomnia in both simple and complex functions based on the *ADORA2A* gene (Bodenmann *et al.*, 2012; Baur *et al.*, 2021). Nevertheless, as discussed in Chapter 2, only the CC genotype group of the rs5751876 SNP was included in one study (Baur *et al.*, 2021) and the second study included only male subjects (Bodenmann *et al.*, 2012).

A possible reason for the absence of genotype differences in the present investigation is the limited representation of homozygous *ADORA2A* T allele carriers in

our sample, as only one participant fell into this category. Consequently, we had to group all T allele carriers together. This categorisation left us without a distinct group of caffeine-sensitive individuals based on previous research. However, it is noteworthy that the study conducted by Carswell *et al.* (2020), despite having a more balanced representation of sensitive and non-sensitive groups, also failed to reveal a significant modulatory effect of the *ADORA2A* genotype on the impact of caffeine on cognitive performance.

4.4.2. Caffeine metabolism

Our overall findings are strengthened by the saliva caffeine assay findings that systemic caffeine levels were higher during caffeine phases of the study, while being at negligible levels during placebo phases.

However, our results showed that there were no between-genotype differences in caffeine metabolism. The absence of an effect of genetic metabolism on cognitive performance in the present study is therefore possibly due to the observed lack of any pharmacokinetic differences over the study period (i.e., up to 6 h after caffeine ingestion). Faster clearance of caffeine, as was anticipated to occur in 'fast' metabolisers, would have indicated higher cognitive performance relative to 'slow' metabolisers, because of a build-up of paraxanthine in the brain (Cappelletti *et al.*, 2015; Alsabri *et al.*, 2018). Paraxanthine, the main caffeine metabolite, is a potent adenosine receptor antagonist (Gu *et al.*, 1992) and possesses psychostimulant properties (Graham, 2001).

From the genetics studies up to date on cognition, only the study by Carswell *et al.* (2020) measured caffeine concentration, specifically in serum using high-performance liquid chromatography (HPLC). Although the study by Carswell *et al.* (2020) used a different assessment method for caffeine metabolism compared to our study (serum vs saliva assessed using HPLC vs EMIT assay, respectively), the two methods have been shown comparable, as well as reliable and reproducible (Tripathi *et al.*, 2015). Despite the authors found a difference in performance between 'fast' and 'slow' metabolisers in attention, serum metabolite measures between genotypes were not different, which means that there is currently no evidence to indicate that differences in caffeine metabolism were responsible per se. In fact, this strengthens our argument for possible exercise-induced cognitive enhancements in the study.

Therefore, no between-genotype differences in saliva caffeine concentrations were present up to 6 h post-caffeine consumption in the present study, which may partly explain the lack of significant between-genotype differences in cognitive performance.

4.4.3. Genetic habitual caffeine intake

It is important to highlight that the results of the present study do not confirm that genetics may be involved in individual variability in caffeine consumption at the pharmacodynamic and pharmacokinetic level (Laitala, Kaprio and Silventoinen, 2008; Yang, Palmer and de Wit, 2010). The genetic metabolism variants employed in the current analysis have been shown to be associated with habitual caffeine intake (Cornelis *et al.*, 2011, 2015, 2016), supporting the hypothesis that individuals self-regulate their caffeine intake to achieve a self-perceived optimal level of arousal (Zhou *et al.*, 2010; Harvanko *et al.*, 2015).

Moreover, the TT genotype in the *ADORA2A* rs5751876 has been linked with lower habitual caffeine intake than C allele carriers (Cornelis, El-Sohemy and Campos, 2007). Yet, although we would have expected 'slow' metabolisers (caffeine stays in their system for longer) and sensitive individuals (experience caffeine-induced anxiety) to have a significantly lower habitual caffeine intake compared to 'fast' metabolisers and 'non-sensitive' individuals respectively, no differences were observed. This finding is most probably due to the small sample size in this study. In essence, we replicated this finding for 'fast' vs 'slow' metabolisers, but not for genetic caffeine sensitivity groups in Study 2.

4.4.3. Strengths and limitations

A notable strength of the present study is that cognitive performance was assessed using a test battery of widely used and validated tests for the four key domains of cognition (Stroop, 1935; Lundqvist, Flykt and Öhman, 1998; Owen *et al.*, 2005; Lim and Dinges, 2008). A second strength lies within the protocol, which was designed to control for withdrawal reversal (James, 1994a, 1994b, 1998). Our study was designed to isolate the chronic effects of dietary caffeine, while the mean habitual intake of participants was representative of adult moderate consumers. Further strengths of the current experimental approach include the use of an ecologically valid dose of caffeine prior to performance assessments, previously used in genetics studies

on cognitive performance (Salinero *et al.*, 2017; Carswell *et al.*, 2020) and the validation of caffeine metabolism using saliva samples.

Finally, one of the major strengths of the present study is the selection of an extended testing period using four timepoints to assess cognitive function: pre- and 1, 3 and 6 h post-supplementation. Importantly, caffeine concentrations measured up to an hour after ingestion may be more reflective of caffeine absorption than metabolism (Graham, 2001). While the half-life of caffeine in adults is on average 4–6 h (Nehlig, 2018), it is not yet known to what degree caffeine metabolism is different between 'fast' and 'slow' metabolisers.

Nevertheless, the sample size of this work needs to be considered. The 12 participants in the present study were not sufficient to meet the calculated sample size requirement and the proportion of individuals with fast and slow metabolism or sensitive and non-sensitive genotypes. In fact, this sample size meant that only one participant was homozygous of the *ADORA2A* T allele, and only one participant was homozygous of the *CYP1A2* C allele. As a result, there were no participants who could be classified as 'fast' caffeine metabolisers; instead, they could be categorised as 'intermediate' metabolisers, while no participants were caffeine 'sensitive' (TT homozygous) and were instead grouped with the CT genotype group. This may be one of the reasons for the lack of statistical significance in our results and warrants further investigation using a larger sample size.

Another potential limitation of the present study is that only caffeine and not paraxanthine was measured in saliva. However, clearance of caffeine in plasma or saliva is the gold standard measurement for CYP1A2 enzyme function (Faber, Jetter and Fuhr, 2005). Lastly, participant compliance to the protocol was not confirmed by requesting a saliva sample every afternoon. Although recommended by the protocol from James (1994), daily saliva samples were not requested to avoid an increase in the load of participants, especially since the appropriate conditions of saliva sampling and storage, as recommended by the manufacturer, could not be guaranteed. Instead, adherence was assessed using the method of capsule count and by dichotomising participants into adherent and non-adherent using arbitrary thresholds (Karve *et al.*, 2009; Warren *et al.*, 2013). Although most participants were considered adherent, it needs to be considered that using dichotomous scales means that much of the information was lost. Moreover, studies show that pill counts may overestimate adherence to supplementation (Cramer *et al.*, 1989; Matsui *et al.*, 1994).

4.5. Conclusions

The present study is the first to investigate the separate and combined influence of three SNPs (*ADORA2A* rs5751876 for caffeine sensitivity and *AHR* rs6968554 and *CYP1A2* rs2472297 combined for caffeine metabolism) on the effects of caffeine on cognitive performance, up to 6 h post-supplementation and after controlling for withdrawal reversal symptoms. Genotype was not found to affect caffeine levels in saliva, nor performance in any indices of cognition.

After controlling for withdrawal reversal, caffeine had no significant net enhancing effects on cognitive performance. Our study was designed to identify net effects, independent of any restorative effects due to withdrawal reversal, while the findings are relevant to moderate caffeine consumers. The present results indicate the need for future studies using comparable methodologies in larger samples to understand the role of polymorphisms involved in caffeine pharmacodynamics and pharmacokinetics before any personalised recommendations for the use of this psychostimulant on cognition can be made based on genotype.

Chapter 5. General Discussion

This chapter constitutes the general discussion of this research and highlights the contribution of present findings in advancing current knowledge. This chapter encompasses a comprehensive analysis of both the strengths and limitations inherent in this research, along with the formulation of suggestions for future investigations. Furthermore, this chapter analyses the practical implications arising from this research in shaping individualised recommendations for optimal cognitive performance, considering genetic factors. Additionally, the chapter extends to the potential of this research and future work in the field, to shape not only scientific perspectives but also influence the trajectory of the caffeine supplement industry.

5.1. Thesis key findings and original contribution

Caffeine is considered a powerful psychostimulant, being consumed daily by most adults worldwide to overcome fatigue, enhance alertness, or prolong their capacity to complete everyday activities (Cappelletti *et al.*, 2015; McLellan, Caldwell and Lieberman, 2016). Nevertheless, current research on caffeine effects on cognition remains controversial. Part of the variability in research findings on the topic is being attributed to differences in genes associated with caffeine metabolism and physiological effects (Nehlig, 2018); however, evidence is limited. The aim of this thesis was to investigate the interactions between chronic and acute caffeine intake and cognitive performance in all domains of cognition based on genes that are implicated in caffeine pharmacokinetics and pharmacodynamics.

Considering the research aim, the present thesis comprised four research chapters with its main objectives being:

Objective 1: To identify in the literature up to date the caffeine x gene interactions in brain-related outcomes (Chapter 2).

Objective 2: To explore the associations between variations in genes implicated in caffeine pharmacokinetics and pharmacodynamics, habitual (chronic) caffeine intake and cognitive performance in all key domains of cognition (Chapter 3).

Objective 3: To explore if genetic variations associated with caffeine pharmacodynamics and pharmacokinetics modulate the effect of acute caffeine intake on all key domains of cognition (Chapter 4).

Genetic variants explored in this thesis were the variants in key genes associated with caffeine pharmacodynamics and pharmacokinetics, as discussed in

Chapter 1. Since cognitive decline is so common, it is evident that research should be directed towards the healthy population in whom a deeper understanding of factors associated with cognitive performance may lead to more effective prevention or delay of cognitive decline. Thus, the study participants in this thesis were healthy, predominantly young adults with no symptoms of any neurocognitive disorders.

The key findings and original contribution of this thesis are discussed below.

5.1.1. Key findings

With the above-described, the key findings of this thesis, grouped by the research objectives, are presented in Figure 5.1 and below:

Objective 1

Variability in the *CYP1A2* and the *ADORA2A* genes are associated with brain-related outcomes of caffeine, with *CYP1A2* being mostly linked to cognition and *ADORA2A* mostly linked to anxiety and sleep disturbance.

Objective 2

No significant gene x habitual caffeine interactions were observed for genetic caffeine sensitivity (*ADORA2A* gene) on any of the cognitive measures.

Significant gene x habitual caffeine interactions were observed for genetic caffeine metabolism (*CYP1A2* + *AHR* genes) on social and emotional cognition and executive function:

1) 'fast' metabolisers had a lower performance in emotion recognition compared with 'slow' metabolisers, only among high caffeine consumers ($p = 0.004$).

2) within 'fast' metabolisers, low caffeine consumers performed better in emotion recognition than moderate ($p < 0.001$) and high ($p = 0.013$) caffeine consumers.

3) 'slow' metabolisers had a lower performance in executive function compared with 'fast' metabolisers among moderate caffeine consumers ($p = 0.002$).

Objective 3

There were no differences in performance between sensitive and non-sensitive individuals in any of the conditions and timepoints across all cognitive domains.

There were no differences in performance between 'fast' and 'slow' metabolisers in any of the conditions and timepoints across all cognitive domains.

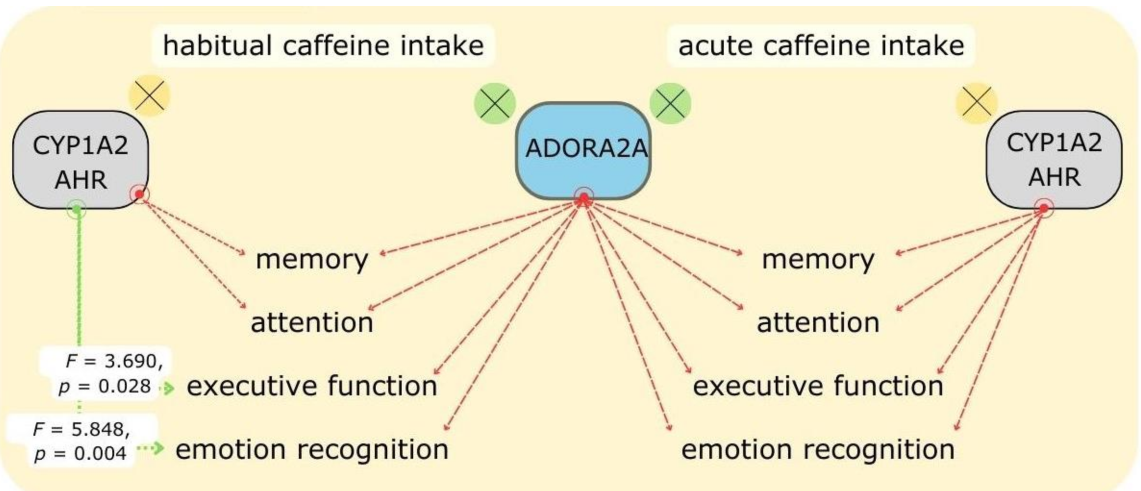

Figure 5. 1 Overview of thesis key findings. Red lines indicate no significant associations between variables and green lines represent significant findings. Yellow circles with X represent the interaction between acute and habitual caffeine intake and genetic caffeine metabolism based on *CYP1A2* and *AHR* genes. Green circles with X represent the interaction between acute and habitual caffeine intake and genetic caffeine sensitivity based on the *ADORA2A* gene. The green lines from top to bottom represent a significant interaction between genetic caffeine metabolism and performance in a) executive function and b) emotion recognition.

5.1.2. Original contribution

While investigating the gene x caffeine interactions on cognitive function, our research yielded a few outcomes that did not reach statistical significance. Although these results might appear inconclusive at first glance, it is important to underscore that the interpretation of our findings should be approached cautiously, considering the specific limitations inherent to our research design, which will be discussed later in this chapter.

This research, although meticulously designed, featured a sample size that fell below the threshold required for capturing subtle effects and achieving statistical power (Gupta *et al.*, 2016). Consequently, the absence of significant results should not be misconstrued as evidence against our initial hypothesis. Rather, it underscores the critical role that sample size plays in clinical research. The importance of appropriately powered studies cannot be overstated, particularly when exploring complex interactions between genetic factors and cognitive processes (Faber and Fonseca, 2014).

It is noteworthy that, due to the distinctive nature of our methodology in both studies, the outcomes of our research may not be directly comparable to other genetics studies of caffeine. This underscores the importance of refining research methodologies to suit the specificities of the variables being investigated. In summary, while our results may not be immediately generalisable to other caffeine studies, they lay the groundwork for an improved and more refined framework for investigating how genes may influence the association between acute and chronic caffeine intake and cognitive function. The results of the present research are briefly discussed below.

5.1.2.1. Genetic caffeine metabolism

Variants in the *CYP1A1/CYP1A2* and *AHR* genes have been previously associated with caffeine metabolism and habitual caffeine intake. In our systematic review, *CYP1A2* was shown to impact caffeine effects on cognition, with 'fast' metabolisers generally having a better performance in attention (Carswell *et al.*, 2020). Interestingly, although results from functional studies (Sachse *et al.*, 1999) and GWAS of caffeine metabolites (Cornelis *et al.*, 2016) have provided evidence on differential caffeine metabolism based on the *CYP1A1/CYP1A2* and *AHR* genes, these findings were not confirmed in previous research (Carswell *et al.*, 2020), or in the present study.

In our second study (Chapter 3), we replicated the finding that 'fast' metabolisers habitually consume higher levels of caffeine compared to 'slow' metabolisers to achieve the desirable psychostimulant effects of caffeine (Cornelis *et al.*, 2016). Moreover, we found significant gene x habitual caffeine interactions for *CYP1A2 + AHR* genes on social and emotional cognition and executive function. 'Fast' metabolisers had a lower performance in emotion recognition compared with 'slow' metabolisers among high caffeine consumers. Moreover, within 'fast' metabolisers, low caffeine consumers performed better in emotion recognition than moderate and high

caffeine consumers. Moreover, 'slow' metabolisers had a lower performance in executive function compared with 'fast' metabolisers among moderate caffeine consumers.

Therefore, our study confirmed previous findings on domain-specific associations between habitual caffeine intake and cognitive performance. Nevertheless, our findings were different from previous genetics studies (Casiglia *et al.*, 2017; Cornelis, Weintraub and Morris, 2020a, 2020b) due to the varied methodologies in cognitive assessment, selection of SNPs and measures of habitual caffeine intake.

With regards to the findings from our final study, we did not find any differences in performance between 'fast' and 'slow' metabolisers in any of the conditions and timepoints across all cognitive domains. This finding was in line with a previous study on cognitive performance (Salinero *et al.*, 2017) that did not control for withdrawal, but contradictory to the findings from a study assessing cognitive performance during exercise (Carswell *et al.*, 2020). Still, considering the methodological disparities between these studies and the limited sample size of the present research, more research is needed to explore this further.

5.1.2.2. Genetic caffeine sensitivity

In our systematic review, the *ADORA2A* gene was found to be associated with caffeine-induced anxiety and insomnia. Moreover, limited data were found on an association of the gene with cognitive performance, specifically in sleep-deprived individuals. In the present research, no *ADORA2A* x habitual caffeine interactions or *ADORA2A* x acute caffeine interactions were observed on any of the cognitive measures. Moreover, in both studies we failed to replicate the finding that caffeine-sensitive individuals habitually consume less caffeine compared to non-sensitive individuals (Cornelis, El-Sohemy and Campos, 2007).

Research has indicated that caffeine attenuates the impairment in cognitive functions such as attention, orienting, memory and executive function during sleep deprivation in individuals who are C homozygous for *ADORA2A* rs5751876 (Baur *et al.*, 2021) and in non-HT4 haplotype carriers of *ADORA2A* compared with the HT4 haplotype (Bodenmann *et al.*, 2012). Moreover, a study on cognition without sleep deprivation showed that the rs5751876 CC genotype performed slower in orienting, while the TT genotype performed slower in motor executive control after caffeine

compared to placebo (Renda *et al.*, 2015). As discussed in Chapters 3 and 4, although the association of *ADORA2A* gene has a biological basis through the adenosine system (Alsene *et al.*, 2003), studies up to date are hampered by varied methodologies and lack generalisability because, for example, they involved only male participants, as in the study by Renda *et al*. (2015).

5.2. Strengths and limitations

In this section, a more comprehensive discussion of the strengths and limitations of this programme of research, which have been presented in the corresponding chapters, will be provided. This will offer a deeper insight into our findings while also acknowledging the challenges and constraints encountered during our research process.

5.2.1. Research strengths

One of the strengths underpinning this research is its comprehensive inception, characterised by a meticulous systematic review that extended beyond the confines of genetics, caffeine and cognition. This approach allowed for an exploration of brain-related outcomes associated with caffeine intake that may exert influence on cognitive function. By embracing a wide spectrum of brain-related effectors, our first study established a robust framework that set the stage for subsequent studies.

Regarding our methodology, this is the first genetics research on caffeine assessing not only one domain, but all key domains of cognition, using a test battery consisting of widely used and validated tests, one for each domain of cognition. Moreover, it is the first genetic association study in an epidemiological setting assessing cognitive performance remotely in real-life conditions on three separate instances, to account for intra-individual differences in cognitive performance.

The remote nature of the observational study (Study 2) had a few strengths. Firstly, it provided access to a larger and more diverse pool of participants across the UK. Recruiting participants from various places, backgrounds and cultures may lead to more diverse and representative data (Craig *et al.*, 2013). Moreover, the nature of the study was more convenient and flexible and eliminated the need for travel, thus was cost-effective for participants, enhancing retention. Since participation could be done anywhere at any time, it was more practical for both researchers and participants.

This convenience was underscored by the study's achievement of enrolling 188% of the target sample size. The study also demonstrated a commendable completion rate of 53%. Comparatively, a recent scoping review encompassing 37 remote health studies spanning less than 12 weeks reported a median participant enrolment of 128% based on target sample size calculations, with a median study completion rate of 48% (Daniore, Nittas and Wyl, 2022). Consequently, our study surpassed this performance.

Finally, the remote nature of this study was able to reduce the risk of bias that can occur during in-person research, such as social desirability bias. Social desirability bias pertains to the tendency of individuals in research to portray themselves or specific situations in a favourable light, aligning with societal norms, rather than providing truthful and accurate responses (Kühne, 2018; Júnior and Patrício, 2022). Driven by diverse influences, participants often magnify behaviours and attitudes that are socially approved, while reducing genuine viewpoints and actions that might be seen as socially undesirable (Kühne, 2018; Júnior and Patrício, 2022).

Considering the shared methodology with Study 2, the cognitive assessment method was one of the strengths of our randomised trial. A second strength lies within the study protocol. The present research has introduced, for the first time in genetics research of caffeine, a previously used protocol designed to control for caffeine withdrawal (James, 1994a, 1994b, 1998), the main methodological challenge in caffeine research. Our study was designed to isolate the chronic effects of dietary caffeine, while the mean habitual intake of participants was representative of the average caffeine consumption among adults in the UK, which was 122-143 mg/day based on the latest data (Fitt, Pell, and Cole 2013).

Further strengths of the current experimental approach include the use of an ecologically valid dose of caffeine prior to performance assessments, previously used in genetics studies on cognitive performance (Salinero *et al.*, 2017; Carswell *et al.*, 2020) and the validation of caffeine metabolism using saliva samples.

Finally, a major strength of Study 3 is the adoption of an extended testing period using four timepoints to assess cognitive function: pre- and 1, 3 and 6 h post-supplementation. This timeframe was selected because a) caffeine concentrations measured up to an hour after ingestion may be indicative of caffeine absorption (Graham, 2001) and b) while the half-life of caffeine in adults is on average 4–6 h

(Nehlig, 2018), it is not yet known to what degree caffeine metabolism is different between 'fast' and 'slow' metabolisers.

5.2.2. Research limitations

One possible limitation of studies 2 and 3 is the modification of the EPIC-FFQ software to assess habitual caffeine intake. To reduce the burden for our participants and subsequent dropout, instead of using an online application such as Nutritics to analyse participant food records, we decided to use the EPIC FFQ. Therefore, we calculated habitual caffeine intake from 11 foods and beverages containing caffeine or cocoa using the volumes and quantities available in the EPIC database (Mulligan *et al.*, 2014) and caffeine content based on published data (Fitt, Pell and Cole, 2013; Malczyk *et al.*, 2021). Although the EPIC FFQ questionnaire is representative of the UK diet and although coffee and tea are the main sources of caffeine in the UK, which are included in the questionnaire and this means that the tool did not omit the most important dietary sources of caffeine, the FETA software is not designed to calculate daily caffeine intake, and this may have introduced misclassification of caffeine assessment.

Nevertheless, as discussed in Chapter 2, caffeine assessment methods for habitual caffeine intake are underreported in genetics studies (Alsene *et al.*, 2003; Retey *et al.*, 2006; Klauke *et al.*, 2012; Casiglia *et al.*, 2017; Erblang *et al.*, 2019), while in the studies reporting on methods, these are variable. Reported caffeine assessment varied from number of caffeinated drinks per day (Nunes *et al.*, 2017), number of cups of coffee and tea per day (Cornelis, Weintraub and Morris, 2020a, 2020b), or caffeine from various sources calculated based on available data (Childs *et al.*, 2008; Rogers *et al.*, 2010). The only genetics study which has used an FFQ was the study from Carswell *et al.* (2020), however the authors did not provide details on the tool.

It should also be noted that, although validated tasks were employed in the test battery, it is possible that only some of the tests may be sensitive enough to identify any performance differences between groups (Hoyland, Lawton and Dye, 2008). For example, deficits in attention performance related to TOT have been reported after 20-min tasks in well-rested subjects, with significant increase of self-reported sleepiness and mental fatigue (Lim *et al.*, 2012; Gui *et al.*, 2015). In both studies of the present research, TOT may have influenced our findings. To account for such effects, we could have requested for self-ratings of several subjective states,

such as fatigue, stress and motivation both before and after the completion of the test battery.

Most limitations in our observational study are inherent to the design and the remote mode of conduct. Observational studies do not differentiate between cause and effect or within the sequence of events (Mann, 2003). Yet, observational studies have greater proximity to 'real life situations', since RCTs have stricter inclusion criteria and rigid protocols that may not reflect real-life conditions (Yang *et al.*, 2010). Therefore, they are useful for identifying associations that can then be more rigorously studied using an RCT (Mariani and Pêgo-Fernandes, 2014). They are also important for creating new hypotheses and establishing the sample size for an RCT (Hannan, 2008). Our observational study was complementary to our trial in designing and testing an online cognitive test battery in a broader population in real-life conditions. Moreover, in our observational study, the tool to assess habitual caffeine intake (modification of FETA software) was developed. Finally, through our observational study, a pool of individuals with known genotype was created and used for recruitment in our trial.

One of the limitations arising from the remote nature of our study was the limited control over the research environment. Evidently, participants were asked to complete the tests across various digital platforms, encompassing tablets, desktops, or smartphones. To standardise this aspect, participants were explicitly instructed to consistently employ the same device during all three test sessions, ensuring a level of control over the technology used. The Gorilla software further augmented our ability to ascertain adherence to this stipulation, as it provided data confirming participant compliance with this instruction by tracking their device usage.

On the contrary, the scrutiny of compliance encountered limitations when it came to assessing the extent to which participants adhered to the requirement of completing the tasks in a quiet room free of distractions. However, whether this indeed limits our study depends on how well it matches our research goals and the real-world situations where cognitive functions happen. It needs to be noted that our study was purposely designed to probe participants under real-world conditions, acknowledging the presence of distractions in daily life.

A potential limitation stems from the limited control we had over technical aspects, which could have affected the quality of our data. While the Gorilla platform provided comprehensive reporting and could identify significant delays caused by internet connection issues, there is a possibility that minor connection-related delays

may not have been identified. Finally, the remote modality of the study precluded a direct interaction between the research team and participants, limiting the opportunity to elaborate on task instructions. Consequently, some participants were excluded from the subsequent analyses due to challenges in comprehending task instructions adequately. A prospective strategy to mitigate such issues could have involved the integration of instructional videos on task completion, analogous to the approach adopted in the second study.

Owing to the exclusion of participant data, the study was not appropriately powered, potentially contributing to the inability to robustly establish caffeine x gene interactions as predictors of cognitive outcomes. Furthermore, the distribution of participants across caffeine groups based on genotypes was not balanced. In general, the greater the disproportion in sample sizes, the lower the overall power of the tests (Rusticus and Lovato, 2014). Therefore, the unbalanced distribution of genotype groups raises questions about the reliability of the findings presented and highlights the need for future studies to thoroughly address this issue.

Another limitation pertains to the requirement for task completion by participants at minimum 5 h after any caffeine consumption. This instruction was predicated on the rationale that participants would be in a state neither influenced by the acute effects of caffeine nor characterised by complete caffeine abstinence during the administration of the test battery (George *et al.*, 1986; Paton, 2005; Casiglia *et al.*, 2017). By adopting this approach, we precluded that the half-life of caffeine, estimated to be 4-6 h in adults (Nehlig, 2018), would have exerted substantial influence. Nonetheless, a complex element of this assumption resides in the yet-undetermined variation in caffeine metabolism between individuals categorised as 'fast' or 'slow' metabolisers.

Furthermore, it is important to highlight that our study, in line with previous investigations, did not confirm participants' adherence to the stipulated 5-hour caffeine abstinence interval. Although the best practice, saliva samples were not requested due to the remote nature of the study, which would not warrant appropriate storage and transfer conditions of samples. To potentially address this challenge, the incorporation of a food diary on the days of the experiments would have provided us information regarding the exact timing and quantity of participants' most recent caffeine intake before the tests.

In Study 3, the 12 participants in our RCT were not sufficient to meet the calculated sample size requirement and the proportion of individuals with 'fast' and 'slow' metabolism or 'sensitive' and 'non-sensitive' genotypes. In fact, this sample size meant that only one participant was homozygous of the *ADORA2A* T allele, and only one participant was homozygous of the *CYP1A2* C allele. Consequently, there were no participants who were indeed 'fast' caffeine metabolisers and instead could be considered 'intermediate' metabolisers and no participants were caffeine 'sensitive' based on previous research but were instead T allele carriers.

Another potential limitation of the present study is that only caffeine and not paraxanthine was measured in saliva. However, clearance of caffeine in plasma or saliva is the gold standard measurement for CYP1A2 enzyme function (Faber, Jetter and Fuhr, 2005). Lastly, participant compliance to the protocol was not confirmed by requesting a saliva sample every afternoon. Although outlined by James (1994a) protocol, daily saliva samples were not requested from participants. This decision was made to prevent overburdening participants, especially because the appropriate conditions of saliva sampling and storage could not be guaranteed, as advised by the manufacturer. Instead, adherence was assessed using the method of capsule count and by dichotomising participants into adherent and non-adherent, based on previous research (Karve *et al.*, 2009; Warren *et al.*, 2013). Although most participants were considered adherent, it needs to be considered that using dichotomous scales means that much of the information is lost. Moreover, studies show that pill counts may overestimate adherence to supplementation (Cramer *et al.*, 1989; Matsui *et al.*, 1994).

5.3. Practical implications

As we explore the implications of our research findings, we are paving the way to a broader understanding of how caffeine and genes interact to influence cognitive abilities. In this section, we extend beyond the confines of our study outcomes and explore what our discoveries and the discoveries of future research in this field could convey in practical terms. In essence, we provide insights that span across scientific understanding in cognitive science and other fields, personalised recommendations and potential policy considerations.

5.3.1. Personalised recommendations for optimal cognition

Nutrigenetics is an emerging field that studies the differential phenotypic response to diet depending on an individual's genotype (Beckett *et al.*, 2017).

Personalised Nutrition (PN) has been defined as an approach that uses individual phenotypic and genetic information to develop tailored nutritional advice (Ordovas *et al.*, 2018). Interestingly, research has shown that although there is high public acceptance of PN (Szakály *et al.*, 2021), there is no difference between PN advice and general recommendations with regards to behaviour change towards healthier lifestyles and disease prevention (King *et al.*, 2023). Nevertheless, the efficacy of gene-based PN in optimising cognitive function is still unexplored.

One of the core principles of PN is to is to target individuals who are most likely to benefit (Mullins *et al.*, 2020). One group which is most likely to benefit from gene-based PN on cognitive function is the ageing population. With the rise in the size of the elderly population globally and in the UK (Office for National Statistics, 2021; United Nations Department of Economic and Social Affairs, 2021), gene-based PN for optimal cognition is of particular interest. Cognitive decline is a natural part of the ageing process and preserving cognitive function is critical in promoting healthy ageing and enhancing the quality of life among older adults (de Jager *et al.*, 2014; Petersen *et al.*, 2018). Moreover, the ability of caffeine to mitigate the effects of fatigue and sleep deprivation can be particularly relevant to individuals who need to stay awake and alert, such as shift workers, long-distance drivers, students and military personnel (Cappelletti *et al.*, 2015; Renda *et al.*, 2015; Carswell *et al.*, 2020).

Caffeine's stimulant properties have been shown to enhance cognitive performance, including improvements in attention, alertness, RT and memory (McLellan, Caldwell and Lieberman, 2016). As reviewed in Chapter 2 of this thesis, the magnitude of caffeine effects in cognition, or the lack of an effect when compared to placebo in research can be partly explained by genetic variations. Nevertheless, data up to date are inconsistent and lack replication.

As we deepen our understanding of the interplay between genes and caffeine metabolism and response, we can start developing gene-based personalised advice on caffeine intake for optimal cognitive function. Therefore, genetic testing could identify an individual's caffeine-related genotypes, with the potential to enable tailored recommendations on dosage, timing and frequency of caffeine consumption. Such personalised strategies could help individuals achieve optimal cognitive performance and mitigate potential adverse brain-related outcomes associated with excessive caffeine intake, such as anxiety and insomnia.

In conclusion, genetics studies on caffeine and cognition can inform personalised recommendations based on individual genetic makeup. The personalised recommendations and precision approaches that may emerge from the present and future research hold promising potential for enhancing cognitive function throughout the life course. Further research is needed to validate the clinical utility and efficacy of this approach.

5.3.2. Informing scientific opinions and position stands

Our research, along with future investigations in this field, has the potential to shape the opinions and position stands of the scientific community in a manner that is both evidence-based and forward-looking. By integrating genetic insights with cognitive outcomes, these perspectives can potentially offer valuable guidance for optimising cognitive function through tailored caffeine consumption. Nevertheless, it is imperative to emphasise that a more comprehensive understanding is required through additional research before establishing these viewpoints.

By uncovering the intricate interplay between genetics, caffeine metabolism and cognitive function, research can provide a robust foundation for evidence-based recommendations on a population level. Scientific opinions and position stands can be shaped by the identification of genetic markers that influence caffeine responsiveness, aiding in the formulation of personalised dietary guidelines for optimal cognitive performance.

After the establishment of such caffeine x gene associations, scientific viewpoints and position stands can elucidate the trade-offs between cognitive benefits and potential health side effects from caffeine consumption. For example, the European Food Safety Authority (EFSA) regarding caffeine intake safe thresholds states that "*Single doses of caffeine up to 200 mg (approximately 3 mg/kg for a 70-kg adult) do not raise safety concerns. The same amount does not give rise to safety concerns when consumed < 2 hours prior to intense physical exercise under normal environmental conditions*." In this context, the statement focuses on safety, which could be underscored by the cognitive advantages associated with caffeine intake based on individual genotype.

Another example is the International Society of Sports Nutrition (ISSN). According to the latest ISSN position stand (Guest *et al.*, 2021):

1. "*Optimal timing of caffeine ingestion likely depends on the source of caffeine."* This statement could be enhanced with information on genetic background, i.e., timing of caffeine ingestion also depends on genetic caffeine metabolism.

2. "Inter-individual differences in sport and exercise performance as well as adverse effects on sleep or feelings of anxiety following caffeine ingestion may be attributed to genetic variation associated with caffeine metabolism, and physical and psychological response." In this statement, information on interindividual differences in alertness and cognitive performance could be added.

3. "Caffeine has been shown to be ergogenic for cognitive function, including attention and vigilance, in most individuals." and

4. "Caffeine may improve cognitive and physical performance in some individuals under conditions of sleep deprivation."

For points 3 and 4, the use of "most individuals" could be altered to "based on genotype in genes associated with caffeine pharmacodynamics and pharmacokinetics".

In essence, research can offer insights into the potential benefits associated with caffeine consumption based on genetics, particularly in relation to cognition. As a result, scientific opinions can endorse the customisation of caffeine intake based on individual genetic profiles, aligning with the emerging paradigm of personalised nutrition to enhance cognitive function.

5.3.3. Informing dietary supplement industry

Research on caffeine x gene interactions in health outcomes including cognition has the potential to inform and contribute to shaping the guidelines for caffeine-containing dietary supplements. A review of labels from 42 caffeine supplements revealed that the prevailing health claims found in caffeine products included "gives energy" and "improves concentration/cognitive improvements" (56.1%). These were closely followed by assertions of "improves focus/alertness" (51.2%) and "reduces physical/mental fatigue" (41.5%) (Estevan Navarro *et al.*, 2021).

Currently, the health claims, "increases strength performance," "increases performance in the short term," "increases performance in endurance sports," "improves performance," "decreases tiredness/feeling of fatigue," "increases concentration," and "increases focus/alertness" can be considered to satisfy the

criteria of the leading scientific institutions (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2015; Thomas, Erdman and Burke, 2016; Maughan *et al.*, 2018) and the effects declared relate to a minimum of 200 mg/dose and day of the product.

The Council for Responsible Nutrition (CRN) is the leading trade association for the dietary supplement and functional food industry (CRN, 2018). By incorporating genetic insights into caffeine metabolism and sensitivity, these guidelines may be refined to offer personalised recommendations. For instance, individuals with certain genetic profiles might be advised on optimal caffeine intake based on their metabolism rates and potential sensitivity. This tailored approach aligns with the goal of dietary supplement guidelines – to ensure the safe and effective use of such products for diverse populations (FDA, 2022). Additionally, genetic information can be leveraged to outline potential interactions between caffeine and other components in dietary supplements, enhancing the guidelines' comprehensiveness.

Thus, genetics research has the potential to offer insights that could refine the precision and relevance of these guidelines in the future, contributing to more informed consumer decisions and promoting overall well-being.

5.4. Future research

Through our research, we have paved the way for a more precise and valid approach in caffeine-related genetics investigations. Our study serves as a pivotal starting point for future research that holds the promise of unravelling the interactions between genetics and caffeine, not only in cognitive responses, but also in other aspects of human health.

Our research, designed to control for caffeine withdrawal, could be adapted and applied to investigate caffeine-gene interactions in different health contexts. This could be of particular interest in gene x caffeine interactions in health outcomes, such as cardiovascular and kidney disease. As recently reviewed, there are a few genes that have been implicated in cardiovascular disease symptoms, however findings in some cases are inconclusive or lack replication (Virgili *et al.*, 2023). There is also recent evidence on gene x caffeine interactions on kidney disease risk (Mahdavi, Palatini and El-Sohemy, 2023). Elucidating the extent to which these genes moderate the association between caffeine and such health outcomes will enable caffeine consumption advice to be tailored to specific individuals to optimise health.

Similarly, there is evidence on the ergogenic effects of caffeine based on genetics (Guest *et al.*, 2018, 2019; Sicova *et al.*, 2021), however some are inconsistent and others lack replication. Our methodology could be employed to verify whether positive results reflect net effects of caffeine on performance and whether negative results represent better controlled studies and thus delineate the influence of genetics on the effects of caffeine on exercise performance.

As the field of nutrigenetics progresses, studies using our innovative protocol are warranted to provide a more comprehensive understanding of the complex dynamics of caffeine and health outcomes. This section will offer recommendations for future research, drawn from the insights provided from our study.

5.4.1. Sample size

5.4.1.1. Sample size calculations

To build upon this work, it is imperative that future studies prioritise the expansion of sample size. Trials with sufficient statistical power will facilitate a more comprehensive understanding of the interplay between genetic factors and caffeine intake on cognitive responses. The use of sample size calculations, after accounting for attrition, can guide the determination of an appropriate sample size (Gupta *et al.*, 2016). It is worth noting that, while small samples can compromise the internal and external validity of a study, very large samples tend to magnify minor group differences into statistically significant differences, even when these lack clinical significance (Faber and Fonseca, 2014). It is also important to acknowledge that effective recruitment and retention strategies following calculations for required sample size must be tailored to the context of each study and the characteristics of the target population (Chaudhari *et al.*, 2020).

5.4.1.2. Participant recruitment

Among the proposed ways to enhance recruitment, our experience highlights the importance of offering participants suitable incentives (Thoma *et al.*, 2010; Bower *et al.*, 2014). In our observational study, we noticed a higher rate of participant engagement when we emphasised that our study would provide a free genetic analysis and a personalised report on study results, including participant performance in cognitive tasks. Moreover, tailoring the communication of study benefits to align with the specific interests of the target audience is essential (Hutchins, 2020). For instance, in the context of our trial, when engaging with fitness centres and Sports Science

students, we directed our focus towards delineating the advantages of caffeine as an ergogenic aid in sports. We therefore highlighted how participants would benefit from insights into their genetic caffeine metabolism and sensitivity.

5.4.1.3. Participant retention

Participant retention is also a crucial matter in research and can be exceptionally challenging, especially in long-term protocols. A number of strategies for participant retention have been proposed (Chaudhari *et al.*, 2020; Desai, 2020; Poongothai *et al.*, 2023). From our experience, we would advise researchers to establish connections with participants by engaging with them and invest time to actively listen to their concerns. Moreover, expressing gratitude for their valuable contributions can also foster a sense of commitment and involvement.

Furthermore, the creation of pleasant and comfortable research environments is of great importance. Recognising that a considerable proportion of our participants were students, offering access to university computers with complimentary Wi-Fi facilitated their participation. Ultimately, it is advisable to provide scheduling alternatives that are adaptable and considerate of participant preferences, thereby reducing obstacles to engagement (Tiersma *et al.*, 2022). In our study, we reserved computer rooms for four days each week over the course of the four-week protocol, ensuring compatibility with our participants' timetables.

Researchers are strongly encouraged to continuously assess and adapt their strategies based on participant feedback and experiences, thereby cultivating an impactful research study.

5.4.2. Prospective recruitment based on genotype

As discussed previously and in the corresponding chapters, a notable limitation of our research lies within the underrepresentation of specific genotypes and the imbalanced distribution of genotype groups. To mitigate such constraints in future genetics studies, it is advisable to meticulously recruit participants based on their genetic profiles, ensuring robust representation across distinct genotype groups.

One promising approach is Genotype-Driven Recruitment (GDR), where research participants are selectively recruited based on their genotype, such as the presence of specific gene variants (McGuire and McGuire, 2008; Beskow *et al.*, 2010). GDR can be particularly valuable for investigating the influence of human genetic

variation on health-related outcomes and responses to interventions (Beskow *et al.*, 2010). Studies employing this design have the potential to enhance the utility of genomic data collected in various settings and accelerate its translation into advancements in human health (McGuire and McGuire, 2008).

In this approach, investigators utilise existing study populations for which genetic analyses have already been conducted to identify and recontact individuals possessing a gene variant of interest (McGuire and McGuire, 2008; Beskow *et al.*, 2010; Michie *et al.*, 2012). This way, GDR could increase the utility of the massive amounts of data generated in GWAS, only a small fraction of which is related to the disease or condition originally under study (McGuire and McGuire, 2008).

In this context, the UK Biobank emerges as a promising repository of data accessible for public use. The UK Biobank encompasses an extensive array of both phenotypic and genotypic data pertaining to a sizable cohort of participants, inclusive of genetic information and data spanning diverse health-related outcomes (Sudlow *et al.*, 2015). Importantly, it also provides opportunities for follow-up phenotyping by re-engaging study participants, as demonstrated in analogous research within the field (Cornelis, Weintraub and Morris, 2020a, 2020b).

Therefore, resources such as the UK Biobank offer an avenue for collecting data on a substantial scale and circumvent the time-consuming and costly process of screening new populations (Chulada *et al.*, 2008).

5.4.3. Control for withdrawal reversal

5.4.3.1. In observational studies

In addition to expanding sample sizes, the integration of observational studies on habitual caffeine intake and cognitive function holds great promise in enhancing our understanding of the multifaceted effects of caffeine. An important improvement in the methodologies for collecting data in such studies is recommended. As discussed in the limitations section of this chapter, previous attempts to control for caffeine intake prior to testing often centred on a) requesting participants to undergo overnight abstinence (Casiglia *et al.*, 2017), a practice that can trigger caffeine withdrawal symptoms and confound results (Juliano and Griffiths, 2004; James and Rogers, 2005; Rogers *et al.*, 2013), or b) report on recent (1 h prior to tests) caffeine drinking (Cornelis, Weintraub and Morris, 2020a, 2020b), attempting to mimic an acute caffeine vs placebo intervention in an observational setting. However, the latter lacks information on

quantity consumed and represents a timeframe which reflects absorption rather than metabolism (Graham, 2001).

In our attempt to avoid both limitations of previous work, we decided to assess participants after having abstained from caffeine for at least 5 h. We hypothesised that during task completion, participants would be in a state neither influenced by the acute effects of caffeine nor characterised by withdrawal symptoms (George *et al.*, 1986; Paton, 2005; Casiglia *et al.*, 2017). However, this requirement raises two issues. Although withdrawal symptoms were unlikely to be present after 5 h for all genotype groups, we cannot reject the possibility that 'slow' metabolisers could have been under the acute effects of caffeine on that time. Secondly, this requirement solely outlined the minimal duration of caffeine abstinence preceding cognitive tasks, without addressing the maximum interval necessary for refraining from any caffeine source prior to task completion. For instance, participants could opt to undertake cognitive tasks in the morning, prior to their first caffeine dose, potentially leading to caffeine withdrawal symptoms.

Instead, a more refined approach can be adopted, wherein participants are queried about their caffeine consumption, encompassing timing and quantity prior to testing, as demonstrated in the Nunes *et al.* (2017) study, investigating caffeine x gene interactions on sleep. To assess the caffeine ingested by individuals on the day of experiments, considering both the quantity and the time elapsed since the last consumption, the authors introduced the concept of a 'caffeine load' index. This index calculates the total number of cups consumed on the testing day divided by the hours elapsed since the last caffeinated beverage was consumed (Nunes *et al.*, 2017). This approach relies on self-reporting of caffeine intake, which has its limitations. However, among other self-reported measures, this approach may offer a more precise evaluation of participant caffeine exposure during testing days, potentially allowing researchers to isolate recent exposure from long-term caffeine intake.

5.4.3.2. In randomised trials

The protocol introduced in our RCT offers a compelling foundation upon which subsequent investigations in genetics can be constructed. Genetics is promising in delineating the inconsistency within caffeine research findings (Nehlig, 2018). However, we need to acknowledge that this potential can only be harnessed effectively when underpinning methodological challenges within the field are rigorously addressed. In this context, we recommend the integration of the protocol introduced

by James (1994a) into future genetics trials investigating the effects of caffeine on cognition.

The protocol introduced by James, as previously discussed, encompasses alternating phases of long-term caffeine and placebo supplementation. This innovative design bears a crucial advantage in experimental trials by effectively controlling for caffeine withdrawal effects. By adopting this innovative protocol, studies can circumvent challenges such as abrupt caffeine withdrawal effects, enhancing the accuracy and reliability of findings.

Notably, the adaptability of this protocol extends beyond cognitive domains and holds promise for a diverse array of outcomes, including sports performance and cardiovascular parameters such as blood pressure.

5.4.4. Standardised methodologies

5.4.4.1. In cognitive assessment

Another recommendation refers to the need for standardised methodologies in cognitive testing. The establishment of standardised protocols for both clinical trials and observational studies can enhance comparability across studies, facilitating the accumulation of knowledge in this field. To our knowledge, there is only one systematic review to date on the use of cognitive tasks in nutritional intervention studies (de Jager *et al.*, 2014). The overall recommendation of this review is that the field of cognitive function assessment needs an alignment of measures in each domain to enable valid comparisons of study outcomes.

Transferring the recommendations by the authors to caffeine research, cognitive task selection should be based on the expected caffeine-cognition relationship. Moreover, chosen tasks should be suitable for repeated use, simple to interpret and match the study sample. Finally, the suitability of a test needs to be assessed considering a) the cognitive domains caffeine is expected to influence and b) whether the test is well-standardised and sensitive to caffeine (de Jager *et al.*, 2014).

5.4.4.2. In habitual caffeine intake assessment

Comparable methods are also recommended for habitual caffeine intake assessment and categorisation. As highlighted in our systematic review, there is lack of standardised methods to assess habitual caffeine intake. Habitual caffeine exposure

may be misclassified in studies not only due to the use of self-reported data, but also because of the variable measures of assessing caffeine content of foods and drinks (Smith, 2002; James, 2014). To address this, we propose future studies to validate the utilisation of the EPIC-FFQ in measuring participants habitual caffeine intake. Additionally, a comparison of the FFQ with the revised Caffeine Consumption Questionnaire (CCQ-R), a tool specifically designed for caffeine intake assessment (Irons *et al.*, 2016), would be prudent. Moreover, we emphasise the importance of authors disclosing the assessment methods employed, enhancing the reproducibility of findings.

Comparable to the classification of caffeine-naïve individuals mentioned in Chapter 1, the categorisation of caffeine consumers into low/moderate and high groups is also subject to the investigator. For example, some researchers define 'low' caffeine intake as 0-150 mg/day (Carswell *et al.*, 2020), while others define it as 0-50 mg/day (Erblang *et al.*, 2019). To ensure the robustness and comparability of findings, there is a need for consistent and uniform approaches to classify caffeine consumption levels across future studies. Such standardisation would not only enhance the reliability of results but also enable researchers to draw more meaningful and generalisable conclusions.

In summary, rigorous comparable methodologies, such as RCTs and well-controlled observational studies, are essential to unravel the complexities of gene x caffeine interactions on cognition and reach safe conclusions. To surpass the challenges posed by the multifaceted nature of caffeine research, collaborative efforts among researchers across diverse fields including geneticists, nutritionists and cognitive health experts are encouraged. By embracing the insights derived from this study and leveraging its innovative protocol, researchers can not only refine our understanding of the effects of caffeine on cognition but also advance caffeine research in other contexts, including exercise performance and cardiovascular outcomes.

5.5. Conclusion

In the present work, it was hypothesised that genetic variants encompassing the *CYP1A2*, *AHR* and *ADORA2A,* which are implicated in caffeine metabolism and sensitivity, may influence the acute and long-term associations between caffeine and the key cognitive phenotypes in humans. Our findings revealed significant interactions

between genetic caffeine metabolism and the domains of social and emotional cognition and executive function: 'slow' caffeine metabolisers had a better performance in social and emotional cognition compared with 'fast' metabolisers, only within the high caffeine intake group. Nevertheless, 'slow' metabolisers had a lower performance in executive function compared with 'fast' metabolisers within moderate caffeine consumers. It is possible that the nature of the different cognitive domains, the duration of the tasks, as well as the differential brain levels of caffeine and paraxanthine may account for the contradicting findings between genotype groups.

This thesis has identified a few methodological considerations and has engaged with innovative strategies. Considering the limitations in caffeine research pertaining to cognition, we have adopted a previously proposed protocol aiming to mitigate the confounding factors related to withdrawal and we have applied it in the context of genetics research for the first time. Future studies are advised to incorporate such protocol to enable an in-depth examination of how genetics influence the impact of acute caffeine intake on cognitive performance based on habitual caffeine intake.

To comprehensively address the questions delineated in this thesis, it is imperative that subsequent studies be conducted using larger sample sizes of balanced genotype groups among diverse populations. By replicating our protocol on a larger scale, researchers will be able to draw robust conclusions regarding the interplay between genetics, caffeine and cognitive responses. Elucidating the extent to which genes associated with caffeine pharmacokinetics and pharmacodynamics moderate the association between caffeine and cognitive performance will enable personalised caffeine consumption advice to individuals for optimal cognition throughout the lifespan.

6. References

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7. Appendices

APPENDIX A: SYSTEMATIC REVIEW PROSPERO REGISTRATION

PROSPERO
International prospective register of systematic reviews

UNIVERSITY of York
Centre for Reviews and Dissemination

Systematic review

Fields that have an asterisk (*) next to them means that they must be answered. Word limits are provided for each section. You will be unable to submit the form if the word limits are exceeded for any section. Registrant means the person filling out the form.

1. * Review title.

Give the title of the review in English

Genetics of Caffeine and Brain-related Effects

2. Original language title.

For reviews in languages other than English, give the title in the original language. This will be displayed with the English language title.

English

3. * Anticipated or actual start date.

Give the date the systematic review started or is expected to start.

16/11/2020

4. * Anticipated completion date.

Give the date by which the review is expected to be completed.

31/08/2021

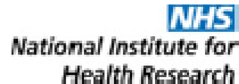
5. * Stage of review at time of this submission.

Tick the boxes to show which review tasks have been started and which have been completed. Update this field each time any amendments are made to a published record.

Reviews that have started data extraction (at the time of initial submission) are not eligible for inclusion in PROSPERO. If there is later evidence that incorrect status and/or completion date has been supplied, the published PROSPERO record will be marked as retracted.

This field uses answers to initial screening questions. It cannot be edited until after registration.

The review has not yet started: No



Review stage	Started	Completed
Preliminary searches	Yes	No
Piloting of the study selection process	Yes	No
Formal screening of search results against eligibility criteria	Yes	No
Data extraction	No	No
Risk of bias (quality) assessment	No	No
Data analysis	No	No

Provide any other relevant information about the stage of the review here.

6. * Named contact.

The named contact is the guarantor for the accuracy of the information in the register record. This may be any member of the review team.

Angie Kapellou

Email salutation (e.g. "Dr Smith" or "Joanne") for correspondence:

Mrs Kapellou

7. * Named contact email.

Give the electronic email address of the named contact.

190551@live.stmarys.ac.uk

8. Named contact address

Give the full institutional/organisational postal address for the named contact.

St Mary's University, Waldegrave Road, Strawberry Hill Twickenham, TW1 4SX

9. Named contact phone number.

Give the telephone number for the named contact, including international dialling code.

+306938406218

10. * Organisational affiliation of the review.

Full title of the organisational affiliations for this review and website address if available. This field may be completed as 'None' if the review is not affiliated to any organisation.

St Mary's University Twickenham

Organisation web address:

https://www.stmarys.ac.uk/home.aspx

11. * Review team members and their organisational affiliations.

Give the personal details and the organisational affiliations of each member of the review team. Affiliation refers to groups or organisations to which review team members belong. **NOTE: email and country now MUST be entered for each person, unless you are amending a published record.**

Mrs Angie Kapellou. St Mary's University Twickenham
Dr Leta Pilic. St Mary's University Twickenham
Dr Yiannis Mavrommatis. St Mary's University Twickenham
Mrs Alexandra King. St Mary's University Twickenham
Dr Catherine Graham. Oxford Brookes University

12. * Funding sources/sponsors.

Details of the individuals, organizations, groups, companies or other legal entities who have funded or sponsored the review.

None

Grant number(s)

State the funder, grant or award number and the date of award

None

13. * Conflicts of interest.

List actual or perceived conflicts of interest (financial or academic).

Yes

The lead reviewer (??) works for iDNA Genomics, a direct-to-consumer (DTC) lifestyle genotyping company.

LP serves as an advisor for DNAFuel Ltd.s. The other authors declare that they have no known conflicts of interest.

14. Collaborators.

Give the name and affiliation of any individuals or organisations who are working on the review but who are not listed as review team members. **NOTE: email and country must be completed for each person, unless you are amending a published record.**

15. * Review question.

State the review question(s) clearly and precisely. It may be appropriate to break very broad questions down into a series of related more specific questions. Questions may be framed or refined using PI(E)COS or similar where relevant.

How do common genetic polymorphisms influence the effect of caffeine on cognitive performance indices?

16. * Searches.

State the sources that will be searched (e.g. Medline). Give the search dates, and any restrictions (e.g. language or publication date). Do NOT enter the full search strategy (it may be provided as a link or attachment below.)

PubMed and EMBASE databases were searched for relevant articles from inception to present day. The search was specific for human studies. We also conducted a manual search of the reference lists of relevant articles, to find any additional papers that were not returned by our initial search. After title and abstract screening, the full-text articles will be reviewed according to predetermined inclusion and exclusion criteria. A

detailed record of all stages of the protocol will be kept.

The search strategy was guided by PRISMA.

Search dates: from December 18, 2020, to April 2, 2021. Searches were re-run prior to the final analysis.

See the uploaded PDF below for further details.

17. URL to search strategy.

Upload a file with your search strategy, or an example of a search strategy for a specific database, (including the keywords) in pdf or word format. In doing so you are consenting to the file being made publicly accessible. Or provide a URL or link to the strategy. Do NOT provide links to your search results.

https://www.crd.york.ac.uk/PROSPEROFILES/257556_STRATEGY_20210527.pdf

Alternatively, upload your search strategy to CRD in pdf format. Please note that by doing so you are consenting to the file being made publicly accessible.

Do not make this file publicly available until the review is complete

18. * Condition or domain being studied.

Give a short description of the disease, condition or healthcare domain being studied in your systematic review.

Brain-related effects

19. * Participants/population.

Specify the participants or populations being studied in the review. The preferred format includes details of both inclusion and exclusion criteria.

Inclusion: Healthy adults

Exclusion: Adolescents (under 18 years of age) and elderly people (over 70), individuals with any diagnosed mental or cognitive decline or impairment, pregnant women and individuals that have not been genotyped.

20. * Intervention(s), exposure(s).

Give full and clear descriptions or definitions of the interventions or the exposures to be reviewed. The preferred format includes details of both inclusion and exclusion criteria.

Caffeine supplementation and/or intake.

21. * Comparator(s)/control.

Where relevant, give details of the alternatives against which the intervention/exposure will be compared (e.g. another intervention or a non-exposed control group). The preferred format includes details of both inclusion and exclusion criteria.

The wild type compared with the risk allele of polymorphisms in genes associated with caffeine metabolism and physiological effect (e.g. ADORA2A and CYP1A2) and different levels of caffeine intake compared with placebo.

22. * Types of study to be included.

Give details of the study designs (e.g. RCT) that are eligible for inclusion in the review. The preferred format includes both inclusion and exclusion criteria. If there are no restrictions on the types of study, this should be

stated.

Both observational (cohort, case-control, cross-sectional) and experimental studies (randomised controlled trials, single or double blinded).

23. Context.

Give summary details of the setting or other relevant characteristics, which help define the inclusion or exclusion criteria.

Studies [illegible]ch, DTC).

24. * Main outcome(s).

Give the pre-specified main (most important) outcomes of the review, including details of how the outcome is defined and measured and when these measurement are made, if these are part of the review inclusion criteria.

Any quantified changes in cognitive performance indices from baseline and after caffeine ingestion, measured as perceived anxiety, insomnia, mood, and changes in memory, attention, decision-making, reasoning, reaction times, alertness and startle response scores.

Measures of effect

Please specify the effect measure(s) for you main outcome(s) e.g. relative risks, odds ratios, risk difference, and/or 'number needed to treat.

Any measure of effect, as reported.

25. * Additional outcome(s).

List the pre-specified additional outcomes of the review, with a similar level of detail to that required for main outcomes. Where there are no additional outcomes please state 'None' or 'Not applicable' as appropriate to the review

None

Measures of effect

Please specify the effect measure(s) for you additional outcome(s) e.g. relative risks, odds ratios, risk difference, and/or 'number needed to treat.

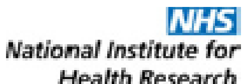
Not applicable

26. * Data extraction (selection and coding).

Describe how studies will be selected for inclusion. State what data will be extracted or obtained. State how this will be done and recorded.

Study selection

Two reviewers will apply eligibility criteria and select studies for inclusion in the systematic review by independently screening records using Covidence software. Researchers will be blinded to each other's decisions. Disagreements between individual judgements will be resolved on a consensus-based discussion between the two reviewers.



Data extraction

The data that will be extracted from study documents will include information about the authors, study design and methodology, participant demographics, baseline characteristics and measures of effect.

Two researchers will be extracting data; one will extract data and the second person will check the extracted data.

Disagreements between individual judgements will be resolved on a consensus-based discussion between the two researchers.

If there are missing data, i.e. if any of this information is missing or unclear, study investigators will be reached via email to ask for clarifications.

Data extraction and management will be conducted using Covidence software.

27. * Risk of bias (quality) assessment.

State which characteristics of the studies will be assessed and/or any formal risk of bias/quality assessment tools that will be used.

Two independent reviewers will be involved in the quality assessment and disagreements between reviewers judgements will be resolved on a consensus-based discussion. The studies included in the systematic review will be assessed for:

1) bias arising from the randomisation process;

2) bias due to deviations from intended interventions;

3) bias due to missing data;

4) bias in outcome measurement;

5) bias in reporting;

6) bias due to confounding;

7) bias in participant selection;

8) bias in classification of interventions.

28. * Strategy for data synthesis.

Describe the methods you plan to use to synthesise data. This **must not be generic text** but should be **specific to your review** and describe how the proposed approach will be applied to your data. If meta-analysis is planned, describe the models to be used, methods to explore statistical heterogeneity, and software package to be used.

A qualitative review of study results is planned for the present systematic review. Studies will be categorised according to the outcomes studied. Summary information on the intervention, study design, participants, outcomes and the duration of follow-up (if any) will be compiled in tables and described in the text. The outcomes, i.e. changes in cognitive performance, will be summarised in terms of the change in reaction times, change in correct answers, or change in number of ocular movements. Information on change in outcomes will be extracted depending on the results reported, such as milliseconds and numbers, and may

be synthesised depending on the number of studies where common outcome metrics can be converted. Two reviewers will be involved in the qualitative review of results, and disagreements between judgements will be resolved on a consensus-based discussion.

29. * Analysis of subgroups or subsets.

State any planned investigation of 'subgroups'. Be clear and specific about which type of study or participant will be included in each group or covariate investigated. State the planned analytic approach.

In the present systematic review, commonalities in findings by participant and study designs will be explored.

30. * Type and method of review.

Select the type of review, review method and health area from the lists below.

Type of review

Cost effectiveness
No

Diagnostic
No

Epidemiologic
No

Individual patient data (IPD) meta-analysis
No

Intervention
No

Living systematic review
No

Meta-analysis
No

Methodology
No

Narrative synthesis
No

Network meta-analysis
No

Pre-clinical
No

Prevention
No

Prognostic
No

Prospective meta-analysis (PMA)
No

Review of reviews
No

Service delivery
No

Synthesis of qualitative studies
No

Systematic review
Yes

Other
No

Health area of the review

Alcohol/substance misuse/abuse
No

Blood and immune system
No

Cancer
No

Cardiovascular
No

Care of the elderly
No

Child health
No

Complementary therapies
No

COVID-19
No

Crime and justice
No

Dental
No

Digestive system
No

Ear, nose and throat
No

Education
No

Endocrine and metabolic disorders
No

Eye disorders
No

General interest
No

Genetics

Yes

Health inequalities/health equity
No

Infections and infestations
No

International development
No

Mental health and behavioural conditions
Yes

Musculoskeletal
No

Neurological
No

Nursing
No

Obstetrics and gynaecology
No

Oral health
No

Palliative care
No

Perioperative care
No

Physiotherapy
No

Pregnancy and childbirth
No

Public health (including social determinants of health)
No

Rehabilitation
No

Respiratory disorders
No

Service delivery
No

Skin disorders
No

Social care
No

Surgery
No

Tropical Medicine
No

Urological
No

Wounds, injuries and accidents
No

Violence and abuse
No

31. Language.

Select each language individually to add it to the list below, use the bin icon to remove any added in error.
English

There is not an English language summary

32. * Country.

Select the country in which the review is being carried out. For multi-national collaborations select all the countries involved.

England

33. Other registration details.

Name any other organisation where the systematic review title or protocol is registered (e.g. Campbell, or The Joanna Briggs Institute) together with any unique identification number assigned by them. If extracted data will be stored and made available through a repository such as the Systematic Review Data Repository (SRDR), details and a link should be included here. If none, leave blank.

34. Reference and/or URL for published protocol.

If the protocol for this review is published provide details (authors, title and journal details, preferably in Vancouver format)

Add web link to the published protocol.

Or, upload your published protocol here in pdf format. Note that the upload will be publicly accessible.

No I do not make this file publicly available until the review is complete

Please note that the information required in the PROSPERO registration form must be completed in full even if access to a protocol is given.

35. Dissemination plans.

Do you intend to publish the review on completion?

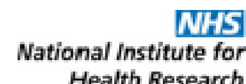
Yes

Give brief details of plans for communicating review findings.?

After completion, the authors intend to attempt publication of the systematic review in a relevant journal in order to increase awareness on the influence of genetics on the effect of caffeine on cognition.

36. Keywords.

Give words or phrases that best describe the review. Separate keywords with a semicolon or new line. Keywords help PROSPERO users find your review (keywords do not appear in the public record but are



included in searches). Be as specific and precise as possible. Avoid acronyms and abbreviations unless these are in wide use.

systematic review

genetics

37. Details of any existing review of the same topic by the same authors.

If you are registering an update of an existing review give details of the earlier versions and include a full bibliographic reference, if available.

38. * Current review status.

Update review status when the review is completed and when it is published.New registrations must be ongoing so this field is not editable for initial submission.
Please provide anticipated publication date

Review_Ongoing

39. Any additional information.

Provide any other information relevant to the registration of this review.

This systematic review is being conducted for the design of human studies to investigate the influence of genetic polymorphisms related to caffeine metabolism and physiological effects on indices of cognition.

40. Details of final report/publication(s) or preprints if available.

Leave empty until publication details are available OR you have a link to a preprint (NOTE: this field is not editable for initial submission). List authors, title and journal details preferably in Vancouver format.

Give the link to the published review or preprint.

APPENDIX B: INSTRUCTIONS FOR BUCCAL SWAB SAMPLE COLLECTION

Instructions for use of buccal swabs with RapiDri Pouch

How to prepare for sample collection

Take your DNA sample at least one hour after eating, drinking or brushing your teeth. For best results, rinse mouth with water immediately prior to sampling.

How to take the test

1	Pull open the package from one end.
2	Remove the swab from the wrapper, taking care not to touch the white swab head with your fingers.
3	Insert the swab into your mouth and rub firmly against the inside of your cheek or underneath lower or upper lip. For standard DNA collection rub for **1 minute** and in all cases rub for a **minimum** of 20 seconds. **Important – use reasonable, firm and solid pressure**
4	Place the swab head first into the RapiDri pouch so that the head sits within the drying area.
5	Fold the RapiDri Pouch at the Fold Line, with the clear side inwards as shown, and use the barcoded sticker to seal.

APPENDIX C: BASELINE QUESTIONNAIRE FOR STUDIES 2 & 3

Page 1: Welcome

Dear Participant,

You have been requested to complete this baseline questionnaire because you have agreed to participate in our study and you have deemed eligible.

The questionnaire includes some questions regarding your health, your lifestyle (smoking, physical activity, sleep and dietary habits), as well as some sociodemographic questions. Please respond to the following questions truthfully, to the best of your knowledge.

We kindly remind you that the information provided to us will be anonymised and there will be no way that this information will be connected with you.

If you have any questions regarding this study, you can contact Angeliki Kapellou, Dr Leta Pilic, or Dr Yiannis Mavrommatis who are conducting the research.

Email: 190551@live.stmarys.ac.uk, leta.pilic@stmarys.ac.uk, yiannis.mavrommatis@stmarys.ac.uk

Page 2: Consent to participate

1. I agree to take part in the research. I have read the Participant Information Sheet. I understand what my role will be in this research, and all my questions have been answered to my satisfaction.
2. I understand that I am free to withdraw from the research at any time, for any reason and without prejudice.
3. I have been informed that the confidentiality of the information I provide will be safeguarded.
4. I am free to ask any questions at any time before and during the study.
5. I have been provided with a copy of this form and the Participant Information Sheet.

Data Protection: I agree to the University processing personal data which I have supplied. I agree to the processing of such data for any purposes connected with the Research Project as outlined to me.

I consent to participate in this study

- Yes
- No

Page 3: Health & Lifestyle Questions

General information

Participant ID (Please provide your assigned participant ID):

Date of birth:

Dates need to be in the format 'DD/MM/YYYY', for example 27/03/1980.

(dd/mm/yyyy)

What is your gender?

- Male
- Female
- Not listed
- Prefer not to answer

If 'Not listed', please specify:

Which of the following best describes your ethnic origin?

- Gypsy or Traveller
- Black or Black British: Caribbean
- Black or Black British: African
- Asian
- Chinese
- Mixed
- Arab
- Other

- White British
- White other

Medical History

Have you ever suffered from any of the following medical conditions?

- Heart Disease or attack
- High or low blood pressure
- Stroke
- Cancer
- Diabetes
- Epilepsy
- High cholesterol
- Asthma
- Allergies
- Other
- No, I have not

Have you ever suffered from any of the following neurocognitive disorders?

- Alzheimer's disease
- Vascular neurocognitive disorder
- Traumatic brain injury (TBI)
- Frontotemporal degeneration
- Parkinson's disease
- Huntington's disease
- Lewy body dementia
- HIV infection-associated neurocognitive disorder
- Substance-induced neurocognitive disorder
- Other
- No, I have not

Please give details of any **medication including supplements and contraceptives** you are currently taking or have taken regularly within the last year. If you are not taking any kind of medication, please respond with N/A.

Are you pregnant or lactating?

- Yes
- No
- N/A, I am a male

Do you smoke?

- Yes
- No

Please indicate how many cigarettes per day:

Physical activity

Think about all the **vigorous** activities that you did in the last 7 days. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, digging, aerobics, or fast bicycling?

- 0
- 1
- 2
- 3
- 4
- 5
- 6
- 7

How much time did you usually spend doing vigorous physical activities **on one of those days (in minutes)**?

Think about all the **moderate** activities that you did in the last 7 days. **Moderate** activities refer to activities that take **moderate** physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

During the last 7 days, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

- 0
- 1
- 2
- 3
- 4
- 5
- 6
- 7

How much time did you spend doing moderate physical activities **on one of those days (in minutes)?**

Think about the time you spent walking in the last 7 days. This includes at work and at home, walking to travel from place to place, and any other walking that you have done solely for recreation, sport, exercise, or leisure.

During the last 7 days, on how many days did you walk for at least 10 minutes at a time?

- 0
- 1
- 2
- 3
- 4
- 5
- 6
- 7

How much time did you spend walking **on one of those days (in minutes)**?

The last question is about the time you spent sitting on weekdays during the last 7 days. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

During the last 7 days, how much time did you spend sitting o**n a weekday (in hours)?**

Employment & education

What is your current employment status?

- Student
- Unemployed
- Self-employed
- Part-time employment/ temporary employment
- Full-time employment
- Prefer not to answer

In what sector is your current job?

- Professionals (doctors, lawyers, professors, scientists, engineers, architects, etc)
- Other non-manual positions (secretaries, stenographers, bookkeepers, typists, office workers, cashiers, tellers, collectors, messengers, and salespersons
- Skilled manual positions (foremen, machinists, electricians, carpenters, mechanics, craftsmen, military enlisted persons, and protective service workers)
- Unskilled manual positions (truck and bus drivers, operatives and apprentices in industry, bartenders, waiters, cooks, other service workers, gardeners, long shoremen, labourers, sharecroppers, and private household workers)
- Prefer not to answer

What is the highest academic qualification you have been awarded to date?

- I do not have any formal qualifications
- GCSE or equivalent (GCSE, NVQ level 1+2, O'level, CSE, etc)
- AS/A level or equivalent (NVQ level 3, International Baccalaureate, BTEC level 3, HSE, etc)
- Foundation Degree
- Bachelor's Degree (undergraduate) or equivalent (BTEC level 4, NVQ level 4, etc)
- Master's degree
- Doctorate
- Other
- Prefer not to answer

What is your current annual household income (place or permanent residence)? If you live in shared rented accommodation, then please answer with your personal annual income.

- £0- 11,850
- £11,851- 46,350
- £46,351- 150,000
- £150,000+
- Prefer not to answer

Page 4: Sleep Habits

The following questions relate to your usual sleep habits during the past month only. Your answers should indicate the most accurate reply for the majority of days and nights in the past month. Please answer all questions.

During the past month....

When have you usually gone to bed?	
How long (in minutes) has it taken you to fall asleep each night?	
What time have you usually gotten up in the morning?	
How many hours of actual sleep did you get at night?	
How many hours were you in bed?	

During the past month, how often have you had trouble sleeping because you:

Please don't select more than 1 answer(s) per row.

Please select at least 9 answer(s).

	Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
A. Cannot get to sleep within 30 minutes	☐	☐	☐	☐
B. Wake up in the middle of the night or early morning	☐	☐	☐	☐
C. Have to get up to use the bathroom	☐	☐	☐	☐
D. Cannot breathe comfortably	☐	☐	☐	☐
E. Cough or snore loudly	☐	☐	☐	☐
F. Feel too cold	☐	☐	☐	☐
G. Feel too hot	☐	☐	☐	☐
H. Have bad dreams	☐	☐	☐	☐
I. Have pain	☐	☐	☐	☐
J. Other reason (s)	☐	☐	☐	☐

If you have had trouble sleeping during the past month because of other reason (s), please describe:

During the past month:

Please don't select more than 1 answer(s) per row.

Please select at least 3 answer(s).

	Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
How often have you taken medicine (prescribed or "over the counter") to help you sleep?	☐	☐	☐	☐
How often have you had trouble staying awake while driving, eating meals, or engaging in social activity?	☐	☐	☐	☐
How much of a problem has it been for you to keep up enthusiasm to get things done?	☐	☐	☐	☐

During the past month:

Please don't select more than 1 answer(s) per row.

Please select at least 1 answer(s).

	Very good	Fairly good	Fairly bad	Very bad
How would you rate your sleep quality overall?	☐	☐	☐	☐

Page 5: Food Frequency Questions

In this section of the questionnaire, you will be asked some information about what you have been eating during the past month.
Please answer every question. If you are uncertain about how to answer a question then do the best you can, but please don't leave a question blank.

For each food there is an amount shown, either a "medium serving" or a common household unit such as a slice or teaspoon. Please select the box to indicate how often, on average, you have eaten the specified amount of each food during the past month.

For the following foods, please estimate your average food use as best you can (select one box per food):

Meat and Fish (medium serving):

Please don't select more than 1 answer(s) per row.

Please select at least 17 answer(s).

	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Beef: roast, steak, mince, stew or casserole	☐	☐	☐	☐	☐	☐	☐	☐	☐
Beefburgers	☐	☐	☐	☐	☐	☐	☐	☐	☐
Pork: roast, chops, stew or slices	☐	☐	☐	☐	☐	☐	☐	☐	☐
Lamb: roast, chops or stew	☐	☐	☐	☐	☐	☐	☐	☐	☐
Chicken or other poultry, e.g. turkey	☐	☐	☐	☐	☐	☐	☐	☐	☐
Bacon	☐	☐	☐	☐	☐	☐	☐	☐	☐
Ham	☐	☐	☐	☐	☐	☐	☐	☐	☐
Corned beef, Spam, luncheon meats	☐	☐	☐	☐	☐	☐	☐	☐	☐
Sausages	☐	☐	☐	☐	☐	☐	☐	☐	☐

Savoury pies, e.g. meat pie, pork pie, pasties, steak & kidney pie, sausage rolls	☐	☐	☐	☐	☐	☐	☐	☐	☐
Liver, liver paté, liver sausage	☐	☐	☐	☐	☐	☐	☐	☐	☐
Fried fish in batter, as in fish and chips	☐	☐	☐	☐	☐	☐	☐	☐	☐
Fish fingers, fish cakes	☐	☐	☐	☐	☐	☐	☐	☐	☐
Other white fish, fresh or frozen, e.g. cod, haddock, plaice, sole, halibut	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oily fish, fresh or canned, e.g. mackerel, kippers, tuna, salmon, sardines, herring	☐	☐	☐	☐	☐	☐	☐	☐	☐
Shellfish, e.g. crab, prawns, mussels	☐	☐	☐	☐	☐	☐	☐	☐	☐
Fish roe, taramasalata	☐	☐	☐	☐	☐	☐	☐	☐	☐

Bread and savoury biscuits (one slice or biscuit):

Please don't select more than 1 answer(s) per row.

Please select at least 5 answer(s).

	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

White bread and rolls	☐	☐	☐	☐	☐	☐	☐	☐	☐
Brown bread and rolls	☐	☐	☐	☐	☐	☐	☐	☐	☐
Wholemeal bread and rolls	☐	☐	☐	☐	☐	☐	☐	☐	☐
Cream crackers, cheese biscuits	☐	☐	☐	☐	☐	☐	☐	☐	☐
Crispbread, e.g. Ryvita	☐	☐	☐	☐	☐	☐	☐	☐	☐

Cereals (one bowl):

Please don't select more than 1 answer(s) per row.

Please select at least 2 answer(s).

	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Porridge, Readybrek	☐	☐	☐	☐	☐	☐	☐	☐	☐
Breakfast cereal such as cornflakes, muesli etc.	☐	☐	☐	☐	☐	☐	☐	☐	☐

Potatoes, rice and pasta (medium serving):

Please don't select more than 1 answer(s) per row.

Please select at least 10 answer(s).

	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Boiled, mashed, instant or jacket potatoes	☐	☐	☐	☐	☐	☐	☐	☐	☐
Chips	☐	☐	☐	☐	☐	☐	☐	☐	☐

Roast potatoes									
Potato salad									
White rice									
Brown rice									
White or green pasta, e.g. spaghetti, macaroni, noodles									
Wholemeal pasta									
Lasagne, moussaka									
Pizza									

Dairy products and fats

Please don't select more than 1 answer(s) per row.

Please select at least 13 answer(s).

	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Single or sour cream (tablespoon)									
Double or clotted cream (tablespoon)									
Low fat yogurt, fromage frais (125g carton)									
Full fat or Greek yogurt (125g carton)									
Dairy desserts (125g carton)									

Cheese, e.g. Cheddar, Brie, Edam (medium serving)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Cottage cheese, low fat soft cheese (medium serving)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Eggs as boiled, fried, scrambled, etc. (one)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Quiche (medium serving)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Low calorie, low fat salad cream (tablespoon)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Salad cream, mayonnaise (tablespoon)	☐	☐	☐	☐	☐	☐	☐	☐	☐
French dressing (tablespoon)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Other salad dressing (tablespoon)	☐	☐	☐	☐	☐	☐	☐	☐	☐

The following on bread or vegetables:

Please don't select more than 1 answer(s) per row.

Please select at least 7 answer(s).

	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Butter (teaspoon)	☐	☐	☐	☐	☐	☐	☐	☐	☐

Block or hard margarine, eg. Stork, Krona (teaspoon)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Polyunsaturated margarine, eg. Flora, sunflower, soya spreads (teaspoon)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Soft margarines, including olive oil based and dairy spreads, e.g. Blue Band, Olivio/	☐	☐	☐	☐	☐	☐	☐	☐	☐
Bertolli, Clover (teaspoon)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Low fat spreads (less than 60% fat), e.g. Outline, Gold (teaspoon)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Very low fat spread (less than 30% fat) (teaspoon)	☐	☐	☐	☐	☐	☐	☐	☐	☐

Sweets and snacks (medium serving):

Please don't select more than 1 answer(s) per row.

Please select at least 18 answer(s).

	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Sweet biscuits, chocolate, e.g. digestive (one)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Sweet biscuits, plain, e.g. Nice, ginger (one)	☐	☐	☐	☐	☐	☐	☐	☐	☐

Cakes e.g. fruit, sponge, home baked	☐	☐	☐	☐	☐	☐	☐	☐	☐
Cakes e.g. fruit, sporge, ready made	☐	☐	☐	☐	☐	☐	☐	☐	☐
Buns, pastries e.g. scores, flapjacks, home baked	☐	☐	☐	☐	☐	☐	☐	☐	☐
Buns, pastries e.g. croissants, doughnuts, ready made	☐	☐	☐	☐	☐	☐	☐	☐	☐
Fruit pies, tarts, crumbles, home baked	☐	☐	☐	☐	☐	☐	☐	☐	☐
Fruit pies, tarts, crumbles, ready made	☐	☐	☐	☐	☐	☐	☐	☐	☐
Sponge puddings, home baked	☐	☐	☐	☐	☐	☐	☐	☐	☐
Sponge puddings, ready made	☐	☐	☐	☐	☐	☐	☐	☐	☐
Milk puddings, e.g. rice, custard, trifle	☐	☐	☐	☐	☐	☐	☐	☐	☐
Ice cream, choc ices	☐	☐	☐	☐	☐	☐	☐	☐	☐
Chocolates, single or squares	☐	☐	☐	☐	☐	☐	☐	☐	☐
Chocolate snack bars e.g. Mars, Crunchie	☐	☐	☐	☐	☐	☐	☐	☐	☐
Sweets, toflees, mints	☐	☐	☐	☐	☐	☐	☐	☐	☐

Sugar added to tea, coffee, cereal (teaspoon)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Crisps or other packet snacks, eg. Wotsits	☐	☐	☐	☐	☐	☐	☐	☐	☐
Peanuts or other nuts	☐	☐	☐	☐	☐	☐	☐	☐	☐

Soups, sauces, and spreads:

Please don't select more than 1 answer(s) per row.

Please select at least 8 answer(s).

	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Vegetable soups (bowl)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Meat soups (bowl)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Sauces, e.g. white sauce, cheese sauce, gravy (tablespoon)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Tomato ketchup (tablespoon)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Pickles, chutney (tablespoon)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Marmite, Bovril (teaspoon)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Jam, marmalade, honey (teaspoon)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Peanut butter (teaspoon)	☐	☐	☐	☐	☐	☐	☐	☐	☐

Drinks:

Please don't select more than 1 answer(s) per row.

Please select at least 14 answer(s).

	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Tea (cup)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Coffee, instant or ground (cup)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Coffee, decaffeinated (cup)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Coffee whitener, e.g. Coffee-mate (teaspoon)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Cocoa, hot chocolate (cup)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Horlicks, Ovaltine (cup)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Wine (glass)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Beer, lager or cider (halt pint)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Port, sherry, vermouth, liqueurs (glass)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Spirits, e.g. gin, brandy, whisky, vodka (single)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Low calorie or diet fizzy soft drinks (glass)	☐	☐	☐	☐	☐	☐	☐	☐	☐

Fizzy solt drinks, e.g. Coca cola, lemonade (glass)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Pure fruit juice (100%) eg. orange, apple juice (glass)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Fruit squash or cordial (glass)	☐	☐	☐	☐	☐	☐	☐	☐	☐

Fruit:

Please don't select more than 1 answer(s) per row.

Please select at least 11 answer(s).

	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Apples (1 fruit)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Pears (1 fruit)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oranges, satsumas, mandarins (1 fruit)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Grapefruit (half)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Bananas (1 fruit)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Grapes (medium serving)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Melon (1 slice)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Peaches, plums, apricots (1 fruit)	☐	☐	☐	☐	☐	☐	☐	☐	☐

Strawberries, raspberries, kiwi fruit (medium serving)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Tinned fruit (medium serving)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Dried fruit, e.g. raisins, prunes (medium serving)	☐	☐	☐	☐	☐	☐	☐	☐	☐

Vegetables (fresh, frozen or tinned, medium serving):

Please don't select more than 1 answer(s) per row.

Please select at least 26 answer(s).

	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Carrots	☐	☐	☐	☐	☐	☐	☐	☐	☐
Spinach	☐	☐	☐	☐	☐	☐	☐	☐	☐
Broccoli, spring greens, kale	☐	☐	☐	☐	☐	☐	☐	☐	☐
Brussels sprouts	☐	☐	☐	☐	☐	☐	☐	☐	☐
Cabbage	☐	☐	☐	☐	☐	☐	☐	☐	☐
Peas	☐	☐	☐	☐	☐	☐	☐	☐	☐
Green beans, broad beans, runner beans	☐	☐	☐	☐	☐	☐	☐	☐	☐
Marrow, courgettes	☐	☐	☐	☐	☐	☐	☐	☐	☐
Cauliflower	☐	☐	☐	☐	☐	☐	☐	☐	☐
Parsnips, turnips, swedes	☐	☐	☐	☐	☐	☐	☐	☐	☐
Leeks	☐	☐	☐	☐	☐	☐	☐	☐	☐
Onions	☐	☐	☐	☐	☐	☐	☐	☐	☐
Garlic	☐	☐	☐	☐	☐	☐	☐	☐	☐

Mushrooms									
Sweet peppers									
Beansprouts									
Green salad, lettuce, cucumber, celery									
Watercress									
Tomatoes									
Sweetcorn									
Beetroot									
Coleslaw									
Avocado									
Baked beans									
Dried lentils, beans, peas									
Tofu, soya meat, TVP, Vegeburger									

Are there any OTHER foods which you ate more than once a week?

- Yes
- No

Please specify (food, usual serving size, number of times eaten each week):

What type of milk did you most often use?

- Full cream/whole
- Semi-skimmed
- Skimmed

- Channel Islands, gold
- Dried milk
- Soya
- None
- Other

Please specify:

How much milk did you drink each day, including milk with tea, coffee, cereals etc?

- None
- Quarter of a pint
- Half a pint
- Three quarters of a pint
- One pint
- More than one pint

Did you usually eat breakfast cereal (excluding porridge and Ready Brek mentioned earlier)?

- Yes
- No

Which brand and type of breakfast cereal, including muesli, did you usually eat?
List the one or two types most often used (Brand, type)

What kind of fat did you most often use for frying, roasting, grilling etc?

- Butter
- Solid vegetable fat
- Lard/dripping

- Margarine
- Vegetable oil
- None

Please give type e.g. corn, sunflower:

What kind of fat did you most often use for baking cakes etc?

- Butter
- Solid vegetable fat
- Lard/dripping
- Vegetable oil
- None
- Margarine

Please give name or type e.g. Flora, Stork:

How often did you eat food that was fried at home?

- Daily
- 1-3 times a week
- Less than once a week
- 4-6 times a week
- Never

How often did you eat fried food away from home?

- Daily
- 1-3 times a week
- 4-6 times a week
- Less than once a week

- Never

What did you do with the visible fat on your meat?

- Ate most of the fat
- Ate as little as possible
- Ate some of the fat
- Did not eat meat

How often did you eat grilled or roast meat (per week)?

How well cooked did you usually have grilled or roast meat?

- Well done /dark brown
- Lightly cooked/rare
- Medium
- Did not eat meat

How often did you add salt to food while cooking?

- Always
- Rarely
- Usually
- Never
- Sometimes

How often did you add salt to any food at the table?

- Always
- Rarely
- Usually
- Never
- Sometimes

Did you regularly use a salt substitute (eg LoSalt)?

- Yes
- No

Which brand?

During the course of last month, on average, how many times a week did you eat the following foods? (medium serving)

	times/week
Vegetables (not including potatoes)	
Salads	
Fruit and fruit products (not including fruit juice)	
Fish and fish products	
Meat, meat products and meat dishes (including bacon, ham and chicken)	

APPENDIX D: ETHICAL APPROVAL LETTER FOR STUDY 3

19 December 2022 SMU_ETHICS_2021-22-319

Angie Kappellou (SAHPS): 'Acute caffeine intake, cognition and DNA study'

Dear Angie

University Ethics Sub-Committee

Thank you for re-submitting your ethics application for consideration.

I can confirm that all required amendments have been made and that you therefore have ethical approval to undertake your research.

This approval is subject to any government and St Mary's University research guidelines relating to Covid19 which may change from time to time.

Yours sincerely

Matthew James
Chair, Ethics Sub-Committee

Cc Yiannis Mavrommatis

APPENDIX E: PARTICIPANT GUIDE

Caffeine & DNA Study - Participant Guide

A. This is a detailed list of the action points you need to be taking during the 4-week experiment.

Run-in days (days 1-6)

Days 1-4

1. Avoid any foods, beverages and supplements listed in the table of section B of this guide, on the left column.

2. Consume freely decaffeinated coffee and tea (provided by the research team) and any foods, beverages and supplements listed in the table of section B of this guide, on the right column.

3. Receive a capsule of caffeine or placebo (provided by the research team) 3 times a day: 9.00am, 11am and 3pm with water freely.

4. Continue your regular daily activities, i.e., your regular diet, exercise, etc.

Day 5

Points 1-4 &

5. Avoid any alcohol intake

Day 6

Points 1-5 &

6. Avoid strenuous exercise

7. Avoid cruciferous vegetables:

- Arugula

- Broccoli

- Brussels sprouts

- Cabbage

- Cauliflower

- Collard greens

- Horseradish

- Kale

- Radish

- Turnips

- Watercress

- Wasabi

Experimental Day (Day 7)

You are requested to arrive at St Mary's computer room M2 (Wednesdays) / M102 (Fridays) at 9am and you will need to remain at St Mary's until 4.30pm. You need to come to the lab after an overnight fast (no breakfast). On the experimental days, the research team will guide you through the experimental procedures. During the breaks between the experiments, you are requested not to drink or eat anything apart from water, decaffeinated beverages and what is provided by the research team and not to engage in vigorous activity. You are free to use the computer rooms to work or study.

B. Sources of caffeine

Find below a list of foods and beverages to avoid (left column) during the 4-week protocol and suggested alternatives.

Beverages

✗	✓
Coffee (instant or ground, espresso, cold brews, cappuccino, latte/iced latte, macchiato, mocha) Tea (English breakfast, black, green, matcha, oolong) Fizzy cola drinks, light fizzy cola drinks (coca cola, Pepsi, Dr Pepper, etc.) Cocoa, hot chocolate (Drinking chocolate, powder)	Herbal tea (check label for caffeine content) Decaffeinated coffee (not mocha) and tea (you will be provided by the research team) Non-cola fizzy drinks (Schweppes, Sprite, 7UP, Fanta, etc.) Decaffeinated hot chocolate

Caffeine-containing energy drinks (monster, red bull, etc.). Coffee Liqueur / espresso martini Kombucha Yerba mate drink Guarana-containing energy drinks	

Foods

❌	✓
Cocoa beans and chocolate (dark and milk chocolate) Chocolate squares Chocolate bars Chocolate croissants Chocolate doughnuts / muffins Foods containing guarana or guarana extract Tiramisu Coffee, mocha, chocolate-flavoured ice cream Coffee-flavoured bagels Chocolate or cocoa breakfast cereal Chocolate chip cookies Chocolate bars Some energy bars	White chocolate-containing foods and beverages Any food not containing dark or milk chocolate (e.g., jam doughnuts, plain croissants and bagels, oats, etc.)

Others

❌	✓
Supplements containing caffeine or guarana (e.g., Berocca boost, slimming pills, etc.) Caffeine-containing chewing gum (e.g., BLOCKHEAD Energy chewing gum) Some pain relievers (e.g., Anadin original)	Non-caffeine containing supplements (e.g., vitamins, electrolytes, etc.) Any regular chewing gum Non-caffeine containing pain relievers (e.g., Nurofen)

For questions contact:

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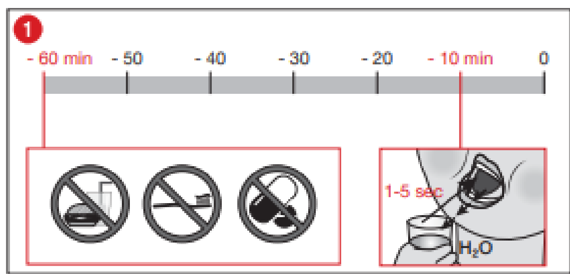
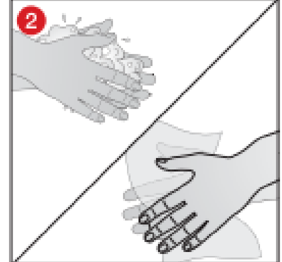
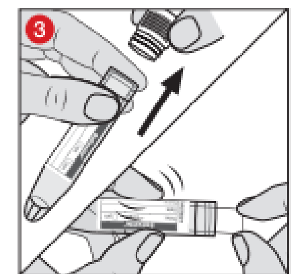
Faculty of Sport, Allied Health and Performance Science
St Mary's University Twickenham

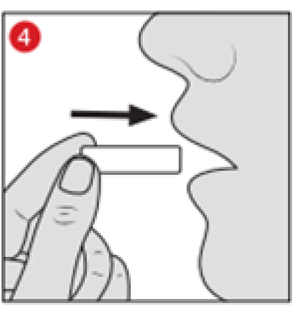
APPENDIX F: INSTRUCTIONS FOR SALIVA CAFFEINE SAMPLING

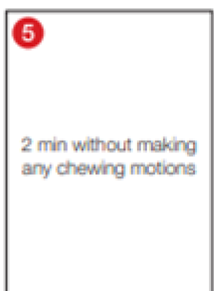
Sample collection

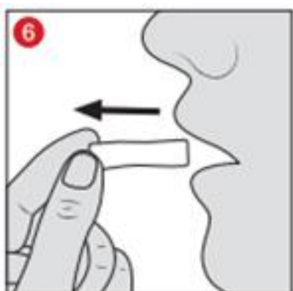












APPENDIX G: 24-HOUR RECALL

Caffeine & DNA Study – 24h recall

Please fill-in the below table regarding your diet, exercise and sleep during the day before the experiment and hand it to a member of the research team. Please provide as much detail as possible.

Date:					Participant ID:	
	Breakfast	Snack	Lunch	Snack	Dinner	Snack
Time						
Food/Drink						
Where?						
Notes						
Exercise						
Wake Up - Bedtime						
To the best of your knowledge, during the past week were you receiving caffeine or placebo?						

The Author’s Declaration

This thesis and the work to which it refers are the results of my own efforts. Any ideas, data or text resulting from the work of others (whether published or unpublished) are fully identified as such within the work and attributed to the originator in the text, bibliography or footnotes.

This thesis has not been submitted in whole or in part for any other academic degree or professional qualification at this or any other institution.

Angeliki Kapellou

30/9/2023