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CAFFEINE INTAKE, COGNITIVE PERFORMANCE, SLEEP QUALITY, AND GLYCEMIC CONTROL

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Summary

Caffeine occurs naturally in coffee, tea and cocoa; and can be added to beverages and medications. It is the most widely used psychoactive drug in the world, and has important effects on subjective alertness and objective cognitive performance. Sleep is a highly regulated and global state controlled by the brain. It is characterised by a reduced awareness to external stimuli, combined with an increased sleep propensity following sleep deprivation. High caffeine intake and disrupted sleep quality impede glycemic control. Type 2 diabetes is a global health problem. Interventions that treat diabetes aim to improve glucose homeostasis and to thus minimise the complications associated with hyperglycemia. Observational research methods can be used to investigate modifiable, health-related behaviours, such as caffeine intake and sleep quality, in healthy and patient populations (Chapter 1).

The two distinct states of wake and sleep result from interplay between the endogenous circadian clock and homeostatic processes. Cognitive performance is influenced by a person's circadian preference, degree of sleep pressure, and accordingly, the time of day that the cognitive testing takes place. The stimulant caffeine ameliorates the negative consequences of sleep deprivation on attentional cognitive processes; but, it is mostly ineffective in mitigating the impact of severe sleep loss on higher-order cognitive functions (memory and executive function). Caffeine blocks adenosine receptors. Thus, aside from adenosine's known role in regulating sleep homeostasis, it may also be important in modulating attention-related cognitive processes (Chapter 2).

In a case-control field study, caffeine consumption and sleep behaviours were investigated in type 2 diabetes patients. The analyses, which applied an age restriction of 40 to 80 years, compared type 2 diabetes (n = 134) and non-type 2 diabetes (n = 230) participants, in terms of demographic status, health, daytime sleepiness, sleep quality and sleep timing, diurnal preference, mistimed circadian rhythms and habitual caffeine intake. Participants also gave saliva for *CYP1A2* genotyping and quantification of caffeine concentration. The results revealed that type 2 diabetes patients reported greater daytime sleepiness, a higher prevalence of sleep apnea and napping, and greater habitual caffeine intake. The greater caffeine intake of the patient group stemmed from the consumption of an extra cup of coffee each day, and was confirmed by higher salivary concentrations of caffeine at bedtime. Statistical modelling demonstrated that type 2 diabetes status was associated with higher self-reported caffeine consumption and higher salivary caffeine. Moreover, next to male

gender, type 2 diabetes status was the strongest predictor of caffeine intake. Interestingly, subjective sleep and circadian estimates were similar between case and control groups; as was the distribution of the genotype-derived, CYP1A2 enzyme-inducibility phenotype. It was concluded that type 2 diabetes patients may self-medicate with caffeine to alleviate daytime sleepiness. However, this high caffeine intake could undermine efforts to control hyperglycemia and reflects a lifestyle factor to be considered when promoting type 2 diabetes management (Chapter 3).

Case-control comparisons, in a non-age restricted sample, revealed that CYP1A2 enzyme activity, and thus speed of caffeine metabolism, was significantly higher in the type 2 diabetes group (case: n = 57; control: n = 146). This was corroborated by higher salivary concentrations of caffeine's major metabolite, paraxanthine, at bedtime. Statistical modelling demonstrated that higher habitual caffeine intake was associated with greater CYP1A2 enzyme activity. It was concluded that high caffeine intake in type 2 diabetes patients may raise CYP1A2 enzyme activity. Thus, again, caffeine intake is potentially an important dietary factor to be considered in the promotion of health in type 2 diabetes populations (Chapter 4).

Despite the negative effects of caffeine on glycemic control and sleep, a recommendation of total caffeine abstinence in type 2 diabetes may be short-sighted. Diabetes patients show cognitive impairments across the three main cognitive domains (attention, memory, and executive function). Based on research in healthy, rested and sleep restricted individuals, acute caffeine administration could improve patients' attentional cognitive deficits. In addition, chronic caffeine intake may protect against their accelerated, late-life cognitive decline. Moreover, epidemiological studies consistently link habitual, high coffee consumption with reduced risk of type 2 diabetes. An alternative approach would be to harness the benefits of caffeine, while avoiding, or at least ameliorating, the negatives. This could potentially be achieved by restricting caffeine intake to healthy / safe levels, and monitoring the timing of caffeine consumption across the day (Chapter 5).

Zusammenfassung

Koffein ist natürlicher Bestandteil von Kaffee, Tee und Kakao und kann zu Getränken und Medikamenten als Zusatz hinzugefügt werden. Es ist die weltweit meistkonsumierte psychoaktive Substanz und hat wichtige Auswirkungen auf die subjektive Aufmerksamkeit und objektive kognitive Leistungsfähigkeit. Schlaf ist ein stark regulierter und globaler Zustand, der vom Gehirn gesteuert wird. Er wird durch eine reduzierte Wahrnehmung äußerer Reize, kombiniert mit einer erhöhten Schlafneigung als Folge von Schlafentzug gekennzeichnet. Erhöhte Koffeinaufnahme und gestörte Schlafqualität behindern die glykämische Kontrolle. Typ-2-Diabetes ist ein internationales Gesundheitsproblem. Eingriffe, die Diabetes behandeln, haben eine Verbesserung der Glukose-Homöostase und eine Minimierung der damit verbundenen Hyperglykämie Komplikationen als Ziel. Wissenschaftliche Beobachtungsmethoden können verwendet werden, um modifizierbare, gesundheitsbezogene Verhaltensweisen, wie die Aufnahme von Koffein und Schlafqualität, bei Gesunden und Patienten zu untersuchen (Kapitel 1).

Die beiden unterschiedlichen Wach- und Schlafzustände resultieren aus einem Wechselspiel zwischen der endogenen zirkadianen Uhr und den homöostatischen Prozessen. Kognitive Leistung wird durch die persönliche zirkadiane Präferenz und den Grad des Schlafdrucks beeinflusst und dementsprechend die Tageszeit, zu der die kognitiven Tests stattfinden. Das Stimulanz Koffein mildert die negativen Folgen von Schlafentzug auf kognitive Aufmerksamkeitsprozesse; aber es ist meist nicht effektiv, um die Auswirkungen von schwerem Schlafmangel auf kognitive Funktionen höherer Ordnung zu mildern (sprich Gedächtnis und exekutive Funktion). Koffein blockiert Adenosin-Rezeptoren. Also, abgesehen von der bekannten Rolle bei der Regulierung der Schlaf-Homöostase durch Adenosin, kann es auch bei der Modulierung von Aufmerksamkeit bezogenen kognitiven Prozessen wichtig sein (Kapitel 2).

In einer Fall-Kontroll-Feldstudie wurden Koffeinkonsum und Schlafverhalten bei Typ-2-Diabetes-Patienten untersucht. Analysen, die eine Altersbeschränkung von 40 bis 80 Jahre eingehalten hatten, verglichen Typ-2-Diabetes (n = 134) und Nicht-Typ-2-Diabetes (n = 230) Teilnehmer in Bezug auf den demographischen Status, Gesundheit, Tagesschläfrigkeit, Schlafqualität und Schlaf Timing, Tagespräferenz, versetzte zirkadiane Rhythmen und Gewohnheiten bei der Koffein Aufnahme. Die Teilnehmer gaben auch Speichel für CYP1A2 Genotypisierung und Quantifizierung von Koffeinkonzentration ab. Die Ergebnisse zeigten, dass Typ-2-Diabetes-Patienten von einer größeren Tagesschläfrigkeit und einer höheren

Prävalenz für Schlafapnoe berichten sowie von Napping. Des Weiteren sind sie sich an einen höheren Koffeinkonsum gewöhnt. Der höhere Konsum von Koffein in der Patientengruppe ergab sich aus dem Verbrauch einer zusätzlichen Tasse Kaffee pro Tag und wurde durch höhere Koffein Konzentrationen im Speichel vor dem Zubettgehen bestätigt. Statistische Modellierung zeigte, dass Typ-2-Diabetes-Status mit höherem selbstberichtetem Koffeinkonsum und höherem Speichel-Koffein assoziiert war. Ausser dem männlichen Geschlecht war darüber hinaus der Typ-2-Diabetes-Status der stärkste Prädiktor für den Konsum von Koffein. Interessanterweise waren die Einschätzungen mit Bezug auf Schlaf und zirkadiane Periodik zwischen den Experimental- und Kontrollgruppen ähnlich, sowie die Verteilung des Genotyp-abgeleiteten CYP1A2 Phänotyps zur Enzym-Induzierbarkeit. Zusammenfassend, versuchen Typ-2-Diabetes-Patienten ihre Tagesschläfrigkeit durch ihren Koffeinkonsum zu lindern. Ein hoher Koffeinkonsum könnte allerdings den Bemühungen, um Hyperglykämie zu bewältigen, untergraben und stellt einen Lifestyle Faktor dar, der bei der Förderung der Typ-2-Diabetes-Behandlung berücksichtigt werden sollte (Kapitel 3).

Fall-Kontroll-Vergleiche in einer Stichprobe ohne Altersbeschränkung ergaben, dass die CYP1A2-Enzymaktivität und die Geschwindigkeit des Koffein Stoffwechsels signifikant höher in der Typ-2-Diabetes-Gruppe waren (Experimental Gruppe: n = 57; Kontrolle Gruppe: n = 146). Dies wurde durch höhere Speichel Konzentrationen des Hauptmetaboliten von Koffein, nämlich Paraxanthin, vor dem Zubettgehen bestätigt. Statistische Modellierung zeigte, dass ein habituell hoher Koffeinkonsum mit größerer CYP1A2-Enzymaktivität einhergeht. Es wurde festgestellt, dass eine hohe Koffeinaufnahme bei Typ-2-Diabetes-Patienten eine CYP1A2 Enzymaktivität erhöhen könnte. Die Aufnahme von Koffein stellt also möglicherweise einen wichtigen Ernährungsfaktor zur Förderung der Gesundheit bei Typ-2-Diabetes-Patienten dar, die berücksichtigt werden muss (Kapitel 4).

Trotz der negativen Auswirkungen von Koffein auf die glykämische Kontrolle und den Schlaf, könnte eine komplette Koffeinabstinenz bei Typ-2-Diabetes zu kurzsichtig sein. Diabetes-Patienten zeigen kognitive Beeinträchtigungen in den drei wichtigsten kognitiven Domänen (Aufmerksamkeit, Gedächtnis und exekutive Funktion). Basierend auf der Forschung von gesunden, ausgeruhten und schlafbeschränkten Probanden, könnte eine akute Verabreichung von Koffein die Defizite bei Kognition und Aufmerksamkeit verbessern. Des Weiteren könnte chronischer Koffeinkonsum das Risiko für einen kognitiven Verfall in fortgeschrittenem Alter verringern. Ausserdem assoziieren epidemiologische Studien konsequent einen angewohnten hohen Kaffeekonsum mit einem reduzierten Risiko von Typ-2-Diabetes. Ein alternativer Ansatz wäre es, sowohl die Vorteile von Koffein zu nutzen, als

auch die Nachteile zu meiden bzw. zu senken. Dies könnte eventuell erreicht werden, indem der Koffeinkonsum auf gesunde bzw. sichere Mengen begrenzt wird, wobei vor allem auf die Zeitpunkte des Konsums über den Tag hinweg geachtet wird (Kapital 5).

Chapter 1:
General Introduction

Caffeine intake

Caffeine

Caffeine occurs naturally in coffee, tea and cocoa; and can be added to beverages and medications. It is the most widely used psychoactive drug in the world, being consumed habitually by eighty percent of adults. Caffeine has important effects on alertness, and there is no doubt that caffeine is widely consumed by persons who need to stay awake or feel sleepy (Fredholm et al. 1999). It is generally accepted that caffeine promotes wakefulness via antagonism of adenosine receptors in the brain (Nehlig et al. 1992). Interestingly, daytime sleepiness is a typical symptom in type 2 diabetes (West et al. 2006); and objective measures of alertness have been shown to be impaired in type 2 diabetes samples (see Awad et al. 2004, for review).

Caffeine is almost completely metabolized in the body by enzyme cytochrome P450 1A2 (CYP1A2) (Gu et al. 1992). Indeed, as illustrated in Figure 1, CYP1A2 exclusively accounts for the metabolism of caffeine to its principal metabolite, paraxanthine (Gu et al. 1992). The CYP enzyme superfamily is involved in the biotransformation of exogenous and endogenous compounds, and accounts for most of phase I drug metabolism (Eichelbaum et al. 2006). Human CYP1A2 is exclusively expressed in the liver (Shimada et al. 1994). *In vivo*, CYP1A2 activity exhibits a significant degree of inter-individual variation (see Faber et al. 2005, for review). Inter-individual variability in CYP1A2 enzyme activity is typically between 5- and 15-fold in healthy humans (Schrenk et al. 1998; Tantcheva-Poór et al. 1999). The observed variability in activity is based on environmental and genetic factors (Faber et al. 2005). Factors determining CYP1A2 activity are summarised in Figure 2, and include caffeine / coffee intake. Faber and colleagues note that in addition, many drugs have a major inhibiting or inducing effect on CYP1A2 activity (Faber et al. 2005). Genetically determined variation in CYP1A2 activity may also contribute to inter-individual differences in enzyme activity (Sachse et al. 2003). For example, a common single nucleotide polymorphism (SNP) (*CYP1A2 -163C>A*) has been associated with greater CYP1A2 activity (i.e. a functional change in activity) in the presence of the inducer, tobacco smoke. That is, in smokers, the A/A genotype was associated with higher CYP1A2 activity (1.6-fold; Sachse et al. 1999).

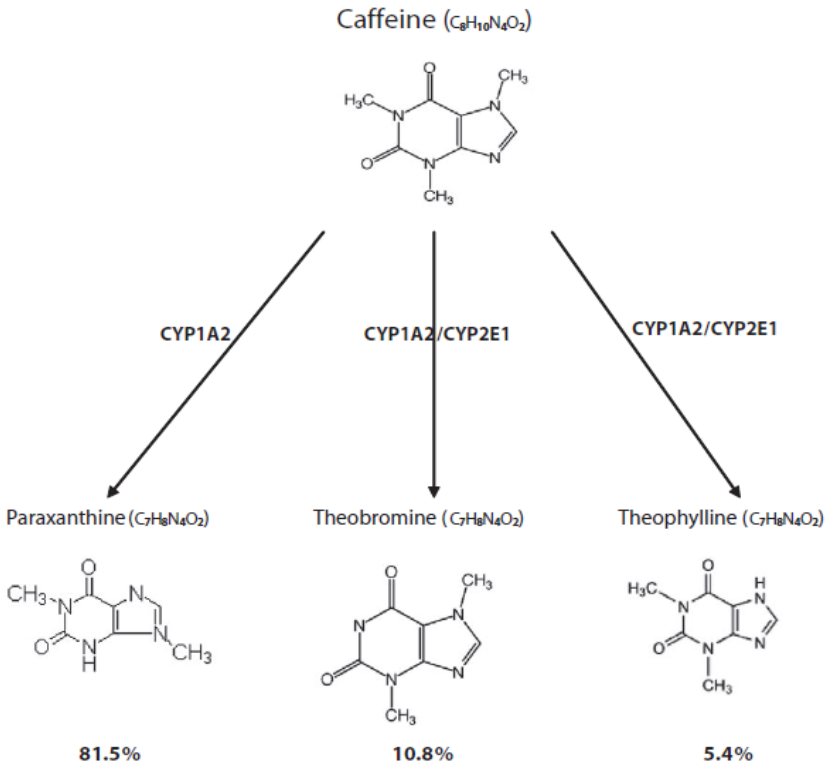

Figure 1: The chemical structures and human metabolic pathways of caffeine to its three primary metabolites. Human CYP1A2 studies have demonstrated that caffeine is predominantly (81.5%) metabolized to paraxanthine exclusively via the CYP1A2 pathway. Caffeine is also metabolized to theophylline (5.4%) and theobromine (10.8%) via enzymes CYP1A2 and CYP2E1. Source: Gu et al. 1992.

Source of variation	Mean resulting change of CYP1A2 activity determined by phenotyping
Cigarette smoking	dose dependent, 1.22-fold, 1.47-fold, 1.66-fold and 1.72-fold for 1–5, 6–10, 11–20 and >20 cigarettes smoked per day
Coffee	1.45-fold per litre of coffee drunk daily
Body mass index	0.99-fold per $kg \cdot m^{-2}$
Female gender	0.90-fold
Oral contraceptives	0.72-fold
Menstrual cycle	1.03-fold up to 1.10-fold (mid-luteal relative to mid-follicular phase)
500 g broccoli daily	1.19-fold
Meat pan-fried at high temperatures	1.40-fold (highly variable, after a controlled 7 day diet)
Chargrilled meat	1.89-fold (variable, after a controlled 6 day diet)

Figure 2: Factors determining CYP1A2 enzyme activity in healthy populations. Source: Faber et al. 2005.

There are high individual differences in caffeine intake, which have been related to variation in genetic (Cornelis et al. 2015; Rodenburg et al. 2012), demographic and environmental factors (Penolazzi et al. 2012; Tran et al. 2015; Wittman et al. 2006). For example, men, smokers and persons with 'impulsive and sensation-seeking' personalities have been shown to consume more caffeine than women, non-smokers and more introverted persons (Penolazzi et al. 2012). Self-reported daytime sleepiness is related to higher caffeine intake (Tran et al. 2015); as is a 'later' body clock or chronotype, as assessed by questionnaire (Munich Chronotype Questionnaire; Wittman et al. 2006). In a genome-wide meta-analysis, variation in the gene encoding the CYP1A2 enzyme (*CYP1A2*) was associated with habitual coffee consumption (Cornelis et al. 2015). Individuals with decreased CYP1A2 enzyme activity, based on *CYP1A2* genotype, have been shown to consume less coffee (SNP ID: rs2472299, which is linked to rs762551; Rodenburg et al. 2012). Interestingly, in a 6-year longitudinal study (Palatini et al. 2015) that followed non-diabetic, hypertensive patients, heavy coffee drinkers (> 3 cups per day) carrying the 'slow' C allele of the *CYP1A2* polymorphism (SNP ID: rs762551), showed a higher adjusted risk of developing impaired fasting glucose compared to coffee abstainers. Such data indicate that in coffee drinkers, the *CYP1A2* genotype may modulate risk of defective glucose metabolism (Palatini et al. 2015).

Caffeine intake and glycemic control

The relationship between coffee and caffeine consumption, and risk of type 2 diabetes, is controversial (Palatini, 2015; Palatini et al. 2015). Several large-scale, prospective studies have reported a negative association between increased coffee consumption and risk of developing the disease (Ding et al. 2014). For example, a systematic review and meta-analysis conducted by Ding and colleagues (Ding et al. 2014), based on 1,109,272 study participants and 45,335 cases of type 2 diabetes, revealed an inverse relationship between habitual coffee consumption and risk of diabetes. That is, compared with no coffee consumption, consumption of 1 cup per day was associated with an 8% lower risk of developing type 2 diabetes. This protective effect then increased in a dose-dependent manner as follows: -15% disease risk for 2 cups/day; -21% for 3 cups/day; -25% for 4 cups/day; -29% for 5 cups/day; and -33% for 6 cups/day. Interestingly, both caffeinated coffee and decaffeinated coffee consumption were linked with reduced type 2 diabetes risk. That is, compared with participants with the lowest level of caffeinated coffee consumption (median consumption 0 cups/day), a median intake of 5 cups per day was related to a 26% lower disease risk; while 4 cups of decaffeinated coffee each day was associated with a 20% reduced risk of diabetes. The association between total coffee consumption and diabetes risk was consistent for men and women, and for European, American and Asian populations (Ding et al. 2014).

Paradoxically, short-term, well-controlled laboratory studies show that both pure caffeine (Greer et al. 2001; Lane et al. 2004) and caffeinated coffee (Moisey et al. 2008) impair glucose metabolism. (See Table 1 for summary of methods and results). Importantly, this impairment is not seen with decaffeinated coffee (Moisey et al. 2008). Such data casts doubts on the protective effects of caffeinated coffee, as implied by epidemiological studies (e.g. Ding et al. 2014), which indeed reflect correlational, but not causal relationships. For example, moderate doses of oral caffeine (375 mg, equivalent to 3-4 cups of coffee), reduced glucose disposal by 23% in lean, healthy volunteers (Greer et al. 2001), and 21% in type 2 diabetes patients (Lane et al. 2004), due to an acute reduction in insulin sensitivity (Greer et al. 2001; Lane et al. 2004). The validity of the findings of Greer (Greer et al. 2001) and Lane (Lane et al. 2004) is supported by the strong, placebo-controlled, crossover study designs; as well as the 'naturalistic' approach of administering caffeine orally (*vs.* intravenously). However, the findings are limited by the small sample sizes (N = 9; N = 14; respectively). Moreover, the use of different methods to assess glycemic control (glucose clamp *vs*. tolerance test) makes it impossible to directly compare the results of the two studies. Moisey and colleagues (Moisey et al. 2008; N = 9) used a mixed meal tolerance test to investigate glucose homeostasis following both caffeinated and decaffeinated coffee. Participants consumed the coffee intervention one hour before ingesting a meal of high or low glycemic index (high sugar breakfast cereal *vs.* low sugar breakfast cereal; served with non-fat milk). Blood sampling at regular time intervals revealed that compared to decaffeinated coffee, intake of caffeinated coffee with a meal of either high or low glycemic index impaired acute blood glucose management due to a reduction in insulin sensitivity, and an inadequate beta cell response. As a result, the authors argued that caffeine and caffeinated coffee should be considered dietary risk factors for poor glycemic control (Moisey et al. 2008). The researchers' 'real life' approach of an instant coffee intervention, rather than pure caffeine, strengthens the ecological validity of the findings; but, the findings are limited by the lack of placebo. Johnston and colleagues also found that blood glucose and insulin levels were higher following caffeinated coffee consumption during a glucose tolerance test (Johnston et al. 2003; N = 9). The circulation of incretin hormones was also measured [glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide–1 (GLP-1)]. Interestingly, compared to the control condition (water + glucose), both caffeinated and decaffeinated coffee significantly altered incretin hormone secretion in a way that would slow intestinal glucose absorption, and thus slow the postprandial rise in blood glucose. That is, GIP secretion was significantly decreased across the 3 hour experimental period; while GLP-1 concentrations were significantly enhanced later in the experimental period. Postprandial secretion of GIP occurs in the proximal (higher) region of the small intestine and is stimulated by the absorption of nutrients from the gut, rather than by their presence in the

gut lumen (Sykes et al. 1980). The rate of absorption of glucose determines the magnitude of the GIP response (Ellis et al. 1995); therefore, coffee may decrease the rate of intestinal absorption of glucose (Johnston et al. 2003). In contrast, GLP-1 is secreted from the distal (lower) portion of the small intestine and responds to the presence of nutrients in the gut lumen, rather than to their absorption (Morgan et al. 1988). GLP-1 secretion can be increased when the absorption of carbohydrate is delayed (Ranganath et al. 1998). Taken together, it was argued that this gastrointestinal hormone data were consistent with delayed glucose uptake in the intestine (Johnston et al. 2003). The authors concluded that chlorogenic acid, a constituent, phenolic compound of coffee, may have mediated this beneficial effect via a variety of mechanisms that may result in an altered pattern of intestinal glucose uptake (Johnston et al. 2003). For example, in rats it has been shown that exposure to phenolic compounds decreases intestinal, brush-border membrane glucose uptake (Welsch et al. 1989).

Overall therefore, coffee components other than caffeine may enhance aspects of glycemic control; and potentially, such compounds may reduce caffeine's acute adverse effects on glucose metabolism. This concept is supported by the results of experimental studies in rats on diet-induced, whole-body insulin resistance. These experiments showed that decaffeinated coffee improved glucose disposal during a hyperinsulinemic–euglycemic clamp method; however, this effect was abolished in the rats that received decaffeinated coffee with added caffeine (Shearer et al. 2003). Coffee beans contain thousands of constituents, including lipids, proteins, carbohydrates, vitamins, and minerals (Tunnicliffe & Shearer, 2008). Aside from chlorogenic acid, other components of coffee may also improve glucose metabolism, including lignans, quinides, and trigonelline (Greenberg et al. 2006; van Dam, 2006; van Dijk et al. 2009).

Table 1: Laboratory studies assessing the effects of caffeine on glycemic control in non-smoking adults.

Reference	Design	Subjects	N	Habitual Caffeine Intake?	Gender	Mean Age (± SD)	Method	Caffeine Intervention	Key Result
Greer et al. (2001)	Randomized, double-blind, placebo-controlled, cross-over	Healthy (sedentary)	9	No	M (9)	25 years (± 0.5)	Glucose clamp*	5 mg caffeine/kg body weight (average dose: 350-400 mg) *vs*. placebo (oral capsule)	*Caffeine*: Insulin sensitivity ↓ Insulin secretion** ↔
Lane et al. (2004)	Randomized, double-blind, placebo-controlled, crossover	T2D (sedentary)	14	Yes	M (11) F (3)	61 years (± 9)	MMTT (liquid meal)	375 mg pure caffeine *vs.* placebo (oral capsule)	*Caffeine*: Insulin sensitivity ↓ Insulin secretion** ↔
Moisey et al. (2008)	Randomized, single-blind, crossover	Healthy (sedentary)	10	Yes	M (10)	23 years (± 1.1)	MMTT (low GI cereal *vs.* high GI cereal)	Caff. *vs.* decaf. coffee (5 mg caffeine/kg body weight) (instant coffee)	*High GI meal with caff. coffee vs. high GI with decaf. coffee:* Insulin sensitivity ↓ Insulin secretion** ↔

Abbreviations: Decaf, decaffeinated coffee; Caff, caffeinated coffee; F, female; GI, glycemic index; M, male; MMTT, mixed meal tolerance test; OGTT, oral glucose tolerance test; T2D, type 2 diabetes.

* *Glucose clamp was a hyperinsulinemic-euglycemic clamp.*

** ↔ *Symbol denotes an inadequate beta cell response / insulin secretion.*

Sleep quality

Sleep

Sleep is a highly regulated and global state controlled by the brain. In its basic form, it is characterised by a reduced awareness to external stimuli, combined with an increased sleep intensity, or drive, following sleep deprivation. By this definition, sleep is observed throughout the animal kingdom and appears to have been conserved throughout evolution (Campbell & Tobler, 1984; Tobler, 2011). Historically, sleep was believed to be a passive state associated with memory impairments and loss of consciousness. However, following the discoveries of rapid eye movement (REM) sleep (Aserinsky & Kleitman, 1953) and later, the cyclic alternations between REM and non-REM (NREM) sleep (Dement & Kleitman, 1957), it was generally accepted that sleep is an active process. Sleep encompasses approximately a third of our daily life (Kripke et al. 2002), and yet, the importance and molecular mechanism(s) of sleep remain poorly understood (Cirelli & Tononi, 2008). Interestingly, although not extensively studied, sleep has been shown to be disturbed in type 2 diabetes patients (Trento et al. 2008; Resnick et al. 2003).

The electroencephalogram (EEG) is an important tool for the non-invasive investigation of the human brain, including sleep. Standardized methods ensure inter-individual comparability for placing EEG electrodes on the head; for example, the 10 - 20 system requires an electrode distance of 10% to 20% of the total frontal-occipital and left-right distance of the skull (see Figure 3, left) (Purves, 2004). The EEG is generated by rapid depolarization of neuronal membranes, mediated by sodium and potassium voltage-dependent ion conductance, and by neurotransmitter-dependent synaptic activation. Consequently, action potentials propagate along axons and dendrites. Since there is no local accumulation of charge, current flow in one direction is compensated by opposite current flows elsewhere. As a result, excitatory or inhibitory postsynaptic potentials are generated. These alternating electric fields can be detected on the scalp surface (Figure 3, right) and are known as field potentials. Field potentials represent summated activity from a large number of neurons and can be recorded with good temporal resolution. Field potentials measured by the EEG are primarily generated by synaptic activity in apical dendrites of cortical pyramidal neurons, oriented perpendicular to the cell surface (Westbrook, 2000).

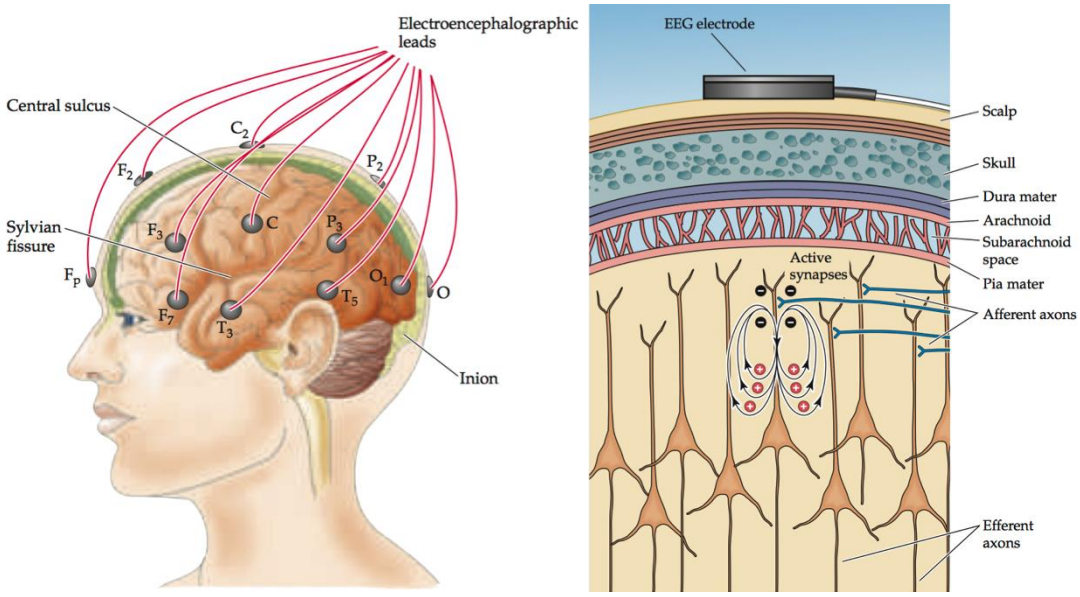

Figure 3: EEG generation and the typical 10 - 20 montage. *Left*: Example of an EEG montage using the 10 - 20 system. Electrodes are positioned according to well-defined criteria with an electrode distance of 10% to 20% of the total frontal-occipital and left-right distance of the skull. Potential differences between electrodes are measured; thus EEG recordings require at least two electrodes. *Right*: The EEG measures field potentials, the combined electrical activity of a large number of cortical pyramidal cells. The activity originates primarily from the apical dendrites orientated perpendicular to the cell surface. Source: Purves, 2004.

The human EEG, together with recordings of the electrooculogram (EOG), electromyogram (EMG) and electrocardiogram (ECG) can discriminate between the vigilance states of REM sleep, NREM sleep, and wakefulness. The EEG is typically divided into bands of waves including slow oscillations (< 1 Hz), delta (1 - 4 Hz), theta (4 – 7.5 Hz), alpha (8 – 13 Hz) and beta (14 - 30 Hz) waves, although these definitions vary considerably across the literature. Slow oscillations are commonly used for the sleep EEG only. Similarly, beta activity during sleep is typically defined at a slightly higher frequency (15-30 Hz) to encompass spindles / sigma (12-15 Hz) activity. Vigilance states, as depicted in Figure 4, can be systematically defined according to the criteria of Rechtschaffen and Kales (Rechtschaffen & Kales, 1968). Here, rested wakefulness (with eyes closed) is typically associated with distinct, low amplitude, alpha activity. NREM sleep is divided into stages 1 – 4 of increasing intensity. Specifically, stage 1 sleep is considered a transitional state between wakefulness and sleep. It is short-lived and characterized by irregular low voltage, mixed frequency EEG activity, associated with a dissipation of alpha waves. Stage 2 sleep is recognized by the transient occurrence of K-complexes and 11 - 15 Hz waxing and waning sleep spindles. Stages 3 and

4 contain moderate (20 - 50%) and high (> 50 %) amounts of slow waves, respectively, with frequencies in the delta range and a peak-to-peak amplitude of >75 □V. Stage 3 and 4 are often combined into so-called slow wave sleep (SWS). Finally, REM sleep, also known as paradoxical sleep, can be recognized by its mixed-frequency EEG activity that resembles active wakefulness. However, REM sleep is associated with strong muscle atonia, where only the eye muscles show rapid activity (Rechtschaffen & Kales, 1968).

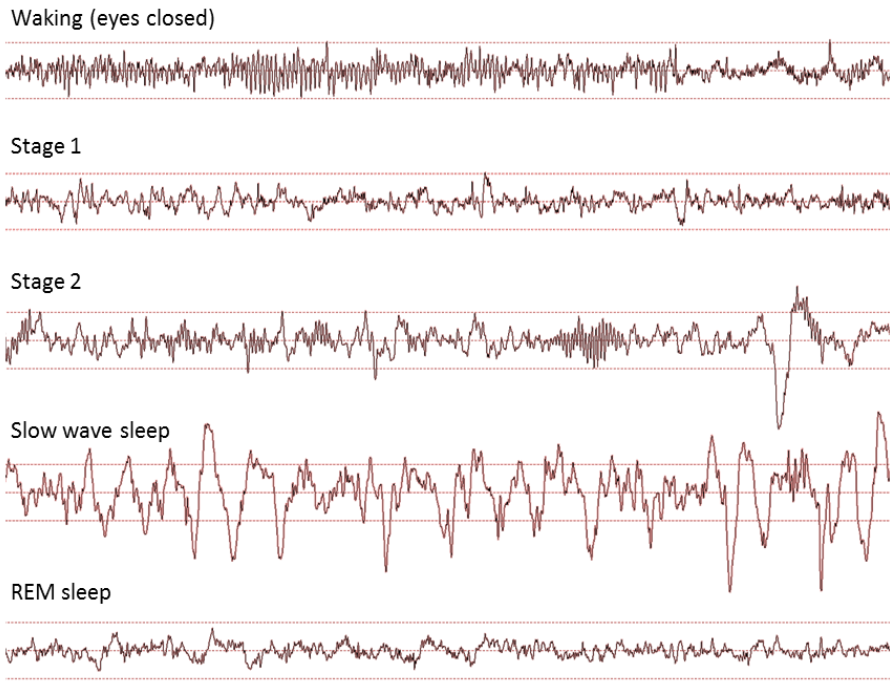

Figure 4: Example of typical EEG oscillations during wakefulness and sleep**.** EEG traces (20 seconds) recorded during a typical 8-hour sleep episode in a representative individual. Waking EEG with eyes closed can be recognized by strong alpha activity around 10 Hz. Stage 1 sleep shows irregular low voltage, mixed frequency EEG. Stage 2 sleep is recognized by the common spindles (~13.5 Hz) and large K-complexes. Slow wave sleep (stage 3 and 4) is illustrated by large slow waves (>75 □V) in the delta frequency range. REM sleep is associated with mixed frequency, low amplitude waves; and by reduced muscle tone and rapid eye movements recognized in the EMG and EOG (not illustrated). Dotted lines illustrate the amplitude criteria for slow waves; ± 37.5 □V. Source: Holst, 2013.

Sleep quality and glycemic control

Well-controlled laboratory studies in humans have demonstrated that sleep restriction (Buxton et al. 2010), disrupted sleep quality (Tasali et al. 2008) and disrupted sleep timing (Leproult et al., 2014) impede glucose homeostasis (see Table 2 for summary). Supporting a link between sleep and metabolism is evidence from large prospective studies that indicates

that sleep duration (Shan et al. 2015), and a mismatch between preferred sleep timing ('chronotype') and work schedule (Vetter et al. 2015), are associated with type 2 diabetes risk. Shan and colleagues (Shan et al. 2015) performed a meta-analysis of ten prospective studies to assess the relationship between sleep duration and risk of developing diabetes. They found a U-shaped association between sleep length and disease risk, with the lowest risk at 7 to 8 hours of sleep per day. Compared with the reference category of 7 hours of sleep, both short and long sleep duration were linked to a significantly increased risk of type 2 diabetes (Shan et al. 2015). Vetter and colleagues (Vetter et al. 2015) examined whether a mismatch of sleep and work timing was associated with type 2 diabetes risk. The study followed 64,615 nurses, from years 2005 to 2011, that undertook rotating night shifts as part of their job. The outcome measure was development of type 2 diabetes (n = 1,452). The results suggested that if work times interfere with preferred sleep timing, shift and day workers may be at an increased risk of type 2 diabetes. Specifically, late chronotypes (persons preferring to fall asleep and wake up later) had significantly higher disease risk if their work schedule included more daytime shifts; while early chronotypes were more at risk if they undertook more night shifts.

In accordance with the epidemiological evidence linking sleep duration and type 2 diabetes risk (Shan et al. 2015), Buxton and colleagues (Buxton et al. 2010) investigated the potential mechanism by which short sleep might impair glucose homeostasis. The team assessed the effects of sleep restriction on glucose tolerance, insulin secretion and insulin sensitivity in healthy men, using a strong, crossover design. The experiment revealed that short sleep reduced glucose tolerance; an impairment that stemmed from an acute decrease in insulin sensitivity. The authors argued that the findings indicate that short-term sleep restriction may contribute to metabolic dysregulation, via a reduction in insulin sensitivity; which, if occurring over the long-term, may result in increased risk for diabetes (Buxton et al. 2010). Previous research has shown that sleep restriction increases self-reported hunger and appetite for carbohydrate-dense foods, with concordant changes in appetite-regulating hormones (anorexigenic-leptin decreases; orexigenic-ghrelin increases) (Spiegel et al. 2004). A strength of the Buxton study was that the protocol did not allow behavioural changes in diet composition, caloric intake, or activity / exercise levels, which could potentially have contributed to the association of reduced sleep duration and metabolic abnormalities. Plus, importantly, resting metabolic rate was similar between sleep interventions (Buxton et al. 2010). However, by controlling food intake, the study did not reflect 'real-life' conditions. Also, the participants were young and healthy - the glucose homeostasis of, for example, older persons or those with impaired glycemic control, may respond differently to sleep restriction, and thus the generalisability of the findings is limited. Nonetheless, if subjects had

been allowed to eat ad-libitum, the impediments of sleep restriction on glycemic control could have been even greater.

The initiation of slow wave sleep (SWS) coincides, temporally, with transient metabolic, hormonal, and neurophysiological changes, which may affect glucose homeostasis. These include decreased brain glucose utilization, stimulation of growth hormone release, inhibition of corticotropic activity, decreased sympathetic nervous activity, and increased vagal tone (Tasali et al. 2008). Thus, Tasali and colleagues (Tasali et al. 2008) investigated whether reduction of sleep depth, by suppression of SWS, might adversely affect glucose homeostasis. In order to suppress SWS, the EEG was continuously monitored. When delta activity, a marker of SWS, was visually noted, the researchers delivered an acoustic tone to the participant via speakers beside the bed. The results showed that this intervention successfully removed SWS (c.90%), but did not influence total sleep time. Suppressing SWS prompted significant decreases in insulin sensitivity, without an adequate compensatory increase in insulin secretion. The authors argued that such data suggest that reduced sleep quality, with low levels of SWS, may lead to reduced glucose tolerance over the long term and thus play a role in diabetes risk (Tasali et al. 2008). This experiment used a small, young, healthy sample, which limits the generalisability of the findings; nonetheless, such results are noteworthy given that obese individuals show reduced sleep quality with low amounts of SWS (even in the absence of sleep-disordered breathing; Vgontzas et al. 1994); and, ageing coincides with reduced slow wave activity (Landolt et al. 1996). Both obesity and ageing are key risk factors for type 2 diabetes (Chen et al. 2012). Thus, obesity- and age-related changes to sleep quality may contribute to the development of metabolic abnormalities in at-risk persons. However, a limitation of the study was the recruitment of both male and female participants, since gender influences measures of postprandial glucose homeostasis (Basu et al. 2006). Thus, including men and women in the same sample reduces the reliability of the findings, unless the data were also analysed separately by gender (such an analysis is not shown). Interestingly, absolute values of insulin sensitivity and insulin secretion differed between the studies of Buxton (Buxton et al. 2010) and Tasali (Tasali et al. 2008); the former study recruited male participants only.

In accordance with epidemiological research relating shift work to type 2 diabetes risk (Vetter et al. 2015), experimental manipulation of both sleep duration and sleep timing revealed that irregular sleep schedules also impede glucose homeostasis (Leproult et al. 2014). This study involved participants completing two experimental conditions: a) sleep restriction with fixed nocturnal bedtimes (‘circadian alignment’); and b) sleep restriction with varied nocturnal bedtimes (‘circadian misalignment’). Assessment of glycemic control revealed that in both

conditions, insulin sensitivity significantly decreased after sleep restriction, without a compensatory rise in insulin release. However, within the male subjects, the reduction in insulin sensitivity was nearly twice as large when the sleep restriction protocol involved misaligned / irregular sleep patterns. The authors argued that such findings highlight that varied sleep-wake schedules, which typically occur during shift work, may increase diabetes risk, independently of sleep loss (Leproult et al. 2014). Nonetheless, a limitation of this finding is evidence that during sleep restriction, the night-time caloric intake of the misaligned group was three times higher than that of the group with the aligned circadian schedule. Eating at night has been related with adverse metabolic consequences (see Gallant et al. 2012, for review), which may have contributed to the more exaggerated impairment to insulin sensitivity in the misaligned group.

Table 2: Laboratory studies assessing the effects of sleep on glycemic control in non-smoking adults.

Reference	Design	Subjects	N	Gender	Age	Method	Sleep Intervention	Key Result
Buxton et al. (2010)	Randomized, crossover	Healthy	11	M (11)	Mean 27 years (SD ± 5.2)	EEG, Glucose clamp*, IVGTT	2 nights of 10 hours sleep *vs*. 5 nights of 5 hours sleep	*Sleep restriction:* Insulin sensitivity ↓ Insulin secretion** ↔
Tasali et al. (2008)	Randomized, crossover	Healthy	9	M (5) F (4)	20 – 30 years (range)	EEG, IVGTT	2 nights of baseline sleep *vs*. 3 nights of SWS suppression	*SWS suppression:* Insulin sensitivity ↓ Insulin secretion** ↔
Leproult et al. (2014)	Parallel-group	Healthy	26	M (10) F (3)	22 – 26 years (range)	EEG, IVGTT	Circadian alignment: 3 nights of 10 hours sleep *vs*. 8 nights of 5 hours sleep	*Circadian alignment & sleep restriction:* Insulin sensitivity ↓ Insulin secretion** ↔
				M (9) F (4)	22 – 25 years (range)		Circadian misalignment: 3 nights of 10 hours sleep *vs*. 8 nights of 5 hours sleep	*Circadian misalignment & sleep restriction:* Insulin sensitivity ↓↓ Insulin secretion** ↔

Abbreviations: EEG, electroencephalogram; F, female; IVGTT, intravenous glucose tolerance test; M, male; SWS, slow wave sleep.

* *Glucose clamp was a hyperinsulinemic-euglycemic clamp.*

** ↔ *Symbol denotes an inadequate beta cell response / insulin secretion.*

Diabetes Mellitus

Diabetes Mellitus is a group of metabolic diseases characterized by hyperglycemia, which results from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia is related to long-term damage, dysfunction, and failure of different organs; in particular, the eyes, kidneys, nerves, heart, and blood vessels. Various pathogenic processes are involved in the development of diabetes. These range from autoimmune destruction of the pancreatic beta cells which leads to insulin deficiency; to abnormalities that result in resistance to insulin action. The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is deficient action of insulin on target tissues. Deficient insulin action results from inadequate insulin secretion and / or reduced tissue responses to insulin at one or more points in the complex pathways of hormone action. Impairment of insulin secretion and defects in insulin action frequently coexist in the same patient, and it is often unclear which abnormality is the primary cause of the abnormally high blood glucose. Symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss and blurred vision. Long-term complications of diabetes include retinopathy, with potential loss of vision; nephropathy, leading to renal failure; peripheral neuropathy, with risk of foot ulcers and amputations; and autonomic neuropathy, causing gastrointestinal, genito-urinary and cardiovascular symptoms, and sexual dysfunction. Moreover, within the diabetes population there is increased incidence of hypertension, atherosclerosis and cardiovascular disease (American Diabetes Association, 2014).

The majority of diabetes cases fall into two broad categories. In one category, type 1 diabetes (5-10% of cases), the cause is an absolute deficiency of insulin secretion, which typically stems from autoimmune destruction of the beta cells of the pancreas. In the other category, type 2 diabetes (90-95% of cases), the cause of high blood glucose is a combination of resistance to insulin action, and an inadequate compensatory insulin secretory response. The diagnosis for diabetes is based on the blood glucose criteria summarised in Figure 5. Categories of increased risk for diabetes are summarised in Figure 6. Glycated haemoglobin (AIC) is a marker of chronic glycemia, reflecting average blood glucose over a 2- to 3-month period.

A1C ≥6.5%. The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay.*
OR
FPG ≥126 mg/dL (7.0 mmol/L). Fasting is defined as no caloric intake for at least 8 h.*
OR
Two-hour plasma glucose ≥200 mg/dL (11.1 mmol/L) during an OGTT. The test should be performed as described by the World Health Organization, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.*
OR
In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose ≥200 mg/dL (11.1 mmol/L).
*In the absence of unequivocal hyperglycemia, criteria 1–3 should be confirmed by repeat testing.

Figure 5: Criteria for the diagnosis of diabetes. (A1C, glycated haemoglobin; DCCT, Diabetes Control & Complications Trial; FPG, fasting plasma glucose; OGTT, oral glucose tolerance test). Source: American Diabetes Association, 2014.

FPG 100 mg/dL (5.6 mmol/L) to 125 mg/dL (6.9 mmol/L) (IFG)
2-h PG in the 75-g OGTT 140 mg/dL (7.8 mmol/L) to 199 mg/dL (11.0 mmol/L) (IGT)
A1C 5.7–6.4%
*For all three tests, risk is continuous, extending below the lower limit of the range and becoming disproportionately greater at higher ends of the range.

Figure 6: Categories of increased risk for diabetes (prediabetes)*. Results below these values are considered 'normal'. (IFG, impaired fasting glucose; PG, plasma glucose; IGT, impaired glucose tolerance). Source: American Diabetes Association, 2014.

Type 2 diabetes

Type 2 diabetes is a global health problem: in 2015, there were 415 million people living with diabetes; 75% of sufferers were in low- and middle-income countries (e.g. India, China); and prevalence is expected to rise to 642 million by 2040. Furthermore, 5 million people died from diabetes in 2015 and disease-related expenditure reached $673 billion (International

Diabetes Federation, 2015). Our modern lifestyle of high calorie diets and physical inactivity, resulting in an epidemic of obesity and the metabolic syndrome (insulin resistance, hypertension, hyperlipidemia and glucose intolerance), is largely blamed for causing the existing diabetes pandemic. However, as illustrated by Figure 7, a complete answer is likely more complex, and involves interactions between environmental and genetic factors, and various pathogenic processes impeding beta cell function. The solution likely requires the integration of research from epidemiologists, geneticists, clinical physiologists and basic researchers (Leahy & Pratley, 2011). Given the economic and societal burden of type 2 diabetes, effective management strategies are considered crucial (Inzucchi et al. 2012).

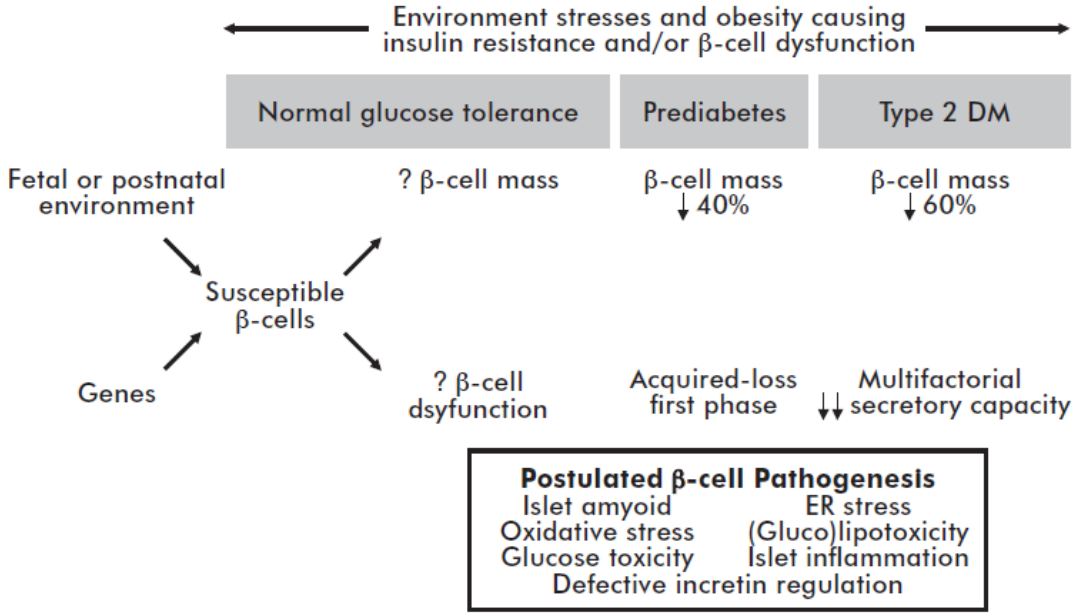

Figure 7: Proposed sequence of the key pathological features of type 2 diabetes. (ER, endoplasmic reticulum). Source: Leahy & Pratley, 2011.

A rise in blood glucose is the net result of glucose inflow exceeding glucose outflow from the blood plasma. In the fasting state, hyperglycemia is directly related to increased hepatic glucose production. In the postprandial state, long-lasting and inappropriate hyperglycemia stems from insufficient suppression of this hepatic glucose output; as well as defective insulin stimulation of glucose disposal in target tissues, especially skeletal muscle (Inzucchi et al. 2012). Abnormal function of pancreatic islet cells is an important feature of type 2 diabetes. In the early stages of the disease, insulin production is normal or increased in absolute terms, but disproportionately low for the level of insulin sensitivity, which tends to

be reduced. However, insulin kinetics, such as the ability of the beta cells to release sufficient insulin in phase with rising glycemia, are impaired (Inzucchi et al. 2012). In accordance with the research of Ferrannini and colleagues (Ferrannini et al. 2005), it is recognised that functional beta cell impairment is the key determinant of hyperglycemia, and ongoing degeneration of function is typical. In addition, pancreatic alpha cells may over-secrete glucagon, which further stimulates hepatic glucose production (Nauck, 2011). Nonetheless, islet dysfunction is not completely irreversible. Indeed, promoting insulin action via any method that promotes normoglycemia, relieves the secretory burden on beta cells; and in some patients, this can ameliorate beta cell dysfunction (Ferrannini, 2010). Typically in type 2 diabetes, especially among obese patients, insulin resistance in target tissues (liver, skeletal muscle, adipose tissue, heart muscle) is a key feature. This resistance promotes both overproduction of glucose from the liver and underutilisation of glucose by muscle groups (Groop & Ferrannini, 1993). More recently, it has been acknowledged that abnormalities in the incretin system are also found in type 2 diabetes. That is, the roles of the incretin hormones (GIP and GLP-1) in the regulation of glucose metabolism and other related physiologic processes (e.g. gut motility and food intake), are disturbed in type 2 diabetes patients (see Nauck, 2009, for review). Hormones GIP and GLP-1 are secreted by the gastrointestinal tract in response to meal ingestion, in particular, carbohydrates. Incretins exercise important gluco-regulatory effects, including the glucose-dependent enhancement of insulin secretion by beta cells. Research on the defective incretin action in type 2 diabetes suggests that in these patients, beta cells have reduced responsiveness to the insulinotropic activity of incretin hormones. However, it is unclear as to whether this abnormality is a primary or secondary defect in the pathophysiology of the disease (Nauck, 2009). Therapeutic approaches for increasing incretin action include degradation-resistant GLP-1 agonists (incretin mimics) and inhibitors of dipeptidyl peptidase-4 (DPP-4) activity (incretin enhancers) (Drucker & Nauck, 2006).

Beta cell function normally varies with the degree of insulin sensitivity (Leahy & Pratley, 2011). That is, while the insulin response to a meal or other stimulus is considerably less in an insulin sensitive person than one who is obese and insulin resistant (Polonsky et al. 1988), both represent normal beta cell function (Leahy & Pratley, 2011). Variances in insulin sensitivity occur in everyday life with puberty, pregnancy, aging and other events; diabetes can thus be viewed as a failure of beta cells to compensate for such variances (Leahy & Pratley, 2011). The relationship between experimentally-determined insulin sensitivity and a measure of beta cell function (first phase insulin response to intravenous glucose) has been mapped out in a large number of non-diabetic subjects to derive the normal curve called the disposition index (Bergman et al. 1981; Kahn et al. 1993; see Figure 8). The hyperbolic lines

are the experimentally-derived normal curve. This index can be used to plot groups of subjects, with varying degrees of glucose tolerance, onto the curve in order to identify the roles of insulin resistance versus beta cell dysfunction (Kahn, 2003).

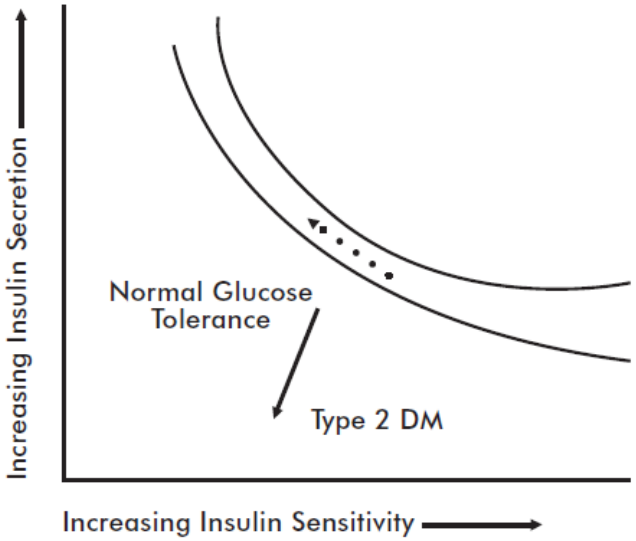

Figure 8: The disposition index: the curved lines show the normal relationship between insulin sensitivity and insulin secretion. The dotted arrow reflects subjects that undergo metabolic stresses such as obesity or aging, without any rise in their blood glucose levels. In these subjects, beta cell compensation is thus adequate and glucose tolerance remains normal. The solid line reflects subjects who develop type 2 diabetes; they typically fall below the curve while still normally glucose tolerant, but show a greater deterioration in beta cell function than in insulin sensitivity. Source: Leahy & Pratley, 2011.

Glucose clamp techniques are considered the gold standard approach for assessing insulin secretion and insulin sensitivity (Tam et al. 2012). The hyperglycemic clamp quantifies beta-cell sensitivity to glucose (DeFronzo et al. 1979). Here, plasma glucose is acutely raised and maintained above basal levels, via an intravenous glucose infusion. Since the plasma glucose concentration is held constant, the glucose infusion rate is an index of insulin secretion and glucose metabolism. Under these conditions of constant hyperglycemia, the plasma insulin response is biphasic with an early burst of insulin release during the first 6 minutes, followed by a gradually progressive increase in plasma insulin concentration (DeFronzo et al. 1979). The euglycemic insulin clamp technique (also called the hyperinsulinemic-euglycemic clamp) quantifies tissue sensitivity to insulin (DeFronzo et al. 1979). Here, the plasma insulin concentration is acutely raised and maintained by intravenous infusion of insulin. The plasma glucose concentration is held constant at basal

levels by a variable glucose infusion. Under these steady-state conditions of euglycemia ('normal' plasma glucose), the glucose infusion rate equals glucose uptake by all the tissues in the body and is therefore a measure of tissue sensitivity to insulin (DeFronzo et al. 1979). However, while clamp techniques give accurate and precise estimates of insulin sensitivity and beta cell function, plasma glucose and insulin concentrations achieved during these protocols are relatively non-physiological (Breda et al. 2001). In order to assess glycemic control under more normal-life conditions, researchers may choose more physiological methods; for example, after an overnight fast, measuring plasma glucose and insulin responses to orally administered stimuli, such as glucose (oral glucose tolerance test, OGTT) or a mixed-meal (mixed-meal tolerance test, MMTT) (Breda et al. 2001).

Interventions in type 2 diabetes

The goal of interventions in the treatment of diabetes is to aid the management of glycemia. Thus, anti-hyperglycemic agents are directed at one or more of the pathophysiological defects of type 2 diabetes; or, they modify physiological processes relating to appetite, nutrient absorption or excretion. Ultimately, type 2 diabetes is a heterogeneous condition in terms of both pathogenesis and clinical manifestation. Consequently, therapeutic options take an individualised approach, and aim to maintain blood glucose as consistently near normal as possible in order to minimise the complications of hyperglycemia; while avoiding undue risk from drug-related side-effects and heavy impingement on quality of life (Inzucchi et al. 2012). In general, the treatment of most type 2 diabetes patients begins with lifestyle changes that aim to reduce adiposity (e.g. dietary change towards healthier, low-fat and low-sugar choices, and increased physical activity); alongside oral therapy of metformin. Metformin lowers blood glucose by reducing hepatic glucose production and increasing peripheral glucose uptake. Due to its low cost, low risk, and weight-neutral profile, as well as its efficacy in reducing hyperglycemia, metformin is considered the optimal first-line drug. A disadvantage of metformin is the initial gastrointestinal side effects (e.g. nausea, diarrhoea). After 3 months, if glycemic targets are not reached, additional oral medications may be added to the treatment program to compliment the actions of metformin. Such classes of drugs include: sulfonylureas, thiazolidinediones, DPP-4 inhibitors and GLP-1 receptor agonists. Subsequently, due to the ongoing beta cell dysfunction that is characteristic of type 2 diabetes, insulin replacement therapy is frequently required as the disease progresses. An initial therapy may be long-acting 'basal' insulin, which provides fairly uniform insulin coverage through the day and night. In later stages of the disease, prandial insulin therapy, with short-acting insulin, may also be required. Insulin therapy is highly efficacious at

reducing blood glucose. However, downsides of insulin administration include weight gain, risk of hypoglycemia and high burden to patients' daily routine (Inzucchi et al. 2012). See Supplementary Figure 1 (Appendix I) for a summary of anti-hyperglycemic therapy recommendations in type 2 diabetes.

Risk factors for type 2 diabetes

As summarised in Figure 9, risk factors for type 2 diabetes stem from modifiable sources, typically related to a person's lifestyle, and non-modifiable sources, such as age and gender. Overall, the global epidemic of type 2 diabetes stems from rising rates of overweight and obesity in adults, as well as in youth (Chen et al. 2012). The prevalence of overweight [body mass index (BMI) 25-30 kg/m^2] and obesity (BMI ≥30 kg/m^2) in the world's adult population is predicted to rise from 33% in 2005 to 58% in 2030 (Kelly et al. 2008). Excess adiposity is the most important predictor of type 2 diabetes (Hu et al. 2001). In particular, visceral adiposity is an independent risk factor for insulin resistance and type 2 diabetes (Lebovitz & Banerji, 2005). Further modifiable risk factors include: physical inactivity and sedentary behaviour, dietary factors (e.g. high fat and high sugar diets), smoking, existing impaired fasting glucose and impaired glucose tolerance, abnormal lipid levels, hypertension, and inflammation (Chen et al. 2012). The intrauterine environment also influences type 2 diabetes risk; for example, low birth weight has been consistently linked with increased disease risk (Whincup et al. 2008). It has been suggested that nutritional deprivation *in utero,* and during infancy, influences later susceptibility to obesity and metabolic abnormalities such as diabetes, through the acquisition of a 'thrifty phenotype'. The thrifty phenotype hypothesis suggests that poor nutrition in early life leads to permanent and detrimental changes in glucose metabolism (Hales & Barker, 2001). Conversely, excessive maternal nutrition and hyperglycemia during pregnancy predisposes the offspring, and the mother, to the development of obesity and diabetes in later life (Chen et al. 2012).

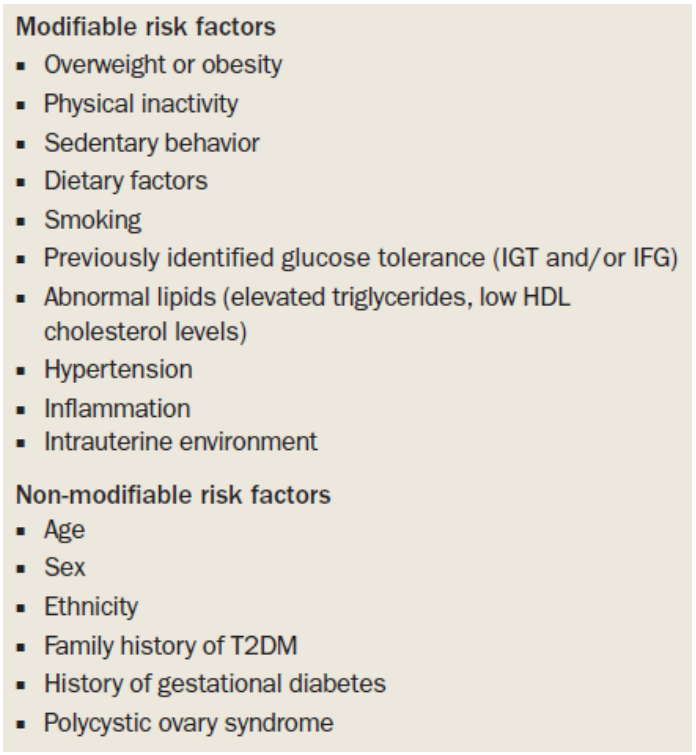

Figure 9: Modifiable and non-modifiable risk factors for type 2 diabetes. (IFG, impaired fasting glucose; IGT, impaired glucose tolerance; HDL, high-density lipoprotein; T2DM, type 2 diabetes mellitus). Source: Chen et al. 2012.

Non-modifiable risk factors of type 2 diabetes include age, gender, ethnicity, family history of diabetes, history of gestational diabetes and polycystic ovary syndrome (Chen et al. 2012). Traditionally, the disease was considered a metabolic disorder of older adults, yet it has become increasingly common in young adults, adolescents, and occasionally, in children (Pinhas-Hamiel & Zeitler, 2005). It is noteworthy that the effect of obesity on lifetime risk of type 2 diabetes is stronger in younger persons (Narayan et al. 2007). Regarding 'gender' as a risk factor: evidence suggests that the sexes are differentially affected by specific, recognised risk factors (Ding et al. 2006). For example, the positive association between adiposity and risk of type 2 diabetes is stronger for women compared with men: the age-adjusted relative risk of developing type 2 diabetes is approximately 10-fold higher for men with a BMI of 30 kg/m^2, relative to men with a BMI of less than 23 kg/m^2 (Colditz et al. 1995). Yet, the risk is even higher for women: overweight women (BMI 30 kg/m^2) are 30 times more at risk than normal weight women (Chan et al. 1994). In addition, while high testosterone levels are associated with higher risk of type 2 diabetes in women, they are linked with lower risk in men (Ding et al. 2006).

Type 2 diabetes is a complex, multifactorial disease fuelled by interactions between multiple susceptible genetic loci and numerous environmental and behavioural factors (Chen et al. 2012). More than three decades ago, it was demonstrated that genetic factors play an important role in the aetiology of type 2 diabetes: in an identical twin study, 48 / 53 twin pairs, living apart, showed concordant diagnoses of the disease (Barnett et al. 1981). Moreover, disparity in the risk of type 2 diabetes between different ethnic groups, after controlling for diverse environmental attributes, highlights a genetic predisposition in the development of the disease (Chen et al. 2012). The gene with a known high risk is transcription factor 7-like 2 *(TCF7L2)* (Sladek et al. 2007*).* Variants of this gene have been shown to alter insulin sensitivity and insulin secretion (Damcott et al. 2006; Saxena et al. 2006). However, despite multiple genetic loci being associated with the risk of type 2 diabetes, the discriminative ability of genetic scores, based on a number of risk alleles, is considered unsatisfactory (McCarthy, 2010). Moreover, disease-risk modelling has shown that the addition of risk alleles only marginally improves the prediction of future type 2 diabetes, compared with risk models based on clinical risk factors or family history of the disease (Chen et al. 2012). Nonetheless, it has been shown that genetic risk markers may be valuable in young adults, where other commonly used phenotypic risk factors (e.g. family history or hypertension) may not yet be expressed (de Miguel-Yanes et al. 2011). Furthermore, diabetes genetic testing can be used to motivate high-risk individuals to change their lifestyle and adhere to necessary preventive measures prior to the onset of clinical phenotypes (Chen et al. 2012).

More recently, there has been increasing research into how aspects of a person's lifestyle might influence and impede their glycemic control. Potential novel, modifiable risk factors to type 2 diabetes have thus been identified; these include caffeine intake (Palatini, 2015; Palatini et al. 2015) and disrupted sleep quality (Chen et al. 2012).

Caffeine intake, sleep quality and glycemic control

The findings of the aforementioned intervention studies, assessing the effects of caffeine (Table 1; Greer et al. 2001; Johnston et al. 2003; Lane et al. 2004; Moisey et al. 2008) and sleep (Table 2; Buxton et al. 2010; Leproult et al. 2014; Tasali et al. 2008) on glycemic control, are limited by the short-term nature of the experimental designs. As a result, it is impossible to determine whether the demonstrated detrimental effects to glucose homeostasis (reduced insulin sensitivity and inadequate insulin secretion) would persist over the long-term, following chronic caffeine intake and / or disturbed sleep.

It is well-established that caffeine reduces sleep duration and sleep quality; and this impediment includes a reduction in total sleep time and slow wave activity (see Clark & Landolt, 2016, for review), which themselves have been linked to impaired glucose homeostasis (Buxton et al. 2010; Leproult et al. 2014; Tasali et al. 2008). Despite these negative consequences, caffeine is the most widely used psychoactive substance in the world. Furthermore, caffeine is commonly consumed to relieve sleepiness (Fredholm et al. 1999), which is a typical symptom of type 2 diabetes (West et al. 2006). The simultaneous effects of caffeine and disturbed sleep on glucose homeostasis have never been assessed in a controlled, laboratory setting. Moreover, it is unknown if type 2 diabetes patients, within a population-based sample, report habitual caffeine intake and subjective sleep quality that differ from the norm. If this was the case, caffeine and sleep may reflect lifestyle factors that could be adapted to improve glycemic control in type 2 diabetes patients. The increasing burden of the diabetes pandemic suggests that such research is warranted.

Methods in observational research

Many questions in health and medical research are investigated in observational studies (Glasziou et al. 2004). For example, much of the research into the cause of diseases relies on cohort, case-control, or cross-sectional studies. Such observational studies, that use quantitative and epidemiological methods, also have a role in research into the benefits and harms of medical interventions (Black, 1996). Randomised trials cannot answer every important question about a given intervention. For example, observational field studies are more suitable to detect rare or late adverse effects of treatments; to provide an indication of what is achieved in daily medical practice; and to provide information regarding real-life, health behaviours (Papanikolaou et al. 2006). On the other hand, randomised, laboratory-based studies have better experimental control, and thus more protection against the effect of confounding variables on the outcome(s). As noted by Black (Black, 1996), randomised, controlled trials and non-randomised, observational studies both have strengths and weaknesses; as such, the two approaches should be seen as complementary. After all, experimental methods depend on observational methods to generate clinical uncertainty; generate hypotheses; identify the processes and outcomes that should be measured in a trial; and help to establish the appropriate sample size for a randomised trial (Black, 1996). Every research strategy within a discipline contributes importantly relevant and complementary information to a totality of evidence upon which rational, clinical decision-making and public policy can be reliably based (Hennekens & Buring, 1994).

To strengthen the reporting of observational research, the STROBE Statement was developed ['Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement'; Vandenbroucke et al. 2007]. The STROBE Statement (see Appendix I) provides guidance to researchers about how to report high quality and transparent observational research, which facilitates critical appraisal and interpretation of findings. The Statement is a checklist of items to be addressed in articles reporting on the three main study designs of analytical epidemiology: cohort, case-control, and cross-sectional studies. These three designs represent different approaches of investigating the occurrence of health-related events in a given population and time period. In cohort studies, the investigators follow people over time. They obtain information about people and their exposures at baseline, let time pass, and then assess the occurrence of outcomes. In case-control studies, investigators compare exposures between people with a particular disease outcome (cases) and people without that outcome (controls). Investigators aim to collect cases and controls that are representative of an underlying cohort or a cross-section of a population. That population can be defined geographically, but also more loosely as the catchment area of health care facilities. In cross-sectional studies, investigators assess all individuals in a sample at the same point in time, often to examine the prevalence of exposures, risk factors or disease (Vandenbroucke et al. 2007).

Assessment of caffeine intake

The field of research regarding the effects of habitual caffeine intake is immense and frequently utilises self-report measures of caffeine use (Addicott et al. 2009). Admittedly, the presence of caffeine in many foods, beverages and medications can make the quantification of daily caffeine use difficult, since within every source of caffeine variability exists in the amount of caffeine per volume, the total volume in each serving (e.g. cup size), and the frequency of servings within one day and across multiple days. Moreover, the brewing method of both coffee and tea affects caffeine content (Bracken et al. 2002). Despite these challenges, however, daily caffeine use is routinely estimated with self-report measures of habitual caffeine intake. The validity of this self-report method is demonstrated by positive correlations between participants' subjective reports and objective, salivary caffeine concentrations (Addicott et al. 2009; James et al. 1989). Saliva is an adequate measure of objective caffeine concentrations since the correlation between salivary and serum caffeine concentrations has been reported to be 0.99, and salivary concentrations are approximately 70% of serum concentrations (Biederbick et al. 1997). The advantage of saliva over blood

assessment is that saliva sampling is low-cost and non-invasive (Faber et al. 2005). Moreover, saliva sampling can reliably be done remotely (i.e. away from the laboratory), as caffeine concentrations in saliva remain stable at room temperature for two weeks (Perera et al. 2010). This gives participants time to post their saliva samples back to the laboratory, without the risk of the caffeine content degrading in the meantime. Caffeine concentrations in saliva can then be determined using a simple, high performance liquid chromatography (HPLC) technique (Fuhr & Rost 1994). Equally, saliva sampling using Oragene™ collection devices, allows a home-based, non-invasive method of DNA collection. Importantly, the saliva receptacles can be mailed using standard postal systems; and they provide sufficient quantity and quality of DNA to assess the genetic characteristics of study participants (Rogers et al. 2007).

Assessment of sleep quality, sleep timing, and sleepiness

Questionnaires can also be used to assess characteristics of sleep (Buysse et al. 1989; Roenneberg et al. 2003). For example, 'sleep quality' represents a complex phenomenon that can be difficult to define and measure objectively. It includes quantitative aspects of sleep, such as sleep duration and sleep latency, as well as purely subjective aspects, such as 'restfulness' of sleep (Buysse et al. 1989). The Pittsburgh Sleep Quality Index (PSQI) (Buysse et al. 1989) (see Appendix I) was developed to provide a reliable, valid and standardised measure of sleep quality (the 'global' PSQI score), that could discriminate between 'good' and 'poor' sleepers. The questionnaire contains nineteen self-rated questions that assess a wide range of variables related to quality of sleep, including estimates of sleep duration and sleep latency, frequency and severity of sleep disturbances, use of sleep medications, and perceived daytime dysfunction. Eighteen months of field testing in healthy and patient populations confirmed that the PSQI provides a sensitive and specific measure of poor sleep quality, when compared to clinical data (e.g. sleep-wake diaries, structured interviews) and to objective sleep data (sleep latency, sleep efficiency and sleep duration) (Buysse et al. 1989). Higher scores on the PSQI, which reflects poorer sleep, have been positively correlated with plasma caffeine concentrations (Curless et al. 1993). The Munich Chronotype Questionnaire (MCTQ) (Roenneberg et al. 2003; Roenneberg et al. 2012) (See Appendix I) measures sleep and wake behaviours on both work and free days separately. Such data yields information regarding sleep duration and preferred sleep timing ('chronotype'); it also highlights any discrepancy between work and free day sleep schedules ('social jetlag').

Excessive daytime sleepiness is a common symptom of many sleep disorders, including sleep-related breathing disturbances (Bloch et al. 1999). Sleep disordered breathing is a highly prevalent condition that involves frequent, intermittent breathing pauses during sleep. The condition is prevalent in the general population, affecting 17% of US adults. This prevalence is expected to rise given that obesity, a causal factor for sleep disordered breathing, is increasing in adults and children (Young et al. 2005). The standardised evaluation of daytime sleepiness by a questionnaire is important for clinical management of affected patients, as well as for research studies. It has advantages over an unstructured interview since it reduces observer bias and facilitates comparisons between different individuals and populations (Bloch et al. 1999). The Epworth Sleepiness Scale (ESS), developed and validated by Johns (Johns, 1991) (See Appendix I), is a simple, self-administered questionnaire, which provides a measurement of a person's general level of daytime sleepiness. Specifically, subjects rate the chances that they would doze off or fall asleep when in eight different situations commonly encountered in daily life (e.g. watching television). Total scores on the scale significantly distinguish normal subjects from patients in various diagnostic groups, including obstructive sleep apnea, narcolepsy and idiopathic hypersomnia. Moreover, ESS scores have been shown to be significantly correlated with sleep latency, as measured by the multiple sleep latency test and during overnight polysomnography; and importantly, ESS scores of participants who simply snore, do not differ from those of healthy control participants (Johns, 1991). The ESS is sensitive to change in clinical status, as evidenced by improvements following treatment of sleep apnea with continuous positive air-way pressure (CPAP) (Johns, 1993). Finally, persons suffering from insomnia report elevated sleepiness, based on ESS scores (Sanford et al. 2006).

The present thesis

The primary aim of the present thesis was to use observational research methods to investigate caffeine intake and sleep quality in type 2 diabetes patients. In Chapter 2, the compound caffeine and the state of sleep are discussed in more detail; with particular emphasis on how sleep restriction and circadian preference influence cognitive performance; and moreover, the ability of caffeine to ameliorate some of the cognitive deficits seen when sleep is disrupted. In Chapter 3, in accordance with STROBE guidelines, a case-control approach is used to assess the relationships between caffeine consumption, sleep quality and sleep timing, and daytime sleepiness in type 2 diabetes patients. CYP1A2 enzyme activity, a measure of speed of caffeine metabolism, is also investigated (Chapter 4). Finally, in Chapter 5, the key findings of the present thesis are set in context with related topics, and are discussed in relation to clinical and cognitive aspects of type 2 diabetes.

Chapter 2:

Adenosine, caffeine, and performance: from cognitive neuroscience of sleep to sleep pharmacogenetics

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Abstract

An intricate interplay between circadian and sleep-wake homeostatic processes regulate cognitive performance on specific tasks, and individual differences in circadian preference and sleep pressure may contribute to individual differences in distinct neurocognitive functions. Attentional performance appears to be particularly sensitive to time of day modulations and the effects of sleep deprivation. Consistent with the notion that the neuromodulator, adenosine, plays an important role in regulating sleep pressure, pharmacologic and genetic data in animals and humans demonstrate that differences in adenosinergic tone affect sleepiness, arousal and vigilant attention in rested and sleep-deprived states. Caffeine - the most often consumed stimulant in the world - blocks adenosine receptors and normally attenuates the consequences of sleep deprivation on arousal, vigilance and attention. Nevertheless, caffeine cannot substitute for sleep, and is virtually ineffective in mitigating the impact of severe sleep loss on higher order cognitive functions. Thus, the available evidence suggests that adenosinergic mechanisms, in particular adenosine A_{2A} receptor-mediated signal transduction, contribute to waking-induced impairments of attentional processes, whereas additional mechanisms must be involved in higher-order cognitive consequences of sleep deprivation. Future investigations should further clarify the exact types of cognitive processes affected by inappropriate sleep. This research will aid in the quest to better understand the role of different brain systems (e.g., adenosine and adenosine receptors) in regulating subjective state and distinct cognitive functions. Furthermore, it will provide more detail on the underlying mechanisms of the detrimental effects of extended wakefulness, as well as lead to the development of effective, evidence-based countermeasures against the health consequences of circadian misalignment and chronic sleep restriction.

Circadian and homeostatic influences permit consolidated periods of wakefulness and sleep

Wakefulness and sleep take place periodically, at specific times, during the 24-hour light-dark cycle. These two distinct states result from the interplay between circadian and homeostatic oscillators, a concept originally described by the two-process model of sleep-wake regulation (Borbély 1982). The circadian process reflects an endogenous, 24-hour variation in the propensity for sleep and wakefulness (Borbély 1982). This latter process is controlled by the suprachiasmatic nuclei (SCN) of the anterior hypothalamus, an anatomical structure considered to be the circadian master clock in mammals. Human research, conducted under a range of experimental conditions (e.g., internal desynchronization of the sleep-wake cycle, forced desynchrony paradigms, fragmented sleep-wake cycles, sleep deprivation and sleep displacement), has highlighted the existence of a robust drive to maintain wakefulness towards the end of the habitual waking day (Lavie 2001). Thus the circadian master clock promotes wakefulness in the early evening, just before habitual time for sleep. The positioning of this 'wake maintenance zone' (Strogatz et al. 1987), at the end of the waking day, may seem paradoxical. However, it is thought that this high circadian-based tendency for wakefulness is what prevents humans from falling asleep during the early evening, when homeostatic sleep pressure reaches its highest level. The homeostatic process represents an hourglass mechanism, which gradually builds up with increasing time awake, and roughly exponentially declines during sleep. Thus circadian and homeostatic systems work in opposition to ensure a consolidated period of wakefulness (Cajochen et al. 2010). This antagonism between the two processes also occurs as the biological night progresses and allows the maintenance of a consolidated sleep episode (Dijk & Czeisler 1995; Dijk & Czeisler 1994). The SCN may promote a circadian increase in sleep tendency, which counteracts the decrease in homeostatic sleep propensity as the individual accumulates sleep.

The endogenous circadian clock modulates cognitive performance

The states of subjective sleepiness and alertness, as well as distinct neurobehavioral functions (e.g. cognitive performance on specific tasks), are also influenced by this interplay between circadian and homeostatic processes (reviewed by Cajochen et al. 2004). Indeed, from a cognitive perspective, the two-process model of sleep-wake regulation implies that neurobehavioral efficiency may change over the day due to the influence of circadian timing on alertness and task performance, due to increasing homeostatic sleep pressure, or due to a combination of both these factors (Carrier & Monk 2000). For example, research

incorporating a 40-hour constant routine protocol revealed a clear circadian modulation of subjective sleepiness (Karolinska Sleepiness Scale [KSS]) (Gillberg et al. 1994) and cognitive performance (psychomotor vigilance task [PVT]) (Dinges & Powell 1985), even in the absence of strong homeostatic sleep pressure (Cajochen et al. 2001; Graw et al. 2004). This protocol permits the manipulation of homeostatic sleep pressure by either sleep depriving (high sleep pressure) or sleep satiating (low sleep pressure) study participants by the allowance of regular nap opportunities throughout the circadian cycle. This circadian modulation of subjective state and neurobehavioral performance is organized in a temporal manner which prompts maximal performance throughout the waking hours, including the wake-maintenance zone. Yet, if testing continues into the biological night (e.g., under sleep deprivation conditions), there is a significant decline in performance, which coincides with the decline of the circadian arousal signal. Importantly, however, performance deterioration moves in line with the circadian cycle, such that an improvement can be observed in the biological morning, once the circadian drive for wakefulness takes center stage once again (Cajochen et al. 2004).

Individual differences in circadian preference modulate human neurobehavioral performance

Forced desynchrony paradigms can be used to separate the influence of the circadian pacemaker from the influence of homeostatic sleep pressure. Here, subjects are isolated from the usual 'zeitgebers' (i.e., time givers such as light) and for weeks are exposed to an artificial sleep/wake schedule with a 'day' duration that is significantly shorter (e.g., 19 hours) or longer (e.g., 28 hours) than the normal 24-hour day. With time, the protocol forces a progressive desynchronization of the artificial sleep-wake cycle from the endogenous circadian cycle. Such research indicates that the extent to which circadian rhythm modulates performance is largely dependent on the extent of homeostatic sleep pressure. Specifically, rising sleep pressure attenuates circadian arousal during the subjective evening hours (Dijk & Archer 2009). As a result, even small changes in the relationship between the two processes may have an important effect on an individual's ability to maintain a consistent cognitive performance during the normal waking day (Cajochen et al. 2010). In fact, as reviewed by Schmidt and colleagues (2007), large differences in circadian parameters can be observed in the temporal disposition of an individual, and this gives rise to differential modulations in cognitive performance across the normal waking day. Prominent inter-individual variation in circadian preference significantly affects the temporal organization of a wealth of human behaviors. Morningness-eveningness is the most substantial source of this variation (Roenneberg et al. 2003), and is expressed by favorite periods for diurnal activities, such as working hours, and specific sleep habits (Taillard et al. 2003). Such behaviors in turn reflect the particular chronotype of the individual. The morningness-eveningness

chronotype can be assessed using self-report questionnaires, such as the Morningness-Eveningness Questionnaire (MEQ) (Horne & Östberg 1976) and the Munich Chronotype Questionnaire (MCTQ) (Roenneberg et al. 2003). At one end of the scale exist extreme morning types, who show a strong preference for waking up early in the morning and find it difficult to remain awake beyond their usual bedtime. At the opposite end of the scale, extreme evening-types prefer to go to bed late at night, and experience great difficulty in getting up in the morning (Schmidt et al. 2007). It has been suggested that these extreme chronotypes are 'phase shifted' according to their circadian rhythmicity. That is, their peaks and troughs of physiological circadian markers (core body temperature [CBT], melatonin) occur either earlier (phase advance, morning types) or later (phase delay, evening types) in relation to the external clock time, as compared to 'neutral' individuals who show no strong preference for morningness or eveningness (Duffy et al. 2001). Importantly, as well as differences in physiological characteristics, the diurnal profile of some neurobehavioral variables is also influenced by chronotype. Accordingly, alertness and performance may peak at different clock times, depending on the chronotype of the individual. For example, some people may be consistently at their best in the morning, while others are more alert and perform better in the evening (Schmidt et al. 2007).

To sum up, subjective sleepiness and alertness, and neurobehavioral performance, are contingent upon the synchronicity between the individual's peak periods of circadian arousal and the time of day at which testing takes place, as well as an individual's chronotype (Schmidt et al. 2007). Accordingly, it could be intuitively assumed that individuals who feel subjectively sleepier and less alert, are more likely to be cognitively impaired (Leproult et al. 2003). However, there is accumulating evidence to contradict this proposal. For example, sleep deprivation protocols have revealed that subjective sleepiness and objective alertness are not always linked to measures of neurobehavioral performance (Leproult et al. 2003). In fact, subjective measures of alertness and performance can differ to a great extent (Van Dongen et al. 2003). Such findings raise the question as to whether different cognitive domains are differentially affected by circadian rhythms, reflected by testing subjects at different times of the day.

Circadian influences differently affect distinct cognitive performance tasks

Cognitive functioning domains range from simple attention to logical reasoning, working memory, long-term memory, and more complex executive functions. A simplified overview and classification of the main cognitive processes (attention, memory and executive functions) can be seen in Figure 1 (adapted from Schmidt et al. 2007).

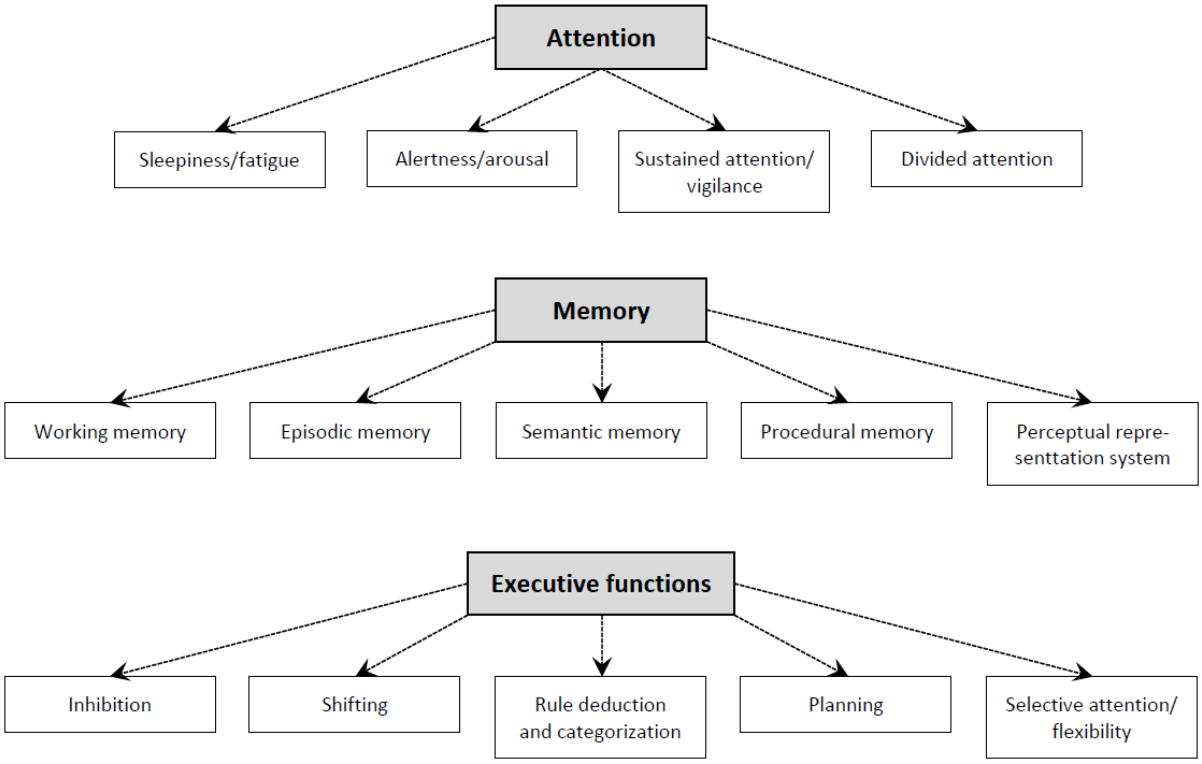

Figure 1: Overview and simplified global classification of the main cognitive processes. (See Schmidt et al. 2007 for more detailed explanations).

Most studies in the circadian domain have focused on the impact of time of day on vigilance and basic attentional parameters (Schmidt et al. 2007). Historically, research revealed a temporal relationship between circadian variations in cognitive performance measures and daily fluctuations in physiological variables such as CBT. That is, when CBT is high and endogenous melatonin is low, alertness and neurobehavioral performance tend to be higher (Kleitman et al. 1938). It was suggested that the circadian-related increase in body temperature would indirectly speed up cognitive processing by increasing metabolic activity in the brain (Kleitman et al. 1938). However, further research highlighted the role of other, external factors, on time-of-day effects in cognition. More specifically, peak and troughs in performance can be attributed to the type and difficulty of the task (e.g., differential working-memory load) (Folkard et al. 1983). While performance speed on simple repetitive and serial search tasks peaks with temperature levels in the evening (Colquhoun 1981; Monk 1982), speed performance on more complex cognitive tasks (e.g., logical reasoning tasks) peaks in the late morning (Folkard 1975), and performance in short-term memory retention peaks in the early to mid-morning (Laird 1925). Thus, Bonnet proposed that the optimal time of day for completing a cognitive test is largely dependent on the specific parameters of the task,

such as its cognitive domain, duration and difficulty, the administration method, and the measured variable (Bonnet 2000). Alternative data, however, revealed that the selected paradigm (e.g., normal sleep/wake conditions *vs.* 40-hours of enforced wakefulness during constant routine) also influences temporal performance (Cajochen et al. 1999). Moreover, compensatory mechanisms, such as motivational factors and expectancy due to experience, also play a role in the outcome (Schmidt et al. 2007).

To date, the picture that emerges is that time-of-day modulations affect performance on a range of cognitive tasks, and these performance fluctuations are additionally contingent upon inter-individual differences in circadian preference (i.e., chronotype). It seems that only highly practiced responses (e.g., constant performance tasks) (Valdez et al. 2005) are rather invariant across the day, with all other responses being vulnerable to the time-of-day effect during normal day-night conditions, as they require a certain degree of control over stimuli and responses. Above attentional processes, higher order cognitive functions, such as working-memory load or executive control, appear to be particularly sensitive to time-of-day modulations (Mikulincer et al. 1989). However, given the current lack of research in this domain, and the varying choices of protocol and experimental control, it is impossible to conclude whether different tasks, involving a range of cognitive processes or differing in difficulty, exhibit genuine differences in time-of-day modulations (Schmidt et al. 2007).

Sleep pressure modulates cognitive performance

Sleep deprivation affects attentional processes

According to the 'wake state instability' hypothesis (Doran et al. 2001), neurobehavioral performance becomes increasingly variable under the influence of elevated sleep pressure due to inadvertent microsleep episodes, with brief moments of low arousal that make it difficult to sustain attention. This unstable state, which fluctuates from second to second, is characterized by increased lapses of attention, errors in response, and increased compensatory efforts resulting in normal reaction times for a short period of time. Over the last two decades, the instrument that has emerged as the dominant assay of vigilant attention in paradigms of sleep loss is the PVT (Dinges et al. 1985). This task has been widely used in human studies to detect the sustained attention (or 'vigilance') deficits associated with different types of sleep loss, including chronic sleep restriction (Belenky et al. 2003; Van Dongen et al. 2003) and sleep deprivation (Doran et al. 2001; Rétey et al. 2006). Importantly, the task is highly sensitive to sleep loss, independent of aptitude, lacks learning effects, and its reliability and validity have been amply demonstrated (Lim & Dinges 2008). The PVT is a test of simple reaction time to a cue that occurs at random inter-stimulus intervals. During the task (standard duration of 10 minutes) subjects are instructed

to attend to a small, rectangular area on a dark screen. They are then required to respond as quickly as possible whenever they perceive the appearance of a bright millisecond counter inside this rectangular area. Stopping the counter allows subjects to view their reaction time, which serves as feedback for that particular trial. Button presses when the counter is not displayed on the screen are counted as false starts, which subjects are instructed to avoid. Four dominant findings have emerged from the use of the PVT in sleep research protocols. First, sleep deprivation results in an overall slowing of responses. Second, sleep deprivation increases the propensity of individuals to lapse for lengthy periods (> 500 ms), as well as make false starts. Third, sleep deprivation enhances the time-on-task effect, the phenomenon whereby performance worsens across the course of a cognitive task owing to fatigue and reduced motivation. Finally, PVT results during extended periods of wakefulness reveal the presence of interacting circadian and homeostatic sleep-regulatory processes (Lim & Dinges 2008).

Sleep deprivation affects higher-order cognitive processes

Sleep deprivation has been shown to have significant adverse effects on a range of higher-order cognitive processes, including memory encoding, consolidation, and retrieval (Walker 2008), behavioral inhibition (Drummond et al. 2006; Harrison et al. 2007), judgement (Killgore et al. 2007a), planning (Horne 1988; Killgore et al. 2009), and divergent thinking capacities (Horne 1988). All such processes are believed to draw heavily upon resources in the prefrontal cortex (Killgore et al. 2011). Moreover, recent research from rodent experiments has highlighted that sleep deprivation is associated with reduced neural activity within brain regions involved in memory (frontal cortex and hippocampus), emotion (amygdala), and regulation of the sleep-wake cycle (anterior hypothalamus and supraoptic nucleus) (Pierard et al. 2007). Disruption of any of these distinct facets of cognition by sleep deprivation may contribute to noteworthy errors in decision-making (reviewed by (Killgore 2010)). Interestingly, however, deficits in executive functions have not been observed universally, particularly during shorter durations of sleep deprivation, such as one night (Pace-Schott et al. 2009). This suggests that the brain's executive function systems may temporarily compensate for brief sleep loss by utilising additional cognitive resources via activation of alternative brain regions (Drummond et al. 2000; Drummond et al. 2005b).

More recently, research has focused on clarifying the ways in which sleep deprivation may influence well-characterized, higher cognitive processes, such as mental heuristics and emotional biases that affect risk assessment and decision-making (Killgore et al. 2012). For example, McKenna and colleagues (2007) revealed that when the possible outcomes from a gambling task were framed in terms of potential gains, sleep deprivation prompted subjects to take more risks compared to when they were well rested. Yet, when the same task was

presented in terms of potential losses, lack of sleep led them to take fewer risks than usual. Such findings indicate that sleep deprivation may lead to greater reliance upon pre-existing cognitive biases. Moreover, functional neuro-imaging studies have highlighted that sleep-deprived individuals show differences within brain-reward circuitry during risky decision making, and this may bias them toward expectations of gains while reducing their focus on losses (Venkatraman et al. 2007).

Another way that prolonged wakefulness affects decision-making is that it appears to reduce the weight that a person places on new information when making choices (Dickinson & Drummond 2008). This suggests that sleep deprived individuals may tend to rely more upon automatic, as opposed to effortful, forms of cognitive processing (Killgore et al. 2012). Emotional biasing is a form of automatic processing that may influence decision making. Indeed, Damasio (1994) proposed that emotional reactions act as a cognitive streamlining function that quickly and efficiently narrows an individual's choice of options. These emotional 'gut reactions' prime a person to make choices based on how rewarding or unpleasant they found a previous similar experience. In an experimental setting, this emotion-guided decision making can be investigated using the Iowa Gambling Task (IGT) (Bechara et al. 1994). During the computerized program, participants are presented with four decks of cards placed face down. Next, players are required to select 100 cards from these four available packs. On card selection, they are immediately informed as to whether the card they selected results in a monetary gain or a monetary loss. Unbeknownst to the subject, however, two of the decks are 'good' decks and lead to small but consistent net gains; while the other two decks are 'bad' decks, and comprise large short-term gains but consistent long-term losses. With regards the results, healthy individuals usually learn from the trial-by-trial feedback and adjust their playing strategy to avoid the risky bad decks in favor of the modest, but consistently advantageous, good decks (Bechara 2004). However, patients with damage to the ventromedial prefrontal cortex (vmPFC) fail to make this adjustment (Bechara 2004). Such findings are in line with evidence that damage to the vmPFC leads to shortsightedness for the future (Bechara et al. 1994), as well as neuroimaging data that indicates that this brain region plays a key role in the decision making process of the IGT (Li et al. 2010).

Importantly, the vmPFC is also particularly affected by sleep deprivation. For example, regional cerebral blood flow in this region correlates with electroencephalogram (EEG) slow-wave activity (SWA; power density in the 0.75-4.5 Hz range) in non-rapid-eye-movement (NREM) sleep (Dang-Vu et al. 2010), which represents the primary physiological marker of sleep homeostasis (Achermann & Borbély 2011). Moreover, significant correlations between positron emission tomography (PET) correlates of brain activity and EEG SWA in NREM sleep are also found in anterior cingulate cortex, basal forebrain, basal ganglia (striatum),

insula, and precuneus (Dang-Vu et al. 2010). In the vmPFC, metabolic activity is drastically reduced after a single night of sleep loss (Thomas et al. 2000), whereas increased activation of this area is correlated to a subject's degree of responsiveness to rewards during sleep-deprived decision-making (Venkatraman et al. 2011). Accordingly, in a series of studies performed by Killgore and colleagues (2006; 2007b), the IGT was used to assess the effects of sleep deprivation on emotionally-guided decision making. As predicted, well-rested subjects rapidly learned the contingencies of the task, and adapted their responses to favor the advantageous, and less risky, decks of cards as the task progressed. However, following 49 hours (Killgore et al. 2006) and 75 hours (Killgore et al. 2007b) of prolonged wakefulness, the same participants showed a significant decline in decision making performance. Specifically, they became progressively more risk-taking and short-sighted in decision making, tending to prefer risky short-term gains at the expense of incurring long-term losses. Overall, such findings indicate that prolonged sleep loss is associated with making choices that begin to favor short-term over long-term outcomes – a pattern paralleling that often observed among patients with lesions to the vmPFC (Bechara 2004). Since the vmPFC is important in several key cognitive-affective processes (Damasio 1994), alterations in vmPFC functioning, or its associated neuro-circuitry following sleep loss, may indeed underlie some of the subtle changes in decision-making observed in the current two studies (Killgore et al. 2012). The findings are also in accordance with evidence suggesting that sleep deprivation leads to difficulty incorporating new information into ongoing decision making processes, implying an overall decline in cognitive flexibility, in favor of greater reliance on automatic cognitive processes (Dickinson & Drummond 2008).

Taken together, the available findings suggest that distinct higher order cognitive processes are impaired by sleep deprivation. The pronounced sensitivity of the prefrontal cortex to the effects of sleep deprivation may also be reflected in distinct regional changes of EEG activity after sleep deprivation. More specifically, not only the increase in SWA in NREM sleep, but also the rise in EEG theta (~5-9 Hz range) activity in wakefulness (Cajochen et al. 1995) is larger over anterior than over posterior cortical areas (Finelli et al. 2000).

Cerebral underpinnings of circadian and homeostatic influences on performance

Accumulating evidence demonstrates that circadian and homeostatic sleep-wake regulatory processes interact in a fine-tuned manner to modulate cognitive performance (Schmidt et al. 2012). Neural connections from the SCN indirectly reach target areas implicated in sleep homeostasis, including ventro-lateral-preoptic area (VLPO), tuberomammillary nucleus

(TMN), lateral hypothalamus (LH), thalamus, and brainstem nuclei via its connections to the dorsal medial hypothalamus (DMH) (Mistlberger 2005). Simultaneously, diffuse monoaminergic activating systems are under circadian control and adjoin with many thalamo-cortical areas, which suggests that the interaction with sleep homeostasis takes place at many different levels (Dijk & Archer 2009).

Research conducted by Aston-Jones and colleagues indicated that the noradrenergic locus coeruleus (LC) plays an important role in the circadian regulation of arousal (2005; 2001). Activity in the LC, combined with its widespread thalamic and cortical connections, may modulate a variety of central nervous system functions, including alertness and vigilance, and also higher order cognitive processes (Cajochen et al. 2010). Moreover, a recent study incorporating behavioral assessments, EEG, and functional magnetic resonance imaging (fMRI) in morning and evening chronotypes indicated that homeostatic sleep pressure exerts an influence on attention-related cerebral activity in key structures crucially involved in generating the circadian wake-promoting signal. More specifically, maintenance of optimal attentional performance in the evening after a normal waking day was associated with higher activity in evening chronotypes than in morning chronotypes in LC and anterior hypothalamus, including the SCN (Schmidt et al. 2009). Furthermore, activity in the anterior hypothalamus decreased with increasing homeostatic sleep pressure, as indexed by EEG SWA in the first NREM sleep episode. These data suggest that circadian and homeostatic interactions contribute to the neural activity that underlies diurnal variations in human behavior. Interestingly, the differential activation pattern was observed only for optimal performance on the PVT (i.e., the fastest 10^{th} percentile of reaction times) (Schmidt et al. 2012), which reflects the phasic ability to recruit the attentional network above normal levels (Drummond et al. 2005a).

The mechanisms by which circadian oscillations in the SCN, as well as circuits controlling for states of wakefulness and sleep, interact at the cerebral level in order to regulate arousal and cognitive behavior, are yet to be clarified (Cajochen et al. 2010). Conceptually, endogenous ‘sleep substances’ may accumulate during wakefulness and modify activity in key areas regulating cortical arousal, including brainstem, hypothalamic nuclei and basal forebrain. During sleep, the ‘sleep substances’ would dissipate. Although the biochemical ‘substrate’ of sleep homeostasis remains poorly understood, it is widely accepted that adenosine, nitric oxide, prostaglandin D_2, tumor necrosis factor alpha, interleukin-1, growth-hormone-releasing hormone, and brain-derived neurotrophic factor may be important mediators of the consequences of prolonged wakefulness (Krueger et al. 2008).

A role for adenosine in homeostatic sleep-wake regulation

Compelling and converging evidence in animals and humans has accumulated over the past two decades to support a role for adenosine and adenosine receptors in sleep-wake regulation (see Krueger et al. 2008; Landolt 2008; Porkka-Heiskanen & Kalinchuk 2011, for reviews). Animal studies suggest that the extracellular adenosine concentration in the brain may increase during prolonged wakefulness, and decline during (recovery) sleep (Porkka-Heiskanen et al. 2000).

Adenosine formation, transport and metabolism

The formation of adenosine in the brain changes in an activity-dependent manner and different mechanisms contribute to the appearance of adenosine in extracellular space. Increased energy demand during wakefulness leads to the break-down of energy-rich adenine nucleosides such as adenosine-tri-phosphate (ATP). Adenosine is formed in neurons by 5'-nucleotidase and transported through plasma and intracellular membranes by specialized transporters, including sodium-driven concentrative (CNT) and equilibrative nucleoside transporters (ENT) (Figure 2). The CNTs use energy to move adenosine into the cell, whereas the ENTs transport adenosine according to the extracellular/intracellular concentration gradient. Elevated intracellular adenosine concentrations following increased utilization of ATP in conditions of high energy demand lead to release of adenosine. In addition, extracellular adenosine is also formed by ecto-nucleotidases through hydrolysis of ATP. Release of ATP from synaptic vesicles occurs along with several other neurotransmitters, including the major excitatory neurotransmitter glutamate (Haydon & Carmignoto 2006). Finally, ATP and glutamate are also released from astrocytes by a recently established process referred to as gliotransmission. Molecular genetic manipulations in mice strongly suggest that glial cells provide a significant source of extracellular adenosine in the brain (Haydon & Carmignoto 2006). Furthermore, astrocyte-derived ATP may activate purinergic (e.g., $P2X_7$) receptors and affect sleep independently from adenosine (Krueger et al., 2008; Krueger et al., 2010). Transgenic mice with a dominant-negative (dn) SNARE domain in astrocytes show reduced gliotransmission (Pascual et al. 2005). In contrast to wild-type littermates, performance in these animals on a novel object recognition task appears to be virtually unimpaired after prolonged wakefulness (Halassa et al. 2009). These data suggest an important role for astrocyte-derived adenosine in modulating cognitive consequences of sleep deprivation.

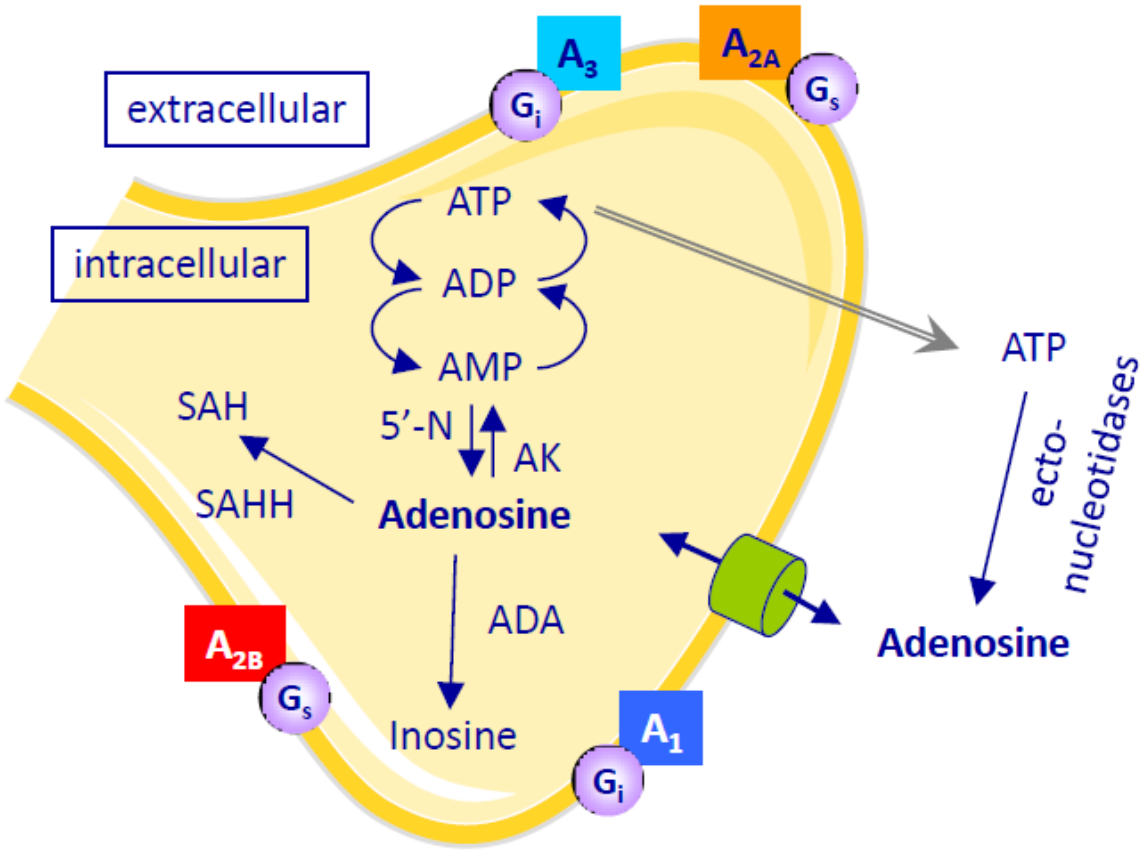

Figure 2: Schematic representation of adenosine formation, metabolism, and transport. Neurons, astrocytes and microglia cells can release adenosine and adenosine-tri-phosphate (ATP; *grey arrow*). All cell types express adenosine receptors, adenosine transporters (*cylinder*) and ecto-nucleotidases that convert ATP into adenosine. A_1, A_{2A}, A_{2B}, A_3 = adenosine receptors coupled to corresponding G-proteins; ADP = adenosine-di-phosphate; AMP = adenosine-mono-phosphate; SAH = S-adenosyl-homocysteine; 5'-N = 5'-nucleotidase; AK = adenosine kinase; ADA = adenosine deaminase; SAHH = S-adenosyl-homocysteine hydrolase.

Clearance of extracellular adenosine mostly occurs through the non-concentrative nucleoside transporters (Fredholm et al. 2005). The main intracellular metabolic pathways of adenosine are the formation of adenosine-mono-phosphate by adenosine kinase (AK), and the irreversible break-down to inosine by adenosine deaminase (ADA). Ecto-ADA also catalyzes the extracellular deamination of adenosine. Mainly due to the high activity of AK, baseline levels of extracellular adenosine usually remain low. The action of ADA, which appears to be more abundantly expressed in astrocytes than in neurons (Fredholm et al. 2005), may be particularly important when elevated concentrations of adenosine have to be cleared, such as after sleep deprivation. Both, molecular genetic manipulations of AK in mice (Palchykova et al. 2010), as well as genetically reduced ADA enzymatic activity in humans (Rétey et al. 2005), increase

deep slow wave sleep and EEG SWA in NREM sleep. These findings provide strong additional support to the idea that adenosine importantly contributes to the homeostatic control of sleep.

Adenosine affects neuronal systems regulating wakefulness and sleep

Adenosine attenuates the activity of wakefulness/vigilance-promoting neurons in brainstem (e.g., LC), basal forebrain (BF), and hypothalamus (e.g., TMN) and may contribute to cortical disfacilitation, a form of inhibition due to reduced activating input from ascending cholinergic and monoaminergic pathways. As suggested by intracellular recordings in non-anaesthetized cats the long-lasting hyperpolarizing potentials in NREM sleep, which provide the cellular substrate of EEG SWA, may represent periods of disfacilition (Steriade et al. 2001; Timofeev et al. 2001). Moreover, adenosine activates hypothalamic VLPO neurons by reducing inhibitory γ-amino-butyric-acid (GABA)-ergic inputs. These neurons fire significantly faster after sleep deprivation than they do during normal sleep, indicating that their activity is modulated by homeostatic mechanisms representing sleep need (Sherin et al. 1996).

One current hypothesis based upon biochemical, pharmacological, electrophysiological, and behavioral studies postulates that elevated adenosine in the BF plays a primary role in mediating the sleep deprivation-induced increase in sleepiness and homeostatic sleep drive (Basheer et al. 2004; Porkka-Heiskanen & Kalinchuk 2011; Strecker et al. 2000). It may be important to note, however, that Blanco-Centurion and colleagues highlighted that the actions of adenosine are not restricted to the BF region (2006). This research team used a lesion and pharmacological approach to reveal that adenosine accumulation in the BF is not necessary for sleep induction, and also that BF cholinergic neurons are not essential for sleep drive. Thus, the available data rather suggest that extracellular adenosine provides a global feedback signal on a neuronal network, including subcortical and cortical structures (Franks 2008), that regulates important functional aspects of wakefulness and sleep.

Adenosine A_1 and A_{2A} receptors mediate effects of adenosine in sleep-wake regulation

The cellular effects of adenosine are mediated via four subtypes of G-protein coupled adenosine receptors: A_1, A_{2A}, A_{2B}, and A_3 receptors. *In vitro* studies indicate that physiological concentrations of endogenous adenosine can activate A_1, A_{2A}, as well as A_3 receptors. Nevertheless, it is widely accepted that the high-affinity A_1 and A_{2A} receptors are primarily involved in mediating the effects of adenosine on sleep and vigilance, at least in humans (Sebastiao & Ribeiro 2009).

Adenosine A_1 receptors and the effects of prolonged wakefulness

The stimulation of A_1 receptors opens several types of K^+-channels, inhibits adenylate cyclase through activation of G_i proteins and inactivates transient voltage-dependent Ca^{2+}-channels. The A_1 receptor is ubiquitously, but not homogenously, expressed in the central nervous system (Bauer & Ishiwata 2009). *In vivo* PET with the selective A_1 receptor antagonist, ^{18}F-CPFPX, revealed highest receptor occupancy in striatum and thalamus, as well as temporo-parietal and occipital cortex (Figure 3A). Pre- and post-synaptic activation of A_1 receptors inhibits excitatory neurotransmission. This receptor subtype, therefore, has long been assumed to play an important role in sleep-wake regulation. Pharmacologic and genetic studies in rats and mice, as well as molecular imaging in humans, partly support this notion. For example, inducible knock-out of neuronal A_1 receptors in mice reduces SWA (3.0-4.5 Hz range) in NREM sleep under baseline conditions, and attenuates the homeostatically regulated rise in SWA after sleep restriction (Bjorness et al. 2009). Moreover, prolonged wakefulness appears to up-regulate A_1 receptor binding in subcortical and cortical brain structures in animals and humans (Elmenhorst et al. 2009; Elmenhorst et al. 2007). Taken together, these data indicate a role for adenosine A_1 receptors in mediating distinct consequences of sleep deprivation.

Adenosine A_{2A} receptors and the effects of prolonged wakefulness

The stimulation of A_{2A} receptors increases adenylate cyclase activity through activation of G_s (or G_{olf} in striatum) proteins, induces the formation of inositol phosphates, and activates protein kinase A. Compared to the A_1 receptor, this adenosine receptor subtype is less widely distributed in the brain (Bauer & Ishiwata 2009). The highest expression in the human central nervous system is found in basal ganglia (particularly in putamen and caudate nucleus) (Figure 3B). Recent studies in rodents, including experiments in knock-out mice, suggest that also A_{2A} receptors contribute to the effects of adenosine on sleep. Local administration of the selective A_{2A} receptor agonist, CGS21680, to the subarachnoid space adjacent to BF and lateral preoptic area increases *c-fos* expression in the VLPO area and promotes NREM sleep (Scammell et al. 2001). Direct activation of sleep-promoting VLPO neurons upon stimulation of A_{2A} receptors could underlie this effect (Gallopin et al. 2005). Interestingly, preliminary data suggested that mice with A_{2A} receptor loss-of-function have reduced sleep and an attenuated sleep rebound after sleep deprivation (Hayaishi et al. 2004), indicating that A_{2A} receptors are part of the neural network that regulates sleep homeostasis in mammals. These findings are supported by recent data in humans, suggesting that genetic variants of the A_{2A} receptor gene (*ADORA2A*) modulate the sleep deprivation-induced increase in EEG SWA in NREM sleep (Bodenmann et al. 2012; Landolt 2012). In conclusion, both adenosine A_1 and A_{2A} receptor subtypes probably mediate functional effects of adenosine after sleep deprivation, whereas distinct effects may be site- and receptor-dependent.

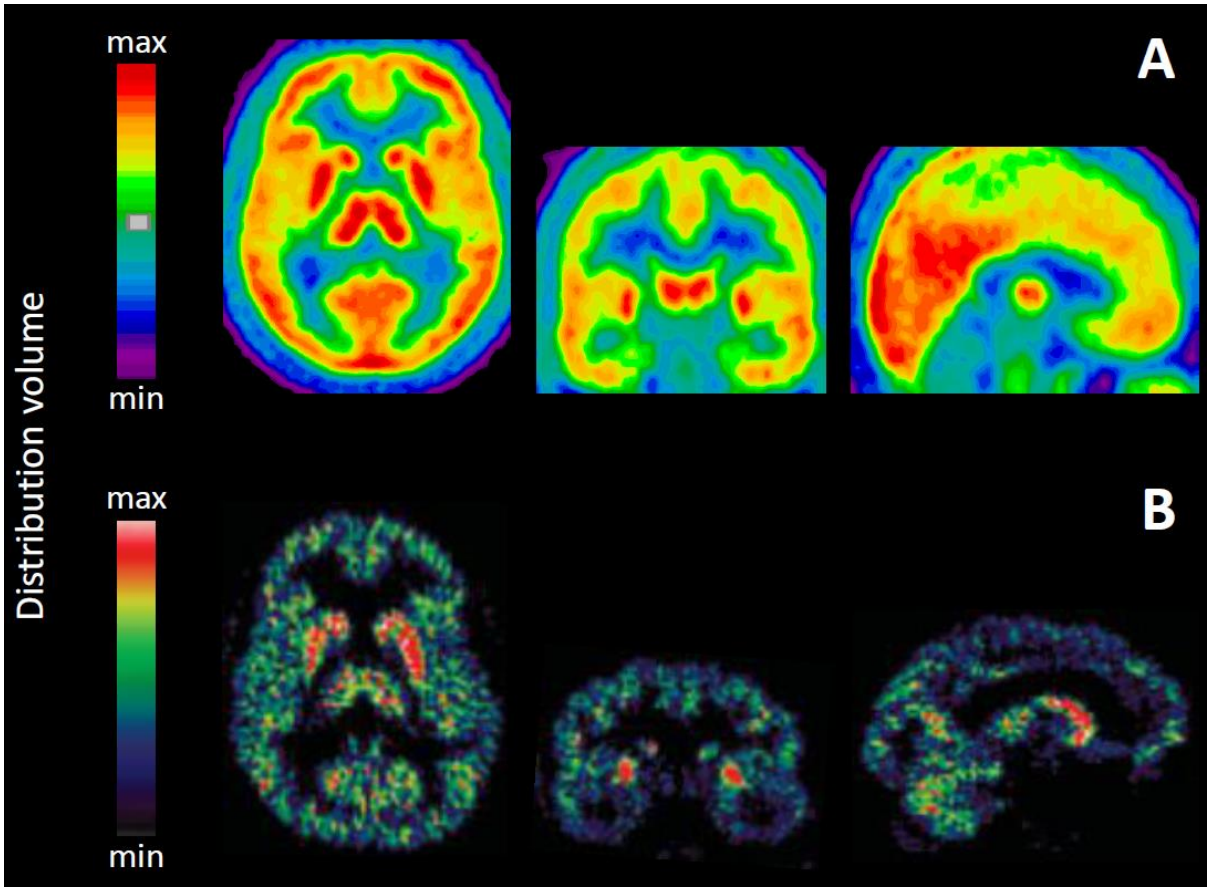

Figure 3: Distribution of adenosine A_1 and A_{2A} receptors in the human brain. (A) Color-coded distribution volumes of the selective A_1 receptor antagonist, ^{18}C-CPFPX (mean values of 10 healthy young men). From *left* to *right*: axial, coronal, and sagittal planes (coordinates according to the Montreal Neurological Institute brain atlas: z = -4, y = -12, x = 0). (Unpublished data). (B) Color-coded distribution volumes of the selective A_{2A} receptor antagonist, ^{11}C-KW6002 (istradefylline), in a healthy male volunteer. From *left* to *right*: axial, coronal, and sagittal sections. (Figure modified from Brooks et al. 2008).

Adenosine and sleep-associated cognitive functions

Sleep deprivation, adenosine, and vigilant attention

While a wealth of evidence supports the concept that modulation of cerebral adenosine contributes to the regulation of wakefulness and sleep, it was not until more recently that research revealed how this manipulation could also alter neurobehavioral performance (Christie et al. 2008). This is poignant given that the BF in particular has been implicated not only in adenosinergic mechanisms of sleep regulation but also in the control of sustained attention (Baxter & Chiba 1999). Thus, the fact that decrements in sustained attention tend

to occur concomitantly with feelings of sleepiness, is consistent with studies indicating that the same mechanisms implicated in the control of the homeostatic sleep drive, are also involved in the regulation of attention (Zaborszky et al. 1997). Moreover, neurons within the BF project to components of the cortical sustained attention network, whose activation is linked with optimal human performance on the PVT (Drummond et al. 2005a). More recently, a rat version of the PVT (rPVT) was developed that enabled invasive investigations of the role of adenosine and the BF in the control of behavioral state and sustained attention (Christie et al. 2008). Christie and colleagues (2008) utilized this task to assess the effects of elevated cerebral adenosine on vigilant performance. The study revealed that rats receiving infusions of adenosine in the BF immediately prior to performing the rPVT showed prolonged response latencies and more performance lapses. The effect was blocked by the co-administration of the A_1 receptor antagonist, 8-cyclopentyl-theophylline, demonstrating that the performance decrements were indeed due to elevated adenosine in the BF, as opposed to other, unrelated factors (Christie et al. 2008). Furthermore, the adenosine-induced impairments in sustained attention were similar to those seen in rats undergoing sleep deprivation (Cordova et al. 2006). These findings are consistent with the hypothesis that sleep loss induces an accumulation of adenosine in the BF, which leads to increased sleepiness and reduced vigilance.

Local cerebral administration of adenosine is not possible in humans. Nevertheless, a *G-to-A* single nucleotide polymorphism (SNP) at nucleotide 22 of the gene encoding ADA underlies an Asp-to-Asn amino-acid substitution at codon 8 of ADA protein (SNP-ID: rs73598374). Compared to G/G homozygotes, carriers of the variant allele show reduced ADA activity *in vitro* (Battistuzzi et al. 1981; Riksen et al. 2008), and presumably elevated tissue adenosine levels *in vivo* (Hirschhorn et al. 1994). This functional SNP not only modulates the duration and intensity of slow wave sleep (Bachmann et al. 2012; Mazzotti et al. 2012; Rétey et al. 2005), but also human attentional performance in rested and sleep deprived states. More specifically, carriers of the G/A genotype (n = 29) performed worse on the d2 focused attention task than G/G homozygotes (n = 191) (Bachmann et al. 2012). The difference was also present between two prospectively matched subgroups of G/A (n = 11) and G/G (n = 11) genotypes. Moreover, sustained attention (Figure 4A) and vigor were reduced, whereas waking EEG alpha activity (8.5-12 Hz), sleepiness, fatigue, and *a*-amylase activity in saliva were enhanced in A-allele carriers when compared to G/G homozygotes. These convergent data demonstrate that genetic reduction of ADA activity in healthy humans not only modulates the quality of sleep, but also the quality of wakefulness, including neurobehavioral performance.

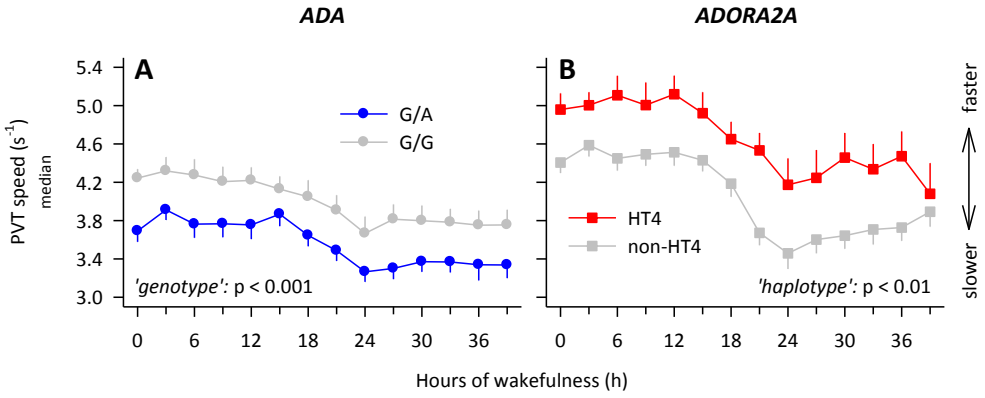

Figure 4: Functional variants of genes contributing to adenosine metabolism (adenosine deaminase, *ADA*) and signal transmission (adenosine A_{2A} receptor, *ADORA2A*) contribute to inter-individual differences in psychomotor vigilance during prolonged wakefulness. Starting 30 minutes after wake-up from the baseline night, a 10-min psychomotor vigilance task (PVT) was administered at 3-hour intervals during 40 hours prolonged wakefulness. Ticks on the x-axis are rounded to the nearest hour. The time courses of median speed (1/reaction times) are illustrated; error bars indicate + or - 1 SEM. Data were analyzed with 2-way ANOVA models with the factors *'genotype'* (G/A, G/G) or *'haplotype'* (HT4, non-HT4) and *'session'* (14 assessments during prolonged waking). (**A**) *Blue circles*: G/A genotype (n = 11). *Grey circles*: G/G genotype (n = 11). The G/A genotype of *ADA* performs worse than the G/G genotype throughout prolonged waking (*'genotype'*: $F_{1,25} = 15.4$, $p < 0.001$; *'session'*: $F_{13,239} = 38.6$, $p < 0.001$; *'genotype'* x *'session'* interaction: $F_{13,146} = 0.3$, $p > 0.9$). (Data were re-plotted from Bachmann et al. 2012). (**B**) *Red squares*: Carriers of HT4 haplotype alleles (n = 6). *Grey squares*: Carriers of HT4 haplotype alleles (n = 17). (See Bodenmann et al. 2012 for details of genetic analyses). Individuals with haplotype HT4 performed faster than non-HT4 allele carriers throughout sleep deprivation (*'haplotype'*: $F_{1,21} = 9.3$, $p = 0.006$; *'session'*: $F_{13,273} = 16.3$, $p < 0.001$; *'haplotype'* x *'session'* interaction: $F_{13,273} = 0.9$, $p > 0.5$). (Data were re-analyzed from Bodenmann & Landolt, 2010).

Sleep deprivation, adenosine, and higher order cognitive functions

As discussed previously, Bjorness and colleagues (2009) revealed that conditional knock-out of the A_1 receptor elicits selective attenuation of the SWA rebound following restricted sleep. The research team also investigated the effects of this genetic manipulation on working memory. It demonstrated that animals lacking the A_1 receptor not only showed a reduced rebound SWA response, but they also failed to maintain normal cognitive function, although this function was normal when sleep was not restricted. Since the attenuation of SWA is associated with compromised working memory performance, this indicates a functional role for adenosine A_1

receptor-dependent SWA homeostasis in maintaining this cognitive ability when sleep is restricted (Bjorness et al. 2009). Here, it is worth noting that while the loss of the A_1 receptors in the conditional gene deletion used in this study are exclusively neuronal (Tsien et al. 1996), the source of the adenosine may include neuronal and non-neuronal cells, or glia cells (Pascual et al. 2005; Studer et al. 2006). Halassa and colleagues (2009) genetically inhibited the release of gliotransmitters in order to investigate if astrocytes play a role in sleep-wake regulation. Using transgenic mice, they found that indeed, restricting gliotransmission attenuated the build-up of sleep pressure, and prevented memory deficits associated with sleep loss. The research team also conducted pharmacological studies, and they concluded that astrocytes modulate the accumulation of sleep pressure and its cognitive consequences through a pathway involving adenosine A_1 receptors (Halassa et al. 2009).

The adenosine receptor antagonist, caffeine, and sleep-loss-associated cognitive impairments

Caffeine is the most widely consumed stimulant in the world. In the μM plasma concentrations reached after moderate consumption, caffeine acts as a non-selective, competitive antagonist at A_1 and A_{2A} receptors (Fredholm et al. 1999). Novel PET imaging findings suggest that intake of 4-5 cups of coffee (corresponding to ~450 mg caffeine) in a 70-kg volunteer can displace endogenous adenosine from 50% of cerebral A_1 receptors (Elmenhorst et al. 2012). By contrast, other effects of caffeine observed in vitro, such as inhibition of phosphodiesterase, blockade of $GABA_A$ receptors, and Ca^{2+} release, require more than 100 times higher doses than adenosine receptor antagonism, and are toxic to humans (Fredholm 1995).

Caffeine counteracts sleep deprivation-induced impaired vigilant attention by interfering with sleep homeostasis

Various studies have examined the effects of caffeine on sustained attention in humans, via performance on the PVT. The psychostimulant has been consistently shown to reverse sleepiness and PVT impairments in sleepy humans (Landolt et al. 2004; Balkin et al. 2004; Kamimori et al. 2005; Rétey et al. 2006; Van Dongen et al. 2001; Wyatt et al. 2004; Landolt et al. 2012). Given that physiological doses of caffeine antagonize adenosine receptors, such findings are consistent with those of the aforementioned rodent study, which revealed decrements in vigilant performance following adenosine administration (Christie et al. 2008).

Wyatt and colleagues (2004) reflected that many studies investigating the neurobehavioral benefits of caffeine during sleep loss confounded the two major processes regulating sleep

and wakefulness: the circadian phase and the duration of prior wakefulness (i.e., homeostatic sleep pressure). Specifically, previous research had not clarified if caffeine's ability to counteract performance deficits related to sleep deprivation was related to its interaction with circadian or homeostatic signals modulating sleep propensity and performance. The authors stress the importance of accounting for variance explained by sleep homeostatic and circadian modulation when interpreting data from protocols in which tests are given in only a single administration, such as typically occurs in traditional clinical and cognitive neuroscience research. As a result, the research team conducted a study to assess the effects of repeated low-dose caffeine administration during a 29-day forced desynchrony paradigm. The period of the sleep-wake cycle was scheduled to be 42.85 hours (28.57-hour wake episodes and 14.28-hour sleep episodes), and thus far removed from the circadian range. This protocol allowed for separate quantification of the circadian, sleep homeostatic, and caffeine contributions to performance deficits and improvements. Moreover, the 42.85-hour cycle simulated the extended wakefulness commonly encountered by medical and military personal, or anyone skipping a night of sleep (Wyatt et al. 2004).

During the study, caffeine was administered during wakefulness at a rate of 0.3 mg per kg per hour. The dosage schedule was designed to increase caffeine blood plasma concentrations in parallel to the rate of increase in sleep homeostatic drive during wakefulness, and also in line with the potential accumulation of adenosine (Porkka-Heiskanen et al. 2000). Polysomnographic recordings were used to monitor each scheduled sleep episode, as well as the majority of each wake episode, in order to detect incidences of slow eye movements and unintentional sleep onsets. During wake periods, mood and subjective sleepiness were assessed at 30-minute intervals using visual analog scales (SCALES) and the Karolinska Sleepiness Scale (KSS). Cognitive performance was tested every 2 hours (Wyatt et al. 2004).

Post completion of the study, comparison of the placebo and caffeine data revealed that rising levels of caffeine significantly reduced wake-dependent deterioration in several measures of cognitive functioning, particularly at the circadian performance nadir (Wyatt et al. 2004). Specifically, caffeine attenuated performance deficits on the PVT such that the caffeine group showed fewer lapses and less impairment in the slowest 10% of reaction times than the placebo group. Caffeine also enhanced the ability of subjects to remain consistently awake for extended periods. That is, the researchers observed inhibition of EEG-verified accidental sleep onsets during scheduled wake episodes. Such findings suggest that individuals receiving caffeine were kept at an earlier, less-severe stage of the sleep-onset continuum (Ogilvie et al. 1988), and this held them back from completing the full transition to sleep. However, the caffeine group also showed impairment of polysomnographically verified sleep during scheduled sleep episodes. Subsequently, the

additional sleep accumulated by the placebo group during scheduled wake and sleep episodes was associated with lower reports of sleepiness, independent of circadian phase or duration of prior scheduled wakefulness. Indeed, subjects receiving caffeine self-reported greater impairment of alertness on the KSS and SCALES measures. A similar paradoxical finding of increased subjective sleepiness in participants receiving caffeine over repeated days has been reported in other studies (Bonnet & Arand 1992). Thus, the wake-promoting effects of caffeine do not replace the restorative effects gained through sleep (Wyatt et al. 2004).

The evidence of a reduction in accidental sleep onsets during caffeine administration supports the concept that caffeine attenuates expression of homeostatic sleep drive. Because the plasma concentrations of caffeine reached in this study can be expected to affect solely adenosine receptors (Fredholm et al. 1999) and because caffeine primarily affects the sleep-wake-dependent modulation of performance, the present findings are in accordance with the proposed role for adenosine in mediating sleep-wake-dependent modulation of sleep propensity and associated variation in neurobehavioral functioning (Wyatt et al. 2004). While further research is required to elucidate whether mechanisms other than adenosine receptor antagonism or a certain degree of tolerance to caffeine over repeated administration could have influenced the experimental outcomes, repeated, low-dose caffeine administration holds potential as a countermeasure to cognitive deficits and unintended sleep attacks, at the cost of increasing subjective sleepiness.

Caffeine ameliorates deficits in vigilant attention from sleep inertia

Another study suggested that caffeine reduces impaired vigilant attention associated with sleep inertia under conditions of sleep loss. Sleep inertia refers to the impaired cognitive performance, grogginess, and tendency to fall back to sleep immediately after waking (Dinges & Orne 1981). Van Dongen & colleagues (2001) administered sustained low-dose caffeine (0.3 mg per kg per hour, except during naps) or placebo to healthy volunteers during the last 66 h of an 88-h period of extended wakefulness, which included seven 2-h naps during which polysomnographical recordings were made. Performance on the PVT was assessed every 2 h of wakefulness, and also during the sleep inertia experienced after awakening from naps. The results revealed that during the placebo condition, testing during sleep inertia was associated with significantly impaired psychomotor vigilance. By contrast, these performance decrements were absent in the caffeine condition. Thus, caffeine was shown to be an effective countermeasure to the impaired sustained attention seen during sleep inertia (Van Dongen et al. 2001).

Many people consume caffeine-containing beverages in the morning, directly after waking, at a time when their homeostatic sleep drive should be reduced. Thus, arguably, there

should be no need to take the stimulant at this time of the day. Nevertheless, it is possible that following rapid awakening from NREM sleep, elevated levels of adenosine, and the corresponding existence of low vigilance and high sleepiness (Virus et al. 1983), could persist until adenosine is removed by reuptake or metabolism, and hence the phenomenon of sleep inertia (Van Dongen et al. 2001). In accordance with this hypothesis, sleep inertia does indeed seem to intensify with prior sleep loss (Dinges et al. 1985), and it is more pronounced when awakening occurs from NREM sleep, rather than from REM sleep (Broughton 1968; Bruck & Pisani 1999). The study of Van Dongen and colleagues (2001) involved less than 4 h of sleep per 24 h. Following the sleep periods, 85 % of awakenings occurred out of NREM sleep in the placebo condition, and subsequent deficits in psychomotor vigilance performance, due to sleep inertia, were consistently recorded. However, during the caffeine condition, sleep inertia after awakening from nap sleep was not apparent. Moreover, when psychomotor vigilance was tested between naps, as opposed to directly afterwards, there was no difference between performance in the two conditions. Such results imply that caffeine's effect was specific to sleep inertia. Overall, such findings are in accordance with the hypothesis that adenosine may be a neurobiological substrate of the sleep inertia phenomenon (Van Dongen et al. 2001).

Caffeine reduces false memories after sleep loss by improving arousal and attention

Human memory is not an exact record of the world and our experiences, but instead is influenced by knowledge representations that already exist in the brain (Bartlett 1932). As a result, what is retrieved from memory can substantially differ from what was originally encoded (Schacter et al. 1998). For example, in some instances, people claim to remember events that in fact never happened. These false memories tend to be semantically linked to actually encoded events, and subjects are usually very confident about the correctness of these memories (Roediger & Mcdermott 1995). Schacter and colleagues (1998) suggest that the development of false memories involves the disruption of the same basic principles of memory formation as the development of correct memories. Memory formation involves three distinct stages: encoding (learning); consolidation (off-line processing and strengthening of memory traces after encoding); and retrieval of the learned material. Research has demonstrated that sleep deprivation may not only impair encoding and consolidation of memory, but also memory retrieval (Harrison & Horne 2000). Impaired memory retrieval associated with reduced source and reality monitoring may be involved in the generation of false memories, and consequently sleep deprivation would be expected to enhance their creation (Diekelmann et al. 2008). In a series of experiments, Diekelmann and colleagues (2008) investigated sleep-associated mechanisms of false memory generation, using the well-established Deese, Roediger, McDermott false memory paradigm (Roediger &

Mcdermott 1995). Here, subjects learned lists of semantically associated words (e.g., ‘‘night,’’ ‘‘dark,’’ ‘‘coal’’). The strongest associate, however, or the ‘‘theme’’ of the list (‘‘black’’ in this example), was not presented during learning. Subsequently, memory retrieval was tested 9, 33, or 44 h after learning. This involved the presentation of the previously viewed ‘‘list’’ words, together with the ‘‘theme’’ word (or ‘‘critical lure’’) and unrelated distracter words. Subjects were required to indicate whether a word had been presented during the learning phase or not. Immediately after learning the words, during the memory consolidation phase, participants either slept or stayed awake. At word retrieval, they were or were not acutely sleep deprived. The study revealed that when participants were sleep deprived during retrieval of stored words, there was a significant increase in the number of false memories of theme words. That is, they reported that they had been presented with a specific word during the learning phase, when in fact they had not. Of particular relevance to the present discussion was the finding that this distortion of memory was removed by administering caffeine to the sleep deprived subjects prior to retrieval testing. Such evidence indicates that adenosinergic mechanisms are involved in the depletion of specific cognitive resources, which elicits the generation of false memories associated with sleep loss (Diekelmann et al. 2008). It is possible that caffeine improved reduced arousal and sustained attention after sleep deprivation, which rely on a prefrontal-parietal network, basal forebrain, and thalamus, and are known to be implicated in memory functions.

Caffeine has weak potency to improve impaired higher-order and executive functions after sleep deprivation

Much research regarding the effects of caffeine on performance during sleep deprivation has focused primarily on measures of simple cognitive processes, as opposed to memory and executive functions. Yet, if a subjective state or cognitive function is impaired by sleep loss, then it may be expected that this decrement would be reversed by caffeine (Wyatt et al. 2004). To test this hypothesis, Wyatt and colleagues (2004) not only studied the effects of caffeine on PVT performance during forced desynchrony, but also assessed short-term memory (Probed Recall Memory Task) and cognitive throughput (Addition Task, Digit Symbol Substitution Task). Indeed, caffeine tended to reduce the wake-dependent impairment of short-term memory and attenuated performance deficits in the two cognitive throughput tasks when compared to placebo. Thus, the potential benefits of caffeine on higher-order cognitive performance warrant further investigation.

Killgore and colleagues (2012) performed an investigation into the potential benefits of stimulants on decision making during sleep deprivation. The protocol required subjects to perform the IGT at four time points throughout a period comprising 61 h of sleep deprivation and 12 h of recovery sleep. After 44 h of wakefulness, participants received a double-blind

administration of caffeine (600 mg), d-amphetamine (20 mg), modafinil (400 mg), or placebo. As predicted, sleep deprivation was found to alter normal decision making, which was consistent with the team's previous research (Killgore et al. 2006, 2007b). Yet, perhaps the most important finding was the fact that although all three stimulants were highly effective at reducing subjective sleepiness and sustaining psychomotor vigilance relative to placebo, none of the pharmacologic agents provided any significant enhancement of decision-making performance on the IGT. In fact, performance was similar to placebo for all stimulant groups (Killgore et al. 2012). It should be noted here that IGT performance was unrelated to self-reported sleepiness or psychomotor vigilance performance during the administration of the stimulants, which implies that the deficits observed in decision making were independent of differences in alertness. That is, despite subjects on stimulants being awake, alert, and able to sustain psychomotor vigilance, they were not any better than placebo on the IGT (Killgore et al. 2012). These findings are consistent with a previous study which evaluated the effect of caffeine on sleep-deprived IGT performance (Killgore et al. 2007b). In that study, repeated doses of caffeine (200 mg every 2 h) during the overnight sessions, up to 3 h before each IGT, had virtually no effect on performance relative to placebo at either 51 or 75 h of sleep deprivation. Similarly, caffeine had no significant effect on the time taken for subjects to make various types of moral judgments after 53 h of prolonged wakefulness (Killgore et al. 2007b).

In fact, other studies have also reported limited effects of various stimulants on higher-order cognition and executive functions during sleep loss. For example, Gottselig and colleagues (2006) revealed that caffeine was effective at restoring simple aspects of cognitive functioning, such as attention. Yet, the stimulant failed to restore a more complex aspect of executive function, random number generation (Brugger et al. 1996), a cognitive process that relies on the prefrontal cortex (Gottselig et al. 2006).

Some evidence indicates that the effectiveness of stimulants, including caffeine, on executive functions may be task-specific, and depend upon the underlying executive function systems targeted by different stimulant (Killgore et al. 2009). For instance, participants' performance on a behavioral measure of risk-taking and impulsive responding (the Balloon Analog Risk Task) was relatively resistant to the effects of sleep loss until about 75 h of continuous wakefulness, at which point there was a clear increase in risky decision making (Killgore et al. 2011). It is noteworthy that caffeine appeared to mitigate this surge in risk-taking at extreme sleep deprivation (Killgore et al. 2011). This finding suggests that the types of executive functions measured by the Iowa Gambling Task, the random number generation task, and the Balloon Analog Risk Task may involve different brain systems that are differentially affected by caffeine, and thus adenosinergic mechanisms.

Inter-individual differences in the effects of caffeine

More recently, there has been increased interest in inter-individual differences in the impairment of neurobehavioral functions from sleep loss and in the effectiveness of common pharmacological countermeasures such as caffeine. The clarification of the mechanisms underlying these differences is relevant because they would reveal insights into the neurophysiological regulation of human wakefulness and sleep. Moreover, they are also of clinical importance because they may highlight individuals at greater risk for impaired neurobehavioral performance and reduced health associated with prolonged wakefulness and shift work (Rajaratnam & Arendt 2001). In humans, sleep loss produces a range of cognitive deficits, including reduction in vigilance, working memory and executive function. Yet, there are large inter-individual differences in these deficits, which account for a substantial portion of the variance. In a study involving repeated exposure to sleep deprivation under controlled laboratory conditions, Van Dongen and colleagues (2004) demonstrated that sleep loss negatively influences measures of subjective sleepiness, fatigue and mood, behavioral alertness (sustained attention), and cognitive processing capability (working memory). While these impairments were stable within individuals, there were significant differences between individuals that were not merely a consequence of variations in the subjects' sleep history. Thus, the authors suggested that these individual differences represented trait-like differential vulnerability to sleep loss.

Adenosinergic mechanisms contribute to inter-individual differences in vigilant attention during prolonged wakefulness

Given the evidence discussed above, Rétey and colleagues (2006) predicted that adenosinergic mechanisms play a role for inter-individual differences in neurobehavioral function during prolonged wakefulness. To test this hypothesis, the research team investigated the combined effects of sleep deprivation and caffeine on PVT speed and EEG activity in individuals that rated themselves as either caffeine-sensitive or caffeine-insensitive. It was previously suggested that subjective differences in the psychostimulant effects of caffeine might reflect genetically determined differences in the adenosinergic system (Alsene et al. 2003; Goldstein et al. 1965). Thus, it was hypothesized that subjects from both ends of the caffeine-sensitivity spectrum would not only react differently to caffeine, but also show different sleep-deprivation induced changes in neurobehavioral function and the EEG (Rétey et al. 2006). The study protocol required the 12 subjectively caffeine-sensitive and 10 caffeine-insensitive subjects to complete two experimental blocks separated by 1 week. Each block consisted of 4 nights and 2 days in the sleep laboratory. After 2 consecutive, 8-h, nocturnal sleep recordings (comprising an ''adaptation'' night and

baseline assessment), the subjects were kept awake for 40 h under constant supervision by members of the research team. During this period of prolonged wakefulness, EEG topography was assessed every 3 h, as well as PVT and random number generation performance. After both 11 and 23 h of sleep deprivation, participants received a capsule with either 200 mg caffeine or placebo, according to a randomized, double-blind, cross-over design. Finally, a 10.5-h recovery night was followed by a final waking EEG, PVT, and random number generation assessment.

Analysis of the results revealed that while there were no differences at baseline in optimal PVT performance (i.e., the fastest 10th percentile of reaction times) between subjectively caffeine-sensitive and caffeine-insensitive men, there were differences in the regional EEG power distribution between these groups in the theta range in waking after a baseline night of sleep. These differences were enhanced by sleep deprivation in the antero-posterior power gradients in the waking EEG, and also induced differences in the PVT (Rétey et al. 2006). Here, prolonged wakefulness impaired PVT speed more in self-rated caffeine-sensitive individuals than in caffeine-insensitive individuals. Such observations are in accordance with functional imaging studies indicating that the vulnerability to sleep deprivation-induced performance decline in working memory is linked with baseline differences in task-related cortical activation (Mu et al. 2005). Taken together, the findings suggest that physiological variables recorded during baseline assessment could be useful future predictors of individual vulnerability to sleep deprivation.

Importantly, caffeine counteracted the sleep-loss-induced PVT differences between the two groups of subjects. Moreover, correlation analyses revealed that those individuals with the largest neurobehavioral impairment from sleep deprivation benefited the most from the stimulant action of caffeine. Interestingly, optimal PVT performance has been shown to activate a cortical-sustained attention network and the motor system including the striatum (Drummond et al. 2005a). This region shows prominent expression of adenosine A_{2A} receptors (Figure 3b) (Bauer & Ishiwata 2009). Furthermore, this adenosine receptor subgroup was shown to be responsible for the wakefulness-promoting effect of caffeine (Huang et al. 2005; Lazarus et al. 2011), and a common c.1976*T* > *C* polymorphism of the A_{2A} receptor gene has been associated with inter-individual differences in EEG theta power during wakefulness and sleep (Rétey et al. 2005). Therefore, Rétey and colleagues suggested that this adenosine receptor subtype plays a role in determining the differences between individuals in their vulnerability to impairments of neurobehavioral performance following sleep loss (Rétey et al. 2006). Indeed, recent preliminary findings indicate that the c.1976*T* > *C* polymorphism of *ADORA2A* impacts neurobehavioral performance during sleep restriction (Rupp et al. 2013).

With regard to the EEG topography data, this study found that the overall effect of sleep loss on the waking EEG was consistent with previous studies (Cajochen et al. 2001). Yet, there were noteworthy differences between the individuals which emerged following analysis of regional power distributions between fronto-central and parieto-occipital EEG derivations. Specifically, both the effects of sleep loss and caffeine on antero-posterior power gradients in the theta range tended to be more prominent in caffeine-sensitive subjects than in caffeine-insensitive participants. These differences mirrored the inter-individual differences in the effects of sleep deprivation and caffeine on sustained vigilant attention. Here it may be important to remember that frontal theta activity reflects the alternative activation of brain regions linked with continuous attention—the prefrontal cortex and anterior cingulate cortex (Asada et al. 1999). Moreover, a combined EEG and fMRI study highlighted a positive correlation between theta activity (5–9.5 Hz range) in waking and the fMRI signal of the right dorso-lateral prefrontal and superior parietal cortices (Foucher et al. 2004). In accordance with the interpretation that these areas are involved in arousal, as well as the maintenance of attention, it has also been reported that optimal PVT speed after sleep deprivation depends on activation of a fronto-parietal sustained attention network and frontal cortical regions (Drummond et al. 2005a). Rétey and colleagues (2006) thus propose that their EEG data support brain imaging studies which show that changes in activation after sleep deprivation in fronto-parietal regions are related to individual differences in attentional impairment from sleep loss, and moreover, that adenosinergic mechanisms may contribute to these differences.

Polymorphisms of ADORA2A modulate the individual response to caffeine after sleep deprivation

More recent researches have demonstrated that in humans, genetic variation of the adenosine A2A receptor gene, ADORA2A, mediates an individuals' susceptibility to panic disorder and individual differences in anxiety-related personality, habitual caffeine consumption, and arousal (Cornelis et al. 2007; Deckert et al. 1998; Hamilton et al. 2004; Hohoff et al. 2010). Furthermore, individual anxiogenic and sleep-disrupting responses to caffeine have been consistently associated with the common *C-to-T* substitution at nucleotide 1976 of *ADORA2A* (Alsene et al. 2003; Childs et al. 2008; Rétey et al. 2007; Rogers et al. 2010). The *T*-allele of this polymorphism predisposes Caucasian individuals to anxiety following caffeine consumption (Alsene et al. 2003; Childs et al. 2008; Rogers et al. 2010), while the *C*-allele seems to relay a tendency toward disturbed sleep following ingestion of the stimulant (Rétey et al. 2007).

In a recent publication, Bodenmann and colleagues (2012) examined the effects of genetic variation of *ADORA2A* and sleep deprivation on subjective sleepiness, PVT, waking and

sleep EEG, and the pharmacogenetic response to the stimulants caffeine and modafinil. The study revealed that the carriers of a distinct *ADORA2A* haplotype (haplotype HT4—these individuals carry a *T*-allele at nucleotide 1976) showed greater vigilance during sleep loss than carriers of non-HT4 haplotype alleles (Figure 4b). Furthermore, caffeine did not counteract the consequences of prolonged wakefulness on psychomotor speed and EEG delta activity in the carriers of haplotype HT4. On the other hand, modafinil, which does not interact with A_{2A} receptors, influenced the effects of prolonged wakefulness irrespective of *ADORA2A* haplotype. It was concluded that genetic variation of *ADORA2A* not only affects psychomotor response speed, but also modulates the effects of caffeine on neurobehavioral and neurophysiological aspects of sleep–wake regulation (Bodenmann et al. 2012).

Conclusions

Consistent findings accumulated over the past few decades which suggest that attentional performance is particularly sensitive to time of day modulations and the effects of sleep loss. Efferent projections from the circadian master clock located in the SCN form connections to the dorso-medial hypothalamus, which sends out afferents to cholinergic and monoaminergic neurons in BF, brainstem, and hypothalamic nuclei involved in promoting behavioral arousal, attention, and cortical activation. The ‘‘sleep substance,’’ adenosine, is released in activity-dependent manner and activates A_1 and A_{2A} receptors located in these and other brain regions, including basal ganglia and cortex. Adenosine induces global cortical disfacilitation by reducing the activating input from the ascending arousal pathways and actively excites sleep-active neurons in the ventro-lateral-preoptic area of the hypothalamus. We, thus, conclude that adenosine contributes to the regulation of brain functions modulated by the sleep–wake cycle, in particular to sleepiness and sustained attention which are heavily affected by sleep loss. Indeed, convergent pharmacologic and genetic data in animals and humans support the notion that differences in adenosinergic tone in the central nervous system affect vigilant attention. The differences appear to be present in rested and sleep-deprived states and do not reflect different accumulation of homeostatic sleep pressure during extended wakefulness or differential vulnerability to the effects of sleep loss.

Further support for a role for adenosine in modulating sleep pressure and associated variation in arousal and attention stems from the effects of the adenosine receptor antagonist, caffeine. Acute and repeated administration of the stimulant attenuate subjective, neurophysiological and neurobehavioral consequences of moderate acute sleep deprivation. It is evident, however, that caffeine cannot substitute for sleep, and commonly consumed doses of the stimulant do not improve higher-order cognitive functions that are compromised

after severe sleep loss. These findings indicate that adenosinergic mechanisms may be particularly important for the initial effects of sleep deprivation and that additional mechanisms contribute to the cognitive consequences of severe sleep deficits.

Caffeine is a non-selective A_1 and A_{2A} receptor antagonist, and these two adenosine receptor subtypes may play different roles in sleep–wake associated brain functions. Recent studies in knock-out animals suggest that the psychostimulant and the arousal effects of the xanthine are mainly mediated by A_{2A} receptors. This conclusion is supported by findings in humans showing that common genetic variation of *ADORA2A* determines individual effects of caffeine on vigilant attention during sleep deprivation. The findings demonstrate a role for A_{2A} receptors in the effects of prolonged wakefulness on vigilant attention.

Perspectives

Further research will aim at elucidating the involvement of adenosine in downstream mechanisms underlying sleep deprivation-induced impairment of cognitive functions and synaptic plasticity. Recent evidence indicates that changes in adenosine during prolonged wakefulness are implicated in plasticity deficits (Dias et al. 2013). Neuronal and glial-derived adenosine may lead to increased sleepiness after sleep loss and signal an increased need for sleep to balance adenosine. Thus, sleep may serve to counteract overstimulation of the brain and excitotoxicity associated with prolonged wakefulness. Adenosine reduces excitatory neurotransmission by stimulating inhibitory A_1 receptors. The A_1 receptor appears to be required for disruption of hippocampal long-term potentiation by a spontaneous slow-frequency EEG pattern, which is typical for deep NREM sleep and could provide a stimulus for plasticity reversal (Dias et al. 2013). Prolonged A_1 receptor activation also induces dynamic changes in the synaptic expression of N-Methyl-Daspartic acid (NMDA) receptors that may reversibly adjust the threshold for plasticity induction (Kopp et al. 2006).

On the other hand, adenosine actively promotes sleep by stimulating excitatory A_{2A} receptors in ventro-lateral preoptic area of the hypothalamus. Activation of A_{2A} receptors by endogenous adenosine is required for hippocampal long-term potentiation by brain-derived neurotrophic factor (BDNF), an established marker of activity-dependent neuronal plasticity (Fontinha et al. 2008). Cortical *Bdnf* in rats is higher after wakefulness than after sleep and increased after sleep deprivation (Conti et al. 2007). Chronic caffeine treatment appears to preserve the levels of BDNF in the sleep-deprived brain (Alhaider et al. 2011). Finally, A_{2A} receptors co-localize with metabotropic glutamate receptors of subtype 5 (mGluR5), which induce BDNF expression and stimulate gliotransmission. The mGluR5 are primarily expressed on post-synaptic neurons and glia cells and contribute importantly to long-term depression (Izumi & Zorumski 2012), but also to long-term potentiation. It was recently found

that sleep loss increases mGluR5 availability in the human brain, and this increase was closely correlated with increased sleepiness after a night without sleep (Hefti et al. 2013).

Whereas many studies investigated the effects of caffeine on the sleep-deprived brain, the possible roles for adenosine, adenosine receptor subtypes, and effects of caffeine in genetically distinct animals and humans on sleep–wake-related neuronal plasticity have only started to be explored. It is suggested that the further development of this avenue of research will permit a better understanding of sleep as a fundamental brain process. This knowledge may then lead to the rational development of more effective treatment and countermeasure strategies, not only of impaired vigilance and attentional processes but also of reduced higher-order cognitive functions, in conditions of sleep deprivation, shift work, and jet-lag, for example. Such strategies are highly important for public health and personal safety.

Chapter 3:

A case-control field study on the relationships among type 2 diabetes, sleepiness and habitual caffeine intake

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Abstract

Objectives: To examine the possible links between type 2 diabetes, daytime sleepiness, sleep quality and caffeine consumption.

Methods: In this case-control field study, comparing type 2 diabetic (n = 134) and non-type 2 diabetic (n = 230) participants, subjects completed detailed and validated questionnaires to assess demographic status, health, daytime sleepiness, sleep quality and timing, diurnal preference, mistimed circadian rhythms and habitual caffeine intake. All participants gave saliva under standardized conditions for *CYP1A2* genotyping and quantification of caffeine concentration. Hierarchical linear regression analyses examined whether type 2 diabetes status was associated with caffeine consumption.

Results: Type 2 diabetic participants reported greater daytime sleepiness ($p = 0.001$), a higher prevalence of sleep apnea ($p = 0.005$) and napping ($p = 0.008$), and greater habitual caffeine intake ($p < 0.001$), derived from the consumption of an extra cup of coffee each day. This finding was confirmed by higher saliva caffeine concentration at bedtime ($p = 0.01$). Multiple regression analyses revealed that type 2 diabetes status was associated with higher self-reported caffeine consumption ($p < 0.02$) and higher salivary caffeine ($p < 0.02$). Next to male sex, type 2 diabetes status was the strongest predictor of caffeine intake. Subjective sleep and circadian estimates were similar between case and control groups.

Conclusions: Type 2 diabetic patients may self-medicate with caffeine to alleviate daytime sleepiness. High caffeine intake could undermine efforts to control hyperglycemia and reflects a lifestyle factor that may be considered when promoting type 2 diabetes management.

Keywords: Vigilance, chronotype, coffee, *CYP1A2* genotype, HPLC, metabolism, sleep apnea.

Introduction

Daytime sleepiness is a typical symptom of type 2 diabetes associated with hyperglycemia (West et al. 2006). Furthermore, daytime sleepiness is a symptom of obstructive sleep apnea, which is prevalent in persons with type 2 diabetes (Foster et al. 2009; West et al. 2006). The disordered breathing of sleep apnea causes repeated hypoxia and sleep fragmentation, and has been linked to increased insulin resistance and glucose intolerance, independent of the confounding effects of obesity (Ip et al. 2002; Punjabi et al. 2002). Elevated sleepiness may also be caused by disrupted sleep quality, sleep restriction, or misaligned circadian rhythms which themselves can impede glucose homeostasis via a decrease in insulin sensitivity (Buxton et al. 2010; Leproult et al. 2014; Tasali et al. 2008).

Caffeine intake reduces elevated sleepiness (Landolt et al. 2004). With this benefit in mind, caffeine is the most widely used psychoactive substance in the world (Fredholm et al. 1999). In the doses typically consumed, however, the stimulant increases the time it takes to fall asleep, shortens sleep duration and reduces sleep depth (Clark & Landolt, 2016; Landolt et al. 1995; Rétey et al. 2007). There may be a bidirectional link between daily caffeine consumption and sleep disturbance, leading to a vicious circle that promotes increased caffeine use: sleep problems lead to daytime sleepiness, and thus higher caffeine intake; yet, equally, caffeine consumption can lead to disturbed sleep and associated sleepiness (Roehrs & Roth, 2008).

There is a controversial discussion about caffeine's influence on glycemic control and type 2 diabetes risk (for review, see Palatini et al. 2015). Laboratory-based studies showed that doses of caffeine equivalent to 3-4 cups of coffee reduced glucose disposal by more than 20 % (Greer et al. 2001; Lane et al. 2004). Conversely, in epidemiology, high coffee consumption (6 cups/day) was related to a 33 % lower risk of type 2 diabetes (Ding et al. 2014). This paradox may reflect coffee's diverse composition, including the constituent compound chlorogenic acid that has beneficial effects on metabolism (Johnston et al. 2003). Susceptibility to the adverse effects of caffeine may also be influenced by speed of caffeine metabolism by enzyme cytochrome P450-1A2 (CYP1A2), which has wide inter-individual variability in activity that is partly regulated by genetic polymorphism c.-163C>A (rs762551) of *CYP1A2* (Han et al. 2001; Sachse et al. 1999). This polymorphism causes inter-individual differences in the inducibility of CYP1A2 enzyme activity, for example by caffeine (Sachse et al. 1999). Slow metabolizers (i.e., C-allele carriers of rs762551) are more exposed to the adverse effects of caffeine, and thus potentially, are more at risk of developing type 2 diabetes following habitual high coffee consumption (Palatini et al. 2015).

The increasing burden of the type 2 diabetes pandemic (International Diabetes Federation, 2013) highlights the need to identify modifiable lifestyle factors that may help to prevent,

treat and manage the progression of the disease. With this in mind, the present field study investigated the possible links between type 2 diabetes, sleep quality, and habitual caffeine consumption; as such relationships remain poorly understood. Based on the evidence presented above, it was hypothesized that type 2 diabetic patients would not only report greater daytime sleepiness, but also shorter sleep duration, poorer sleep quality, greater misalignment of circadian rhythms, and higher caffeine intake than non-type 2 diabetic controls. Moreover, there would be a higher proportion of slow CYP1A2 metabolisers in the type 2 diabetes group than in the controls.

Methods

Study outline

The study used a case-control approach that compared type 2 diabetic and non-type 2 diabetic participants. Subjects completed a series of questionnaires (the 'survey') to assess demographic status; health; sleep and chronotype; and habitual caffeine intake. Participants also gave two samples of saliva at bedtime: one sample was used to genotype participants for single nucleotide polymorphism rs762551 of the gene *CYP1A2*; the second sample was used to quantify the concentration of caffeine in saliva.

Survey data were checked thoroughly. Unrealistic responses were excluded from the analyses and counted as 'missing'; for example, if a subject was required to state a clock time, but instead reported a number greater than twenty four.

Due to genetic variation between populations of different ethnic origin, participants were screened for evidence of non-European descent and subsequently excluded. Subjects reporting type 1 diabetes, as opposed to type 2 diabetes, were also excluded, due to different risk factors associated with the two forms of the disease. To allow reliable assessment of typical sleep estimates across the groups, subjects reporting an extreme sleep schedule were excluded (sleep offset / wake-up time on work days > 12:00 / midday).

Subjects

The study was approved by the review board of the ethics committee at the Swiss Federal Institute of Technology (ETH) Zürich (ethics number: EK 2012-N-53). Experimental protocols were conducted according to the principles of the Declaration of Helsinki. The data were analyzed anonymously and individual results kept confidential. All participants gave written informed consent.

Participants were recruited via advertisements at hospitals, in magazines and at public seminars throughout German-speaking Switzerland. A total of 374 participants were

recruited and their data collected. Ten participants were excluded based on: type 1 diabetes (n=2); non-European descent (n=7); and extreme sleep schedule (n=1). The remaining participants were split into the type 2 diabetic cases group (n = 134) and the non-type 2 diabetic controls group (n = 230). Of these participants, 11 cases and 27 controls chose not to provide saliva, only questionnaire data.

The type 2 diabetes status was determined by self-report. Specifically, an affirmative response to the question: "Over the past 12 months, have you suffered from type 2 diabetes?". This self-diagnosis was considered confirmed if the following criteria were met: *1)* The participant answered the question: "If you suffer from type 2 diabetes, when did you receive your diagnosis?"; *2)* The participant reported a diabetes-appropriate treatment regime of oral medication and/or insulin.

Questionnaire assessment

The self-administered survey contained 6 questionnaires. Participants were instructed to complete the 20-minute survey in its entirety and to the best of their ability either online (2ask® survey software) or in paper form.

The first questionnaire gathered information regarding demographic and sociodemographic status, health and dietary behaviors, and chronic physical and mental health. Questions were based on those of a recent epidemiological study (Stamatakis et al. 2007).

Subjective sleep quality, sleep timing and chronotype assessment

The Pittsburgh Sleep Quality Index (PSQI) (Buysse et al. 1989) assessed subjective sleep quality and habitual sleep timing over the previous month. A higher score on the 0-21 scale reflects poorer subjective sleep quality (scores >5 indicate poor sleep). The Munich Chronotype Questionnaire (MCTQ) (Roenneberg et al. 2003) assessed sleep timing on work days and free days separately, and circadian variables. 'Chronotype' was determined as the midpoint between sleep onset and wake time on free days, corrected for 'sleep debt' accumulated during the work week (Roenneberg et al. 2012). A behavioral indicator of circadian misalignment ('social jetlag') was computed as the absolute difference between the midpoint of sleep on work days and free days (Roenneberg et al. 2012) (for further details and calculations, see supplementary information in Appendix II).

Daytime sleepiness and well-being assessment

The Epworth Sleepiness Scale (ESS) (Johns, 1991) measured participants' general degree of daytime sleepiness. A higher score on the 0-24 scale reflects greater subjective sleepiness (scores > 10 indicate excessive sleepiness). The World Health Organization Well-Being Index (WHO-5) (World Health Organization, 1998) assessed participants' general

well-being and quality of life. A higher score on the 0-100 scale reflects greater well-being (scores < 50 indicate risk of depression).

Habitual caffeine intake assessment

This questionnaire was an extended version of the caffeine intake questionnaire of the sleep laboratory of the University of Zurich (Rétey et al. 2007). Participants were asked to report how frequently (per day or per week) they usually consumed a given range of caffeine-containing foods, drinks, medications and supplements. Supplementary Table 1 (Appendix II) displays the estimated caffeine content (mg/serving) of each item in the questionnaire. These data were used to calculate participants' daily habitual caffeine intake.

Saliva sampling

Participants gave two samples of saliva at home and then posted them back to the laboratory in a pre-paid envelope. Beforehand, participants were posted a parcel containing detailed information, a checklist (to record time/date of sampling and caffeinated products consumed that day), and two saliva receptacles [*1)* Salivette® swab (Sarstedt, Nümbrecht, Germany); *2)* Oragene DNA kit (DNA Genotek Inc., Ottawa, Canada)]. Participants were instructed to give both saliva samples at bedtime, and without eating, drinking, chewing gum or smoking in the thirty minutes beforehand. Contact details of the research team were available in case participants needed assistance.

Genomic assessment with salivary DNA

Oragene receptacles were stored at room temperature until genomic DNA was extracted from saliva according to DNA Genotek Inc.'s instructions. Participants were genotyped for the functional rs762551 polymorphism of the *CYP1A2* gene, and labelled 'highly inducible' or 'less inducible' caffeine metabolizers (A/A genotypes = 'highly inducible'; A/C and C/C = 'less inducible'). All genetic analyses were replicated at least once for independent confirmation of the results. Experimental protocols are described in Appendix II.

HPLC assessment of salivary caffeine

The saliva samples were delivered to the laboratory at room temperature. Upon receipt, the salivettes were stored immediately at -20°C. The stability of salivary caffeine concentrations over 14 days at room temperature has previously been confirmed (Perera et al. 2010). Salivary caffeine concentrations were quantified by high performance liquid chromatography (HPLC) coupled to a UV detector (Fuhr & Rost, 1994). Experimental protocols are described in Appendix II.

The stability of salivary caffeine concentrations during long-term storage at -20°C was confirmed in a sub-sample (N=7). Saliva was analyzed at two time points, ten months apart. Statistical comparisons revealed that there was no significant difference between the caffeine concentrations at the two time points: 2.590 ± 1.573 (SD) *vs.* 2.523 ± 1.351 µg/ml ($p > 0.8$; paired-sample t-test).

Statistical analyses

Analyses were performed with Microsoft Excel 2010 (Microsoft Corp., Seattle, USA) and IBM SPSS Statistics 22 (IBM Corp., Armonk, USA). Mean values (± standard deviations) of raw data are reported and significance was set at $\alpha < 0.05$.

Continuous variables that were not normally distributed were transformed to approximate a normal distribution. The decision was based on visual inspection of the histogram and observation of the SPSS-derived skewness score. A skewness of less than -1 or greater than 1 reflects an unacceptable degree of skewness (Field, 2013). Table legends indicate successful transformation method.

Data from type 2 diabetes and non-type 2 diabetes groups were compared by Fisher's Exact Test (nominal data); independent samples t-test (normally distributed continuous data); or Mann-Whitney U test (not normally distributed continuous data that failed transformation attempts). Habitual caffeine intake of case and control groups was also analyzed by sex and smoking status (independent samples t-tests). HPLC-determined salivary concentrations of caffeine were compared to survey estimates of habitual caffeine intake using the Pearson's product-moment correlation. If data were missing for a variable, the smaller sample size for that variable was reported with the results.

Hierarchical multiple regression analysis was used to test the association between type 2 diabetes and habitual caffeine intake, after controlling for demographic, genetic, sleep, circadian, and work-structure variables. The outcome variable was self-reported habitual caffeine intake, transformed by the square root. The sixteen initial predictor variables were selected based on previous research (Cornelis et al. 2015; Penolazzi et al. 2012; Rodenburg et al. 2012). Predictors in the base model were nominal (binary) or continuous (normally distributed). Demographic variables were entered in the first step [age, sex, smoking, body mass index (BMI), well-being (WHO-5), sleep apnea, long-term medication, alcohol intake]. The *CYP1A2* genotype was entered in the second step. Sleep, circadian, and work-structure variables were added in the third step [subjective sleep quality (PSQI), napping, chronotype, night work, shift work, daytime sleepiness (ESS)]. In the final step, type 2 diabetes status was assessed. To achieve a parsimonious final model and avoid 'overfitting' (Field, 2013), statistically insignificant predictors were systematically tested to ascertain their contribution

to the model (based on adjusted R^2). The final model was tested to ascertain that it met the statistical assumptions of multiple regression.

Results

Table 1 and Supplementary Table 2 (Appendix II) report and compare the type 2 diabetic cases and non-type 2 diabetic controls. Demographic and sociodemographic characteristics were similar between cases and controls; with the exception of male sex, BMI, relationship status and shift work. Within the type 2 diabetes group: 70.1% took oral anti-diabetic medication; 38.8% administered insulin; 43.6% were diagnosed 10 or more years ago.

Table 1: Characteristics of type 2 diabetes and non-type 2 diabetes groups.

Variable	**Type 2 Diabetes (n=134)**	**Non-Type 2 Diabetes (n=230)**	***P*-value**
DEMOGRAPHIC DATA			
Age (years)	64.1 (±9.7)	63.8 (±9.9)	0.738
Male sex (%)	63.4	36.5	**<0.001**
Body mass index (BMI; kg/m^2)	28.8 (±5.5)	23.9 (±3.5)	**<0.001**
Night work (% yes)[a]	8.3	3.5	**(0.054)**
Shift work (% yes)[b]	6.0	1.8	**0.036**
HEALTH BEHAVIOURS			
Smoking (% yes)	14.9	6.1	**0.008**
Alcohol intake (% yes)[c]	31.3	43.5	**0.026**
Physical activity (% yes)[d]	50.0	56.5	0.233
Avoid caffeine to avoid sleep disruption (% yes)[e]	46.0	61.0	**0.009**
DIETARY BEHAVIOURS			
Beverage consumed when thirsty (% water)[f]	52.2	43.9	0.129
Most frequently consumed beverage (% water)[f]	51.5	52.2	0.914
Sugar in hot drinks (% yes)[g]	6.5	20.5	**<0.001**

Artificial sweeteners in hot drinks (% yes)[h]	26.8	10.4	**<0.001**
Dietary regime (%)			
○ None	66.4	80.0	**0.006**
○ Calorie-controlled or low sugar	28.4	8.3	**<0.001**
○ Vegetarian, vegan or 'other'	5.2	11.7	**0.041**
Nutritional, herbal, vitamin supplements (% yes)[i]	23.5	35.4	**0.024**
CARDIOVASCULAR HEALTH			
High blood pressure (% yes)	47.8	17.4	**<0.001**
High cholesterol (% yes)	35.8	14.3	**<0.001**
Heart disorder / problem (% yes)	10.4	6.1	0.154
MENTAL HEALTH			
Mental health disorder (% yes)	20.1	12.2	**0.048**
Depression (% yes)	15.7	8.7	**(0.058)**
Well-being / Quality of life (WHO-5)	49.3 (±25.5)	53.2 (±26.0)	0.166
MEDICAL INTERVENTION			
Advised to avoid to caffeine (% yes)[p]	2.2	0.9	N/A
Long-term medication (% yes)	97.8	54.3	**<0.001**
Oral medication for diabetes (% yes)	70.1	-	N/A
Insulin injections for diabetes (% yes)	38.8	-	N/A
CIRCADIAN ESTIMATES (MCTQ)			
Chronotype metric[k]	03:18 (±01:02)	03:22 (±00:52)	0.592
Social jetlag (min)[l]	40.8 (±43.4)	41.4 (±36.6)	0.483
Average sleep duration (h)[m]	7.15 (±1.14)	7.15 (±1.02)	0.989
ESTIMATES OF SLEEP QUALITY			
Habitual sleep duration (PSQI, h)[n]	6.93 (±1.30)	6.90 (±1.09)	0.790
Habitual time in bed (PSQI, h)[n]	8.14 (±1.33)	8.14 (±1.14)	0.955
Sleep efficiency (PSQI, %)[n]	85.9 (±11.9)	85.3 (±11.0)	0.568
Sleep latency (PSQI, min)[o]	19.3 (±19.1)	20.2 (±18.4)	0.283
Subjective sleep quality (PSQI)[p]	6.2 (±3.5)	5.6 (±3.5)	0.114

Trouble sleeping (% yes)[q]		50.4	46.3	0.513
Insomnia (% yes)		25.4	28.7	0.544
Sleep apnea (% yes)		11.9	3.9	**0.005**
Napping (% yes)[r]		33.1	20.0	**0.008**
Daytime sleepiness (ESS)[s]		8.4 (±4.2)	6.8 (±3.5)	**0.001**
***CYP1A2* GENETIC CHARACTERISTICS**[t]				
Allele frequency (%)	A	74.8	71.0	0.317
	C	25.2	29.0	
Genotype frequency (%)	A/A	59.7	50.7	0.180
	C/A	30.3	40.5	
	C/C	10.1	8.8	
Enzyme inducibility (%)[u]	High	59.7	50.7	0.133
	Less	40.3	49.3	

Abbreviations: T2D, type 2 diabetes; Non-T2D, non-type 2 diabetes; N/A, not applicable; MCTQ, Munich Chronotype Questionnaire; PSQI, Pittsburgh Sleep Quality Index; ESS, Epworth Sleepiness Scale; SNP, single nucleotide polymorphism. Data for continuous variables are means (± standard deviation) of raw data. *P*-values (2-tailed) were calculated using independent samples t-tests, comparing T2D and Non-T2D groups. . Data for categorical variables are %. *P*-values (exact; 2-tailed) were calculated using Fisher's exact test.

Raw data of age and BMI were log10-transformed to achieve a normal distribution. [a] T2D: n=133; NonT2D: n=230; [b] T2D: n=133; NonT2D: n=228; [c] Consume 3 or more alcoholic drinks per week; [d] Physical activity raising breathing and heart rate at least 3 times per week; [e] T2D: n=124; NonT2D: n=210; [f] Options: water, milk, cola/energy drink (caffeinated), diet cola/energy drink (caffeinated), soda (caffeine free), diet soda (caffeine free), fruit juice, herbal tea, coffee, black tea, decaffeinated coffee, decaffeinated tea, beer, wine, spirit; [g] T2D: n=123; NonT2D: n=210; [h] T2D: n=123; NonT2D: n=211; [i] T2D: n=132; NonT2D: n=226; [n] T2D (n=133); [j] T2D: n=134; NonT2D: n=228; [k] T2D: n=99; NonT2D: n=132; [l] T2D: n=85; NonT2D: n=95. Raw data transformation: Reciprocal; [m] T2D: n=99; NonT2D: n=135; [n] T2D: n=124; NonT2D: n=226; [o] T2D: n=132; NonT2D: n=229. Raw data transformation: Log10; [p] T2D: n=125; NonT2D: n=222. Raw data transformation: Square root; [q] T2D (n=133); NonT2D (n=229); [r] Habitually nap during the day at least 3 times per week. T2D: n=133; NonT2D: n=230; [s] T2D: n=125; NonT2D: n=209. Raw data transformation: Square root; [t] SNP rs762551.T2D: n=119; NonT2D: n=205; [u] Highly inducible = genotype A/A; Less inducible = genotypes A/C and C/C.

The type 2 diabetic cases were in poorer physical and mental health than controls. For example, cases reported a greater incidence of high blood pressure, high cholesterol, and mental health disorder (Table 1). While reported physical activity and choice of water as most frequently consumed beverage did not differ between the groups, the type 2 diabetic participants reported lower alcohol consumption, less addition of sugar to hot drinks, and greater adherence to calorie-controlled or low-sugar diets. However, a greater proportion of type 2 diabetic cases were smokers.

Circadian and sleep estimates

Self-reported circadian and sleep estimates were similar between type 2 diabetic cases and controls (Table 1). However, sleep apnea and napping were more common in the type 2 diabetes group, and they reported greater daytime sleepiness.

Caffeine consumption

Estimated habitual caffeine consumption and salivary concentrations of caffeine are shown in Table 2. The type 2 diabetic cases reported consuming roughly 80 mg more caffeine each day than non-type 2 diabetic controls. Assessing men and women separately revealed that male type 2 diabetic patients consumed more caffeine than male controls (378.9 ± 212.1 *vs.* 277.5 ± 172.6 mg/day; $p < 0.002$); the difference was not significant in females (291.1 ± 183.4 *vs.* 251.0 ± 133.1 mg/day; $p > 0.14$) (Figure 1A). Separate assessment of non-smokers and smokers also revealed that non-smoking type 2 diabetic patients habitually consumed more caffeine each day than non-smoking controls (333.4 ± 203.3 *vs.* 257.1 ± 147.3 mg/day; $p < 0.001$) (Figure 1B). In smokers, the two groups did not differ (423.2 ± 208.4 *vs.* 315.5 ± 169.0 mg/day; $p > 0.14$). Greater reported caffeine intake in the type 2 diabetes group was corroborated by HPLC-assessment of saliva. At bedtime, salivary caffeine concentrations were higher in type 2 diabetic participants than in controls (2.69 ± 2.50 *vs.* 2.05 ± 1.94 µg/ml; $p = 0.01$). The self-reported caffeine intake was positively correlated with the salivary caffeine concentration ($r = 0.317$; $p < 0.001$) (Supplementary Figure 1; Appendix II). The higher caffeine intake of type 2 diabetic cases stemmed from a greater intake of coffee, such that diabetic cases habitually consumed an extra cup of coffee each day (Table 2). Consumption of decaffeinated coffee was negligible. Supplementary Figure 2 (Appendix II) illustrates dietary sources of caffeine for cases and controls.

Table 2: Caffeine consumption based on self-reported caffeine intake (survey) and objective salivary caffeine concentrations (HPLC-derived).

Caffeine Consumption	**Type 2 Diabetes (n=134)**	**Non-Type 2 Diabetes (n=230)**	***P*-value**
Total habitual caffeine intake (mg/day)[a]	346.8 (±205.8)	260.7 (±148.9)	**<0.001**
○ Caffeine from coffee (mg/day)[ab]	292.9 (±206.1)	211.1 (±151.7)	**<0.001**
○ Total cups of coffee per day[cd]	3.47 (±2.46)	2.53 (±1.69)	**<0.001**
○ Cups of caffeinated coffee per day[c]	3.43 (±2.47)	2.47 (±1.69)	**<0.001**
○ Cups of decaffeinated coffee per day	0.04 (±0.18)	0.06 (±0.21)	0.243
Salivary caffeine concentration (µg/ml)[ae]	2.69 (±2.50)	2.05 (±1.94)	**0.010**

Abbreviations: T2D, type 2 diabetes; Non-T2D, non-type 2 diabetes. Data are means (± standard deviation) of raw data. *P*-values (2-tailed) were calculated using independent samples t-tests, comparing T2D and Non-T2D groups. Raw data was transformed to achieve a normal distribution (method of transformation noted in legend). The exception was 'decaffeinated coffee cups/day' data which failed transformation attempts; here, the *P*-values (exact 2-tailed) reflect Mann-Whitney U test.
[a] Raw data transformation: Square root; [b] Includes caffeine from decaffeinated coffee (4.5 mg/cup); [c] Raw data transformation: Log10; [d] Includes caffeinated and decaffeinated coffee; [e] T2D (n=123); Non-T2D (n=203).

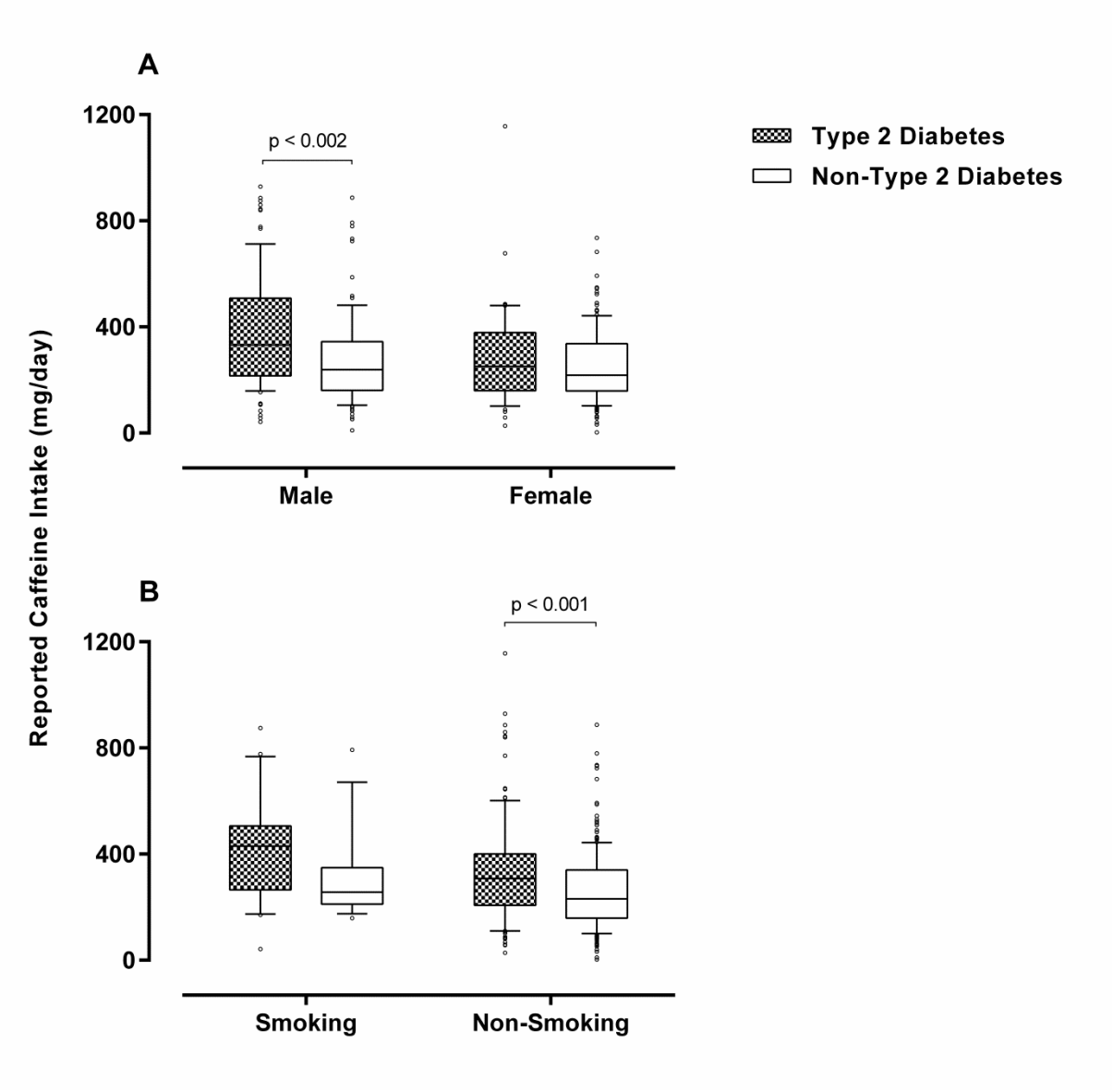

Figure 1: Total habitual caffeine intake of type 2 diabetes and non-type 2 diabetes groups split by sex (A) and smoking status (B). Boxplots represent self-reported total habitual caffeine intake (box: 25th percentile, median and 75th percentile; whiskers: 10th to 90th percentiles; dots: individual data points outside of the whisker range). The estimates of caffeine consumption were based on the caffeine content reported by manufacturers of Swiss products or the website 'Caffeine Informer' (Supplementary Table 1; Appendix II). Statistics compared type 2 diabetes (male: n = 85; female: n = 49; non-smoking: n = 114; smoking: n = 20) and non-type 2 diabetes (male: n = 84; female: n = 146; non-smoking: n = 216; smoking: n = 14) groups with independent samples t-test on square-root data (2-tailed).

CYP1A2 metaboliser status

The distribution of *CYP1A2 -163C>A* genotypes was similar in type 2 diabetes and non-type 2 diabetes groups (Table 1). The allele and genotype frequencies were comparable to published frequencies in older, non-patient control groups, of European descent (Popat et al. 2011).

Association between habitual caffeine intake and type 2 diabetes assessed by hierarchical multiple regression

The base model with 16 predictors (see Methods) predicted total habitual caffeine intake (ANOVA: $F_{16,181} = 3.7$; $p < 0.001$; adjusted $R^2 = 0.18$). Five predictors (age, sleep apnea, long-term medication, subjective sleep quality, night work) were subsequently excluded because they reduced the explanatory power of the model. The final parsimonious model predicted total habitual caffeine intake ($F_{11,189} = 5.7$; $p < 0.001$). The 11 predictors accounted for roughly 25 % of the variation in self-reported caffeine intake (Table 3, model 4)

Demographic variables explained 17.9 % of the variance in caffeine intake (model 1). The genetic predictor, *CYP1A2* genotype (model 2), did not significantly improve the explanatory power of the model ($p > 0.18$). By contrast, sleep, chronotype and work-structure variables (model 3) improved the predictive power of the model ($p = 0.04$). Daytime sleepiness did not help to predict caffeine intake ($p > 0.7$). The type 2 diabetes status was added in the final step (model 4) and was associated with total caffeine intake ($p < 0.02$). That is, when the effects of the other predictors were held constant, type 2 diabetes was associated with an increase in habitual caffeine intake. Furthermore, type 2 diabetes contributed to the model's explanation of the variance in habitual caffeine intake, such that compared to model 3, model 4 explained an additional 2.3 % of the variance in caffeine intake ($p < 0.02$). Overall, male sex was the strongest predictor of caffeine intake (Beta = -0.260), followed by type 2 diabetes status (Beta = 0.179) and then smoking status (Beta = 0.173). When salivary caffeine was substituted into the final model as the outcome variable, type 2 diabetes status was again associated with an increase in salivary caffeine ($p < 0.02$).

Table 3: Hierarchical regression analysis to predict total habitual caffeine intake (N=200).

CATEGORY	**VARIABLE**	**MODEL 1**			**MODEL 2**			**MODEL 3**			**MODEL 4**		
		B	*Beta*	*P*	*B*	*Beta*	*P*	*B*	*Beta*	*P*	*B*	*Beta*	*P*
DEMOGRAPHIC	Sex *(Ref: Male)*	-2.605	-0.236	**0.001**	-2.654	-0.240	**0.001**	-3.123	-0.283	**<0.001**	-2.870	-0.260	**<0.001**
	Smoking *(Ref: No)*	3.084	0.167	**0.012**	3.167	0.172	**0.010**	3.367	0.183	**0.006**	3.189	0.173	**0.008**
	Body Mass Index	17.098	0.251	**<0.001**	16.936	0.249	**0.001**	14.916	0.219	**0.003**	9.455	0.139	0.079
	Well-Being	-0.030	-0.131	**0.049**	-0.029	-0.131	0.050	-0.026	-0.114	0.087	-0.022	-0.100	0.129
	Alcohol Intake *(Ref: No)*	0.911	0.080	0.243	0.963	0.084	0.217	0.895	0.078	0.245	0.977	0.085	0.199
GENETIC	*CYP1A2* Inducibility *(Ref: High)*				-0.956	-0.086	0.189	-0.816	-0.073	0.259	-0.661	-0.059	0.356
SLEEP, CIRCADIAN & WORK STRUCTURE	Napping *(Ref: No)*							-1.592	-0.109	0.105	-1.614	-0.111	0.096
	Chronotype							0.529	0.093	0.153	0.563	0.099	0.124
	Shift Work *(Ref: No)*							3.855	0.137	**0.044**	3.669	0.130	**0.053**
	Daytime Sleepiness							-0.200	-0.023	0.732	-0.274	-0.031	0.634

BLOOD GLUCOSE REGULATION	Type 2 Diabetes *(Ref: Non-Type 2 Diabetes)*										1.994	0.179	**0.017**
	R^2	*0.179*			*0.186*			*0.228*			*0.251*		
	Adjusted R^2	*0.158*			*0.161*			*0.187*			*0.207*		
	Change in R^2				***0.007***		*0.189*	***0.041***		***0.041***	***0.023***		***0.017***

Abbreviations: B = unstandardized coefficient; Beta = standardized coefficient; *P* = *P*-value; Ref = reference.

Table represents final model of 11 variables predicting survey-derived total habitual caffeine intake. Continuous variables were either normally-distributed raw data [well-being (WHO-5), chronotype metric] or raw data transformed to achieve a normal distribution [total habitual caffeine intake (square root), body mass index (Log10), daytime sleepiness (ESS; square root)]. Categorical variables were binary.

The base model contained 16 predictors: Demographic variables: age, sex, smoking, body mass index, well-being, sleep apnea, long-term medication, alcohol intake. Genetic variable: CYP1A2 extent of inducibility. Sleep, Chronotype and Work Structure variables: subjective sleep quality, napping, chronotype, night work, shift work, daytime sleepiness. Blood Glucose Regulation: type 2 diabetes.

Discussion

The main finding of this field study is that habitual coffee consumption is an extra cup higher in type 2 diabetic patients compared to a control group. This result was confirmed by the analysis of salivary caffeine concentrations at bedtime. This finding is pertinent given the ongoing controversy over the relationship between coffee intake and risk of type 2 diabetes (Freedman et al. 2012; Palatini et al. 2015). It supports our prediction that the diabetes group would consume more caffeine than controls.

Elevated sleepiness is a typical feature of type 2 diabetes (West et al. 2006). In line with this notion, the type 2 diabetic participants of this study reported greater daytime sleepiness than the control group, which was corroborated by the higher tendency to nap. The mean score on the Epworth Sleepiness Scale was similar to previous reports in type 2 diabetes samples (Foster et al. 2009; West et al. 2006).

Obstructive sleep apnea is also linked to sleepiness (West et al. 2006). Sleep apnea was more prevalent (12 %) in the present diabetes sample than in the controls (4 %), and the prevalence in the patient group was comparable to published reports (West et al. 2006). Type 2 diabetes and sleep apnea typically co-exist, and may have a bidirectional association by which each condition exacerbates the other (Moon et al. 2015). This field study lacked objective sleep measurements, and relied on questionnaires to assess the presence of sleep apnea. Consequently, it is impossible to differentiate the sleepiness derived from the poor nocturnal breathing in sleep apnea, from the sleepiness associated with diabetes. Both hyperglycemia and sleep apnea may be contributing to the increased sleepiness in the type 2 diabetes group.

Daytime sleepiness can also result from disturbed sleep and misaligned circadian rhythms which, in turn, can result in impaired glycemic control (Buxton et al. 2010; Leproult et al. 2014; Tasali et al. 2008). The diabetes group reported greater shift work than the control group, and shift work can lead to irregular sleep schedules and misalignment of circadian rhythms (Leproult et al. 2014). Nevertheless, self-reported sleep duration, subjective sleep quality, and a behavioral indicator of circadian misalignment (‘social jetlag’) were consistent between cases and controls. On average, participants slept for 7 hours and social jetlag was 41 minutes. These findings are in accordance with a large population-based, European sample, and are age-typical (Roenneberg et al. 2012). Similarly, both groups showed marginally poor sleep quality (PSQI > 5), but this is also a feature of normal aging (Buysse et al. 1989; Bliwise et al. 2005). In conclusion, the congruence of the case and control groups’ subjective sleep and circadian estimates implies that within the present sample, these behavioral factors may not play a key role in impeding glucose homeostasis. Equally, the similarity between groups of the distribution of the CYP1A2 enzyme inducibility suggests that

this factor may not be a key determinant of reduced glycemic control in our type 2 diabetes sample.

It is plausible that the diabetic patients were self-medicating with caffeine to alleviate their sleepiness (Clark & Landolt, 2016; Roehrs & Roth, 2008). Indeed, statistical modelling revealed for the first time that type 2 diabetes status independently predicted higher habitual caffeine intake, both relying on self-report and measured caffeine levels in saliva. It has previously been shown that men and smokers tend to consume more caffeine than women and non-smokers (Penolazzi et al. 2012). These findings were reflected in the present model, with male sex and positive smoking status making significant contributions to the prediction of caffeine intake. Importantly, greater caffeine intake was reported by the male diabetic patients compared to the male control participants, suggesting that the higher caffeine use of the type 2 diabetes group does not reflect a bias of the higher male proportion in the patient group. Moreover, despite the higher prevalence of smoking in the type 2 diabetes group, there was no significant difference in caffeine consumption between case and control participants who currently smoke. The correlation coefficient between self-reported caffeine intake and salivary caffeine concentration in this study was similar to those reported in the literature (James et al. 1989) and validated the caffeine intake questionnaire.

After controlling for demographic, genetic, sleep, circadian and work structure variables already known to influence caffeine consumption (Cornelis et al. 2015; Penolazzi et al. 2012; Rodenburg et al. 2012), our model explained 25 % of the variation in caffeine intake. The negative contribution of daytime sleepiness to caffeine intake (Table 3) indicates that lower sleepiness was related to higher caffeine use. Although this contribution was weak and non-significant *per se*, this finding may suggest that across the entire cohort, the stimulatory effects of caffeine are experienced by participants. However, the occurrence of greater sleepiness and greater caffeine consumption in type 2 diabetic patients implies that within this group, caffeine's stimulation may not be sufficient to outweigh the sleepiness associated with their condition.

On average, the Swiss population consumes 288 mg caffeine per day (Fredholm et al. 1999). Whereas the control group almost exactly matched the population average (261 mg), the present type 2 diabetic participants reported to consume substantially more (86 mg or 33%) at 347 mg caffeine per day. This amount is higher than the caffeine content in a regular cup of coffee (instant coffee = c. 57 mg) and most caffeine-containing, over-the-counter medications (Supplementary Table 1; Appendix II). Importantly, objective caffeine quantification corroborated that salivary caffeine was 31% higher in the patient group. Such findings are clinically relevant because while a normal fasting blood glucose is 70-100 mg/dl, an elevated blood glucose, which signals diabetes, is a reading over 125 mg/ dl (American Diabetes Association, 2014); that is, an elevation of ≥ 25 %. The type 2 diabetes status was

the strongest predictor of high caffeine intake, after male sex. Thus, above and beyond demographic, genetic, sleep, circadian and work structure variables, type 2 diabetes status contributed significantly to the variance in reported caffeine intake (Table 3).

Apart from the male sex bias and the relatively small number of female controls, which may have precluded a statistically significant difference between patient and control study participants in women, the study lacked objective measurements of glycemic control, sleep and circadian regulation. Given the observational nature of the investigation, the findings cannot address the direction of causality between variables, only highlight correlations and suggest plausible rationale for such relationships. Moreover, replication of this study, in a large, population-based sample, is recommended to assess the stability of the findings. Nonetheless, laboratory studies demonstrate that doses of caffeine (375 mg), such as those reported in the type 2 diabetes group, impair glucose metabolism in resting humans through a transient increase in insulin resistance (Greer et al. 2001; Lane et al., 2004). Although the exact underlying mechanisms remain poorly understood, caffeine may interfere with extra- and intracellular processes of glucose signalling (Shearer & Graham, 2014).

Conclusion

The results of this study may indicate that type 2 diabetic patients consume large amounts of caffeine to alleviate daytime sleepiness. High caffeine intake could undermine efforts to treat hyperglycemia. On the other hand, the majority of the caffeine intake in this sample came from coffee and coffee contains bioactive compounds that may ameliorate the negative impact of caffeine on glycemic control (Johnston et al. 2003). In the present study, only 3 of 134 type 2 diabetic patients were advised to avoid caffeine. The American Diabetes Association and the European Association for the Study of Diabetes do not provide recommendations related to caffeine or coffee consumption for the management of type 2 diabetes (Inzucchi et al. 2012). Overall, caffeine and coffee consumption is potentially an important lifestyle factor that should be considered in the promotion of type 2 diabetes management.

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Declaration of conflicting interests

The authors declare no potential conflict of interest, financial or otherwise.

Contribution statement

HPL proposed the trial concept and design and secured the funding. EU, GAS, WL and HPL contributed to recruitment of patients and controls, data collection, data analyses and interpretation, and discussion of the results. AJ (caffeine quantification in saliva), SCH and WB (genotyping) contributed analytic tools. EU and HPL wrote the manuscript. All authors contributed to the reviewing and editing of the manuscript, and approved the final version.

Chapter 4:

Assessment of CYP1A2 enzyme activity in relation to type 2 diabetes and habitual caffeine intake

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Abstract

Background: Coffee consumption is a known inducer of cytochrome P450 1A2 (CYP1A2) enzyme activity. We recently observed that a group of type-2 diabetes patients consumed more caffeine (coffee) on a daily basis than non-type-2 diabetes controls. Here, we investigated whether type-2 diabetes cases may metabolize caffeine faster than non-type-2 diabetes controls.

Methods: To estimate CYP1A2 enzyme activity, an established marker of caffeine metabolism, we quantified the paraxanthine/caffeine concentration ratio in saliva in 57 type-2 diabetes and 146 non-type-2 diabetes participants in a case-control field study. All participants completed validated questionnaires regarding demographic status, health and habitual caffeine intake, and were genotyped for the functional -163C>A polymorphism of the *CYP1A2* gene.

Results: In the diabetes group, we found a larger proportion of participants with the highly inducible *CYP1A2* genotype. Furthermore, the paraxanthine/caffeine ratio, time-corrected to mitigate the impact of different saliva sampling times with respect to the last caffeine intake, was higher than in the control group. Participants who reported habitually consuming more caffeine than the population average showed higher CYP1A2 activity than participants with lower than average caffeine consumption. Multiple regression analyses revealed that higher caffeine intake was potentially an important mediator of higher CYP1A2 activity.

Conclusions: Estimated CYP1A2 enzyme activity, and thus speed of caffeine metabolism, was higher in our type-2 diabetes group; this was possibly due to higher intake of caffeine, a known inducer of CYP1A2 enzyme activity. Given the fairly small sample sizes, the results need to be considered as preliminary and require validation in larger populations.

Keywords: Caffeine, Paraxanthine, Phenotyping, HPLC.

Background

Caffeine is almost completely metabolized in the body by cytochrome P450 1A2 (CYP1A2). This enzyme accounts for the metabolism of caffeine to its principal metabolite, paraxanthine (Gu et al. 1992). In vivo, CYP1A2 activity exhibits a significant degree of inter-individual variation (see, Faber et al. 2005, for review). Inter-individual variability in CYP1A2 enzyme activity is typically between 5- and 15-fold in healthy humans (Schrenk et al. 1998; Tantcheva-Poór et al. 1999), possibly due to environmental and genetic factors. For example, coffee consumption and cigarette smoking both induce CYP1A2 activity, in a dose-dependent manner (Tantcheva-Poór et al. 1999). Interestingly, rodent models demonstrate that the blood-glucose-regulatory hormone insulin also acts as an inducer of CYP1A2 activity (Barnett et al. 1992). While this relationship has not been directly assessed in humans, a correlational study revealed a positive relationship between CYP1A2 activity and endogenous insulin levels in premenopausal women (Hong et al. 2004). Also functional variations in the CYP1A2 gene may contribute to inter-individual differences in enzyme activity (Sachse et al. 2003). Indeed, a single nucleotide polymorphism (SNP) (-163C>A) of CYP1A2 has been associated with increased enzymatic activity in smokers (Sachse et al. 1999).

Systemic caffeine clearance is considered the gold-standard approach to estimating CYP1A2 activity (Fuhr et al. 1996), which reflects the combined effects of genetic, environmental and endogenous factors (Streetman et al. 2000). However, this method requires extensive blood sampling, which is expensive, invasive and time consuming (Faber et al. 2005). A validated alternative is to determine the concentration ratio of paraxanthine to caffeine in a saliva sample collected 6 hours post caffeine dose (Fuhr & Rost, 1994; Fuhr et al. 1996).

In a case-control field study, we recently found that type-2 diabetes patients consumed more caffeine than non-type-2 diabetes controls, possibly to attenuate daytime sleepiness typically associated with the disease (Urry et al. 2016). Based on the above presented evidence that caffeine and insulin act as possible inducers of CYP1A2 activity, we hypothesized that the type-2 diabetes patient group would show higher CYP1A2 enzyme activity than the non-type-2 diabetes control group. To our knowledge, only one study has previously assessed CYP1A2 enzyme activity in type-2 diabetes patients, and no difference was found between case and control groups (Matzke et al. 2000). The findings, however, are limited by the small sample size (n = 16 patients and controls), the long time frame between caffeine ingestion and provision of saliva (8 hours) (Fuhr & Rost, 1994; Fuhr et al. 1996), and the lack of data regarding habitual caffeine/coffee intake, smoking, and CYP1A2 genotype. All these factors may impact CYP1A2 activity in patients and controls.

Methods

Subjects

A total of 445 study participants were recruited. Two type-1 diabetes participants were excluded due to the different pathophysiology of type-1 and type-2 diabetes; 7 participants of non-European descent were excluded due to genetic variation between populations of different ethnic origin. In addition, 179 participants who did not report a 3-12 hour time interval between their final caffeine portion and saliva sampling, were excluded. This is because the paraxanthine/caffeine ratio only reliably measures CYP1A2 enzyme activity when there is a 3-12 hour time interval between caffeine intake and provision of the saliva sample, due to the non-linear kinetics of caffeine metabolism (Spigset et al. 1999). Two participants were excluded due to missing saliva. In 17 participants, the HPLC measurements were below the quantification limit (BQL) of caffeine (BQL = 0.077 µg/ml; n = 5), paraxanthine (BQL = 0.024 µg/ml; n = 5) or both analytes (n = 7); and in 23 participants, the corrected paraxanthine/caffeine ratio was negative (see below). Finally, 14 participants were excluded because of technical difficulties with the HPLC quantification. The final sample comprised 203 participants (57 type-2 diabetes cases and 146 non-type-2 diabetes controls).

The type-2 diabetes status was determined by an affirmative response to the question: “Over the past 12 months, have you suffered from type-2 diabetes?”. As well as, an answer to the question: “If you suffer from type-2 diabetes, when did you receive your diagnosis?”; and a report of a diabetes-appropriate treatment regime of oral medication and/or insulin.

Questionnaire assessment

Questionnaires gathered information regarding demographic status, health and habitual caffeine intake. The survey was completed either online (2ask® survey software) or in paper form. Habitual caffeine intake was assessed using an extended version of the caffeine intake questionnaire of the sleep laboratory of the University of Zurich (Rétey et al. 2007). Participants were asked to report how frequently (per day or per week) they usually consumed a given range of caffeine-containing foods, drinks, medications and supplements. Supplementary Table 1 (Appendix II) displays the estimated caffeine content (mg / serving) of each item in the questionnaire. These data were used to calculate participants’ daily habitual caffeine intake.

Saliva sampling

Participants gave two samples of saliva at home and then posted them back to the laboratory in a pre-paid envelope (Tyvek® material; DuPont). Beforehand, participants were

posted a parcel containing detailed information, a checklist (to record time/date of sampling and caffeinated products consumed that day), and two saliva receptacles [*1)* Salivette® swab (Sarstedt, Nümbrecht, Germany) to determine caffeine and paraxanthine concentrations; *2)* Oragene® DNA kit (DNA Genotek Inc., Ottawa, Canada) for DNA extraction and genotyping]. Participants were instructed to give both saliva samples at bedtime, and without eating, drinking, chewing gum or smoking in the thirty minutes beforehand; and also to complete the checklist. Contact details of the research team were available in case participants needed assistance.

Genomic assessment with salivary DNA

Oragene receptacles were stored at room temperature until genomic DNA was extracted from saliva according to DNA Genotek Inc.'s instructions. Participants were genotyped for the functional rs762551 polymorphism of the *CYP1A2* gene, a demonstrated determinant of inducible CYP1A2 activity (Han et al. 2001; Sachse et al. 1999), and labelled 'highly inducible' or 'less inducible' caffeine metabolizers (A/A genotypes = 'highly inducible'; A/C and C/C = 'less inducible'). All genetic analyses were replicated at least once for independent confirmation of the results. Experimental protocols are described in Appendix II.

HPLC assessment of salivary caffeine and paraxanthine

The saliva samples were delivered to the laboratory at room temperature. Upon receipt, the salivettes were stored immediately at -20°C. The stability of salivary caffeine and paraxanthine concentrations over 14 days at room temperature has previously been confirmed (Perera et al. 2010).

After thawing, saliva was extracted from the Salivette® according to the manufacturer's instructions (centrifugation for 2 minutes at 1,000g). Salivary caffeine and paraxanthine concentrations were quantified by HPLC, coupled to a UV detector, essentially as described by Fuhr and Rost (Fuhr & Rost, 1994), but with minor modifications as described in Appendix II.

The stability of salivary caffeine and paraxanthine concentrations during long-term storage at -20°C was confirmed in a sub-sample (n = 7). Saliva was analyzed at two time points, ten months apart. Statistical comparisons revealed that there was no significant difference between the caffeine concentrations at the two time points: 2.590 ± 1.573 (SD) *vs*. 2.523 ± 1.351 µg/ml ($p > 0.8$; paired-sample t-test). There was also no significant difference between the paraxanthine concentrations at the two time points: 0.920 ± 0.461 (SD) *vs*. 0.789 ± 0.341 µg/mL ($p > 0.2$).

Determination of corrected paraxanthine/caffeine ratio

The present field study participants reported varied caffeine consumption on the day of saliva sampling, and varied time intervals between their last caffeine intake and the saliva sampling. While Perera and colleagues (Perera et al. 2011) demonstrated that CYP1A2 activity can be reliably assessed without a 24-hour period of caffeine abstinence, assessment of CYP1A2 phenotypes is very time-dependent (Grant et al. 1983; Kalow et al. 1993; Labedzki et al. 2002; Rostami-Hodjegan et al. 1996). Correlation analyses between immunoreactive CYP1A2 in the liver, intrinsic clearance for caffeine-3-demethylation to paraxanthine, and various plasma, saliva, and urine based CYP1A2 metrics showed that the saliva paraxanthine/caffeine ratio 6 hours after caffeine intake had the best correlation to intrinsic caffeine-to-paraxanthine clearance, which is the "gold standard" for CYP1A2 activity assessment (Fuhr et al. 1996). That is, six hours post caffeine dose, the molar concentration ratio of salivary paraxanthine to caffeine provides the most valid estimate of CYP1A2 enzyme activity (Fuhr & Rost, 1994; Fuhr et al. 1996). We therefore developed a method to adjust the CYP1A2 activity ratio values to the optimal 6-h post-dose sampling time point, and to thus allow direct comparison within and between groups.

Spigset and colleagues (Spigset et al. 1999) investigated the relationship between sampling time and individual salivary paraxanthine/caffeine ratios, after intake of a single oral dose of 200 mg caffeine, in 12 healthy, young men in a controlled, laboratory setting. Based on inspection of Figure 2 in their publication, the lowest and highest paraxanthine/caffeine ratio, at each time point, was recorded. The mean of the lowest and highest ratio was then calculated and entered in GraphPad Prism (La Jolla, California, USA). After fitting a curve to the data set, the equation $y = 0.016 + (0.141 * x) + (-0.004 * x^2)$ was used to estimate the participants' paraxanthine/caffeine ratio ('y'), if the time span between last caffeine intake and provision of saliva ('x') was known. A time span of 6 hours equates to a mean paraxanthine/caffeine ratio of 0.725.

Figure 1 shows the relationship between the time interval between caffeine intake and saliva sampling, and the paraxanthine/caffeine ratio for both the mean observed ratios based on the data of Spigset et al. (Spigset et al. 1999), and the ratio based on the fitted curve using the above-mentioned equation. The fitted curve explained roughly 70% ($R^2 = 0.702$) of the variance in the observed ratio data.

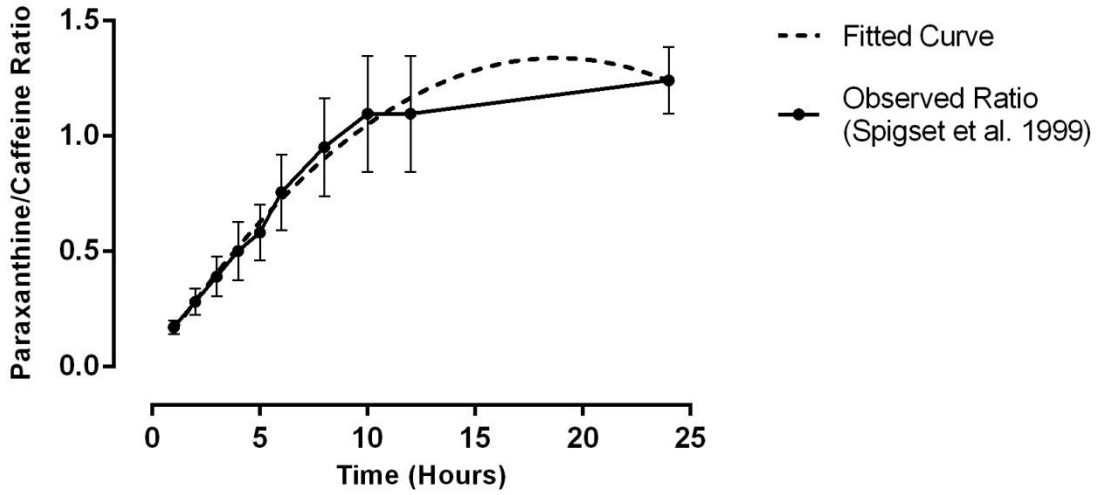

Figure 1: Relationship between sampling time and salivary paraxanthine/caffeine ratio. Solid line: mean observed ratio estimates based on data of Spigset et al. (Spigset et al. 1999). Error bars show standard deviation across the mean of observed ratio data (n = 12). Dotted line: ratio based on fitted curve (dotted line) using a second-order polynomial model: $Y = A + (B \times X) + (C \times X^2)$. Best-fit values (95% confidence intervals): A = 0.016 (-0.206 - 0.238); B = 0.141 (0.090 - 0.191); C = -0.004 (-0.006 - -0.002). Equation: $Y = 0.016 + (0.141 \times X) + (-0.004 \times X^2)$; Y = paraxanthine/ caffeine ratio; X = time interval between final caffeine intake and saliva sampling.

To estimate the participants' paraxanthine/caffeine ratio adjusted to the 'ideal' time interval of 6 hours, several steps were taken. First, based on the molar concentrations of paraxanthine and caffeine, the 'actual' paraxanthine/caffeine ratio was calculated for each participant. Next, the Spigset-derived equation was used to estimate the 'correct' mean ratio, based on participants' reported time interval between caffeine intake and saliva sampling. The difference between the 'actual' ratio and the 'correct' mean ratio was then calculated. Finally, participants' time-corrected paraxanthine/caffeine ratio, to assume a 6 hour 'ideal' time span, was determined by adding this difference to the equation's estimate of the paraxanthine/ caffeine ratio at a 6-hour time interval (0.725). Graphically spoken, the individual ratio value was shifted on a curve with the slope of the Spigset data-derived mean curve to the ideal time of 6 hours. The paraxanthine/caffeine ratio data, derived from our time-correction technique, ranged from 0.00 to 2.85 (mean: 0.577 ± 0.411 [SD]; n = 203).

Statistical analyses

Analyses were performed with Microsoft Excel 2010 (Microsoft Corp., Seattle, USA) and IBM SPSS Statistics 22 (IBM Corp., Armonk, USA), and adhered to documented statistical

principles (Field, 2013). Mean values (± standard deviations) of raw data are reported and significance was set at $\alpha < 0.05$. Continuous variables that were not normally distributed (based on visual inspection of histogram and SPSS-derived skewness score $-1 > x < 1$) were transformed to approximate a normal distribution. Table legends indicate the successful transformation method. If data were missing for a variable, the smaller sample size for that variable was reported with the results. Data from type-2 diabetes and non-type-2 diabetes groups were compared by Fisher's Exact Test (nominal data) and independent samples t-test (normally distributed continuous data). To assess the validity of the paraxanthine/caffeine ratio correction, independent samples t-tests were used to compare the corrected ratio data grouped by variables that were previously reported to influence CYP1A2 enzyme activity, including age (≤ mean age of 59.3 years *vs*. > mean age), body mass index (underweight/healthy ≤ 24.9 *vs*. overweight/obese > 24.9), habitual caffeine intake [lower/normal habitual caffeine intake (≤ Swiss average of 288 mg/day) *vs*. higher habitual caffeine intake (> Swiss average)], contraceptive pill (no *vs*. yes), *CYP1A2* -163C>A genotype (A/C and C/C allele carriers *vs*. A/A allele carriers), gender (male *vs*. female), insulin administration (no *vs*. yes), long-term medication (no *vs*. yes) and smoking (non smoking *vs*. smoking). Within the type-2 diabetes group, the corrected paraxanthine/caffeine ratio data was compared between selected binary covariates (habitual caffeine intake, CYP1A2 inducibility, gender, insulin administration, smoking) by independent samples t-test on raw data that approximated a normal distribution.

If there was a statistical difference in the results of the independent samples t-tests across the whole sample, which compared the corrected ratio data grouped by variables previously reported to influence CYP1A2 activity, then that covariate was included in a multiple regression analysis (simultaneous entry). The regression model tested the association between the selected variables and the paraxanthine/ caffeine ratio across the whole group. The outcome variable was the corrected paraxanthine/caffeine ratio, transformed by square root to approximate a normal distribution. The two predictors were type-2 diabetes status (nominal; binary) and high (> Swiss average of 288 mg/day) caffeine intake (nominal; binary). 'Insulin' was not included as a predictor variable since it was only administered by type-2 diabetes patients, and thus its inclusion would confound the results. The model was tested to ascertain that it met the statistical assumptions of multiple regression (Babyak, 2004; Field, 2013).

Results

Table 1 reports the characteristics of the type-2 diabetes cases and the non-type-2 diabetes controls. The groups differed in age, gender, body mass index, long-term medication intake and oral contraceptive use.

The total self-reported habitual caffeine intake was 96.5 mg higher per day in the type-2 diabetes group (Table 1). Coffee was the major source of caffeine for both groups. Despite the shorter time interval between saliva sampling and the final portion of caffeine intake in the patients, the mean salivary concentration of paraxanthine was significantly higher in the type-2 diabetes patient group than in the control group (Table 1).

The -163C>A allele frequencies of the *CYP1A2* gene were similar in type-2 diabetes and non-type-2 diabetes groups (Table 1). However, compared to the control group, there was a higher proportion of diabetes participants with the highly-inducible A/A genotype and a lower proportion of diabetes participants with the less-inducible A/C and C/C genotypes (Table 1).

Table 1: Characteristics of whole sample, and split by type-2 diabetes and non-type-2 diabetes group [continuous variables: mean (± standard deviation); categorical variables: frequency (% of total)].

Variable	Whole Sample (n=203)		Type-2 Diabetes Cases (n=57)	Non-Type-2 Diabetes Controls (n=146)	*p*-value between groups
Age (years)	59.3 (±15.9)		63.9 (±9.9)	57.4 (±17.4)	**0.008**
Male Gender (%)	87 (42.9%)		38 (66.7%)	49 (33.6%)	**<0.001**
Body Mass Index (BMI; kg/m^2) [a]	25.1 (±4.5)		28.6 (±5.3)	23.7 (±3.3)	**<0.001**
Overweight/Obese BMI (%) [b]	84 (41.8%)		42 (73.7%)	42 (29.2%)	**<0.001**
Smoking (% yes)	21 (10.3%)		9 (15.8%)	12 (8.2%)	0.127
Alcohol Intake (% yes) [c]	78 (38.4%)		16 (28.1%)	62 (42.5%)	0.077
Long-Term Medication (% yes)	129 (63.5%)		55 (96.5%)	74 (50.7%)	**<0.001**
Oral Medication for Diabetes (% yes)	42 (20.7%)		42 (73.7%)	0 (0%)	**<0.001**

Insulin Injections for Diabetes (% yes)		19 (9.4%)		19 (33.3%)	0 (0%)	**<0.001**
Contraceptive Pill (% yes)		9 (4.4%)		0 (0%)	9 (6.2%)	0.064
Total Habitual Caffeine Intake (mg/day) [d]		295.8 (±158.1)		365.2 (±191.3)	268.7 (±134.4)	**<0.001**
Caffeine from Coffee (mg/day) [de]		240.3 (±162.2)		306.9 (±195.7)	214.3 (±139.4)	**0.001**
Higher Habitual Caffeine Intake (% yes) [f]		64 (31.5%)		28 (49.1%)	36 (24.7%)	**0.001**
Salivary Caffeine Concentration (µmol/l) [d]		11.0 (±7.7)		11.9 (±8.2)	10.6 (±7.6)	0.259
Salivary Paraxanthine Concentration (µmol/l) [d]		5.2 (±3.4)		6.0 (±3.4)	4.9 (±3.4)	**0.024**
Time between saliva sample and final caffeine portion (h)		6.7 (±2.6)		5.8 (±2.6)	7.1 (±2.5)	**0.001**
Gene Cytochrome P450-1A2 (*CYP1A2*) [g]						
Allele Frequency (%)	**A**	276 (69.0%)		83 (74.1%)	193 (67.0%)	0.186
	C	124 (31.0%)		29 (25.9%)	95 (33.0%)	
Genotype Frequency (%)						**0.036**
	A/A	97 (48.5%)		34 (60.7%)	63 (43.8%)	**0.040**
	C/A	82 (41.0%)		15 (26.8%)	67 (46.5%)	**0.011**
	C/C	21 (10.5%)		7 (12.5%)	14 (9.7%)	0.610
Enzyme Inducibility (%)	**High**	97 (48.5%)		34 (60.7%)	63 (43.8%)	**0.040**
	Less	103 (51.5%)		22 (39.3%)	81 (56.3%)	

Abbreviations: T2D, type-2 diabetes; Non-T2D, non-type-2 diabetes; Data for continuous variables are means (± standard deviation) of raw data. P-values (2-tailed) were calculated using independent samples t-tests, comparing T2D and Non-T2D groups, on raw data. If raw data was abnormally distributed, the data was transformed to achieve a normal distribution before the t-test was applied (method of transformation noted in legend). Data for categorical variables are frequencies (%). P-values (exact; 2-tailed) were calculated using Fisher's exact test.

[a] Raw data transformation: Log10; T2D (n=57); Non-T2D (n=144). [b] Overweight/Obese BMI >24.9 vs. Underweight/Healthy BMI ≤24.9; T2D (n=57); Non-T2D (n=144). [c] Consume 3 or more alcoholic drinks per week. [d] Raw data transformation: Square root. [e] Includes caffeine from decaffeinated coffee (4.5mg/cup). [f] Higher habitual caffeine intake (> Swiss average of 288mg/day) vs. Lower/Normal habitual caffeine intake (≤ Swiss average). [g] SNP ID: rs762551. T2D (n=56); Non-T2D (n=144). Highly inducible = genotype A/A. Less inducible = genotypes A/C and C/C.

As illustrated in Figure 2, the mean time-corrected paraxanthine/caffeine ratio was significantly higher in type-2 diabetes cases than in non-type-2 diabetes controls (type-2 diabetes patients: 0.700 ± 0.426; non-type-2 diabetes controls: 0.529 ± 0.396; p = 0.010, two-tailed t-test). This finding indicates a higher mean CYP1A2 enzyme activity in the group of type-2 diabetes patients. When those patients who reported to take insulin were excluded, the difference was no longer significant (0.664 *vs*. 0.529; p = 0.121). Indeed, CYP1A2 enzyme activity was significantly faster in participants who administered insulin (Table 2). When only type-2 diabetes patients were assessed, however, the difference was not significant (0.664 *vs.* 0.770; p = 0.382).

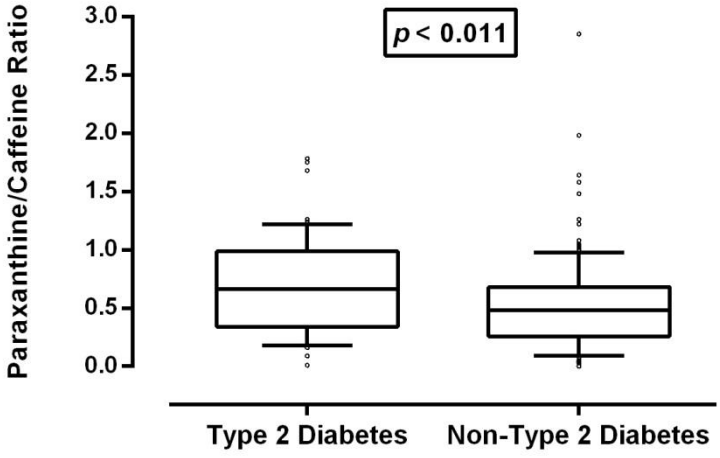

Figure 2: Paraxanthine/caffeine ratios in type-2 diabetes patient and non-type-2 diabetes control groups. Boxplots represent paraxanthine/caffeine ratios corrected to an "ideal" time interval between last caffeine intake and saliva sampling of 6 hours (box: 25th percentile, median and 75th percentile; whiskers = 10th to 90th percentiles; dots: individual data points outside of the whisker range). Statistics compared type-2 diabetes patient (n = 57) and non-type-2 diabetes control (n = 146) groups by independent samples t-test on square-rooted data (2-tailed; equal variances assumed). Statistical

analysis with the non-parametric Mann-Whitney U-test on non-transformed corrected paraxanthine/ caffeine ratios confirmed the robustness of the result: T2D *vs.* non-T2D: mean rank 120.93 *vs.* 94.61; exact sig. 2-tailed: p = 0.004.

It is estimated that the Swiss population consumes roughly 288 mg caffeine per capita and day (Fredholm et al. 1999; Urry et al. 2016). Participants who reported habitually consuming higher amounts of caffeine (> 288 mg/day), showed significantly faster CYP1A2 enzyme activity, compared to participants consuming less caffeine (≤ 288 mg/day) (Table 2). Within the type-2 diabetes sample, patients who reported habitually consuming more caffeine than the population average showed a numerically faster CYP1A2 activity (0.763 *vs*. 0.638), but not to a significant degree (p = 0.276).

The CYP1A2 genotype, gender and smoking status had no significant effect on the mean time-corrected paraxanthine/caffeine ratio (Table 2).

Table 2: Independent samples t-tests comparing time-corrected paraxanthine/caffeine ratios by age, body mass index, caffeine intake, contraceptive pill, *CYP1A2* inducibility, gender, insulin administration, long-term medication, smoking status.

	Group	N	Time-Corrected Paraxanthine/Caffeine Ratio	*p* value
Age	**≤ 59.3 years**	77	0.604 (±0.364)	0.290
	> 59.3 years	126	0.561 (±0.438)	
BMI	**≤ 24.9 kg/m^2**	117	0.560 (±0.415)	0.580
	> 24.9 kg/m^2	84	0.603 (±0.408)	
Caffeine Intake	**≤ 288mg/day**	139	0.535 (±0.431)	**0.010**
	> 288 mg/day	64	0.669 (±0.350)	
Contraceptive Pill	**No**	194	0.579 (±0.417)	0.979
	Yes	9	0.545 (±0.282)	
***CYP1A2* Inducibility**	**Less (C/A & C/C genotypes)**	103	0.552 (±0.430)	0.284
	High (AA genotype)	97	0.609 (±0.394)	

Gender	**Male**	87	0.631 (±0.467)	0.237
	Female	116	0.537 (±0.360)	
Insulin Administration	**No**	184	0.557 (±0.414)	**0.016**
	Yes	19	0.770 (±0.336)	
Medication	**No**	74	0.510 (±0.311)	0.187
	Yes	129	0.616 (±0.456)	
Smoking Status	**Non Smoking**	182	0.573 (±0.410)	0.896
	Smoking	21	0.617 (±0.430)	

Values are given as mean (±SD). Independent samples t-tests were applied to time-corrected paraxanthine/caffeine ratios transformed by square root to approximate a normal distribution. Statistical data reported assumed equal variances. P-values reflected a 2-tailed test. Results are reported to 3 decimal places.

Multiple regression analysis was used to predict the paraxanthine/caffeine ratio across the whole sample (Table 3). The two selected predictors of CYP1A2 enzyme activity, i.e., type-2 diabetes status and higher caffeine intake (see Methods), significantly predicted the paraxanthine/caffeine ratio ($F_{2,200}$ = 5.580, p = 0.004). While they accounted for only 5.3% of the variation in the data, both made statistically significant contributions to the prediction (T2D status: Beta = 0.146; p = 0.040; higher caffeine intake: Beta = 0.146; p = 0.041). When the regression model was run with caffeine intake as a continuous variable, the model also predicted the paraxanthine/caffeine ratio (ANOVA: $F_{2,200}$ = 5.298, p = 0.006), with type-2 diabetes being a significant predictor (Beta = 0.145; p = 0.044) and caffeine intake exhibiting a strong trend to predict the ratio (Beta = 0.137; p = 0.056).

Table 3: Multiple regression analysis to predict the paraxanthine/caffeine ratio (N=203).

MODEL SUMMARY	**COVARIATES**	**Unstandardized Coefficients**		**Standardized Coefficients**	***p* value**
		B	**Std. Error**	**Beta**	
	T2D status *(Ref: non-T2D)*	0.088	0.043	0.146	**0.040**
	Higher caffeine intake *(Ref: no)*	0.085	0.041	0.146	**0.041**
R^2	*0.053*				
Adjusted R^2	*0.043*				
Model ANOVA	***p = 0.004***				

Abbreviations: Ref = reference; T2D = type-2 diabetes; Non-T2D = non-type-2 diabetes.

Table represents multiple regression analysis to predict the corrected paraxanthine/caffeine ratio. The 2 predictor variables were entered simultaneously. Continuous variables were raw data transformed by square root to achieve a normal distribution (corrected paraxanthine/ caffeine ratio. Categorical variables were binary (T2D status, higher caffeine intake). ['Higher' caffeine intake > 288 mg/day (Swiss daily average caffeine intake)]

Discussion

In support of our hypothesis, the main finding of this field study was that CYP1A2 enzyme activity was significantly higher in a type-2 diabetes group compared to a control group. Since caffeine is almost completely metabolized by CYP1A2 (Gu et al. 1992), this faster enzyme activity indicates a faster metabolism of caffeine in the type-2 diabetes participants. Indeed, patients' salivary concentrations of paraxanthine, caffeine's major metabolite (Gu et al. 1992), were significantly higher at bedtime. The results indicate that the previously described inducing effect of caffeine on its own CYP1A2-mediated metabolism may also be present in type-2 diabetes patients.

We used a novel correction technique to adjust participants' paraxanthine/caffeine ratios, an established marker of CYP1A2 enzyme activity, to account for the varied reported time intervals between last caffeine intake and saliva sampling. Based on the equation we derived from published data (Spigset et al. 1999), the paraxanthine/caffeine ratio of the present participants was adjusted to reflect a ratio that would stem from the 'ideal' time interval of 6 hours (Fuhr & Rost, 1994; Fuhr et al. 1996). The time-corrected paraxanthine/caffeine ratios obtained with this method were comparable to published reports (Fuhr & Rost, 1994), which supports the validity of our approach.

In accordance with previous research (Tantcheva-Poór et al. 1999), participants who habitually consumed higher amounts of caffeine showed higher paraxanthine/caffeine ratios, and thus faster CYP1A2 enzyme activity. Participants administering insulin also showed faster CYP1A2 activity. While this relationship has previously not been directly assessed in humans, rodent models demonstrate that insulin induces CYP1A2 activity (Barnett et al. 1992). Moreover, an observational study in humans linked higher CYP1A2 activity with higher endogenous insulin levels (Hong et al. 2004). These results further help to support the validity of our correction technique. Nevertheless, our technique needs to be validated in larger and stringently controlled samples, alongside comparison with systemic caffeine clearance data. If these future studies are successful, the correction could be applied, for example, in epidemiological settings where varied time frames between caffeine intake and saliva sampling are allowed. Here we used this correction only for data of participants who reported caffeine consumption within the time window of 3 to 12 hours before saliva sampling. This is because, compared to the 'gold standard' approach of estimating CYP1A2 enzyme activity [systemic caffeine-to-paraxanthine clearance from blood (Fuhr et al. 1996)], the salivary paraxanthine/caffeine ratio only accurately reflects CYP1A2 activity during this time interval (Spigset et al. 1999).

Interestingly, we found that the time interval between the final caffeine portion and saliva sampling was shorter in the patient group than in the control group. Thus, if both groups had

equal CYP1A2 enzyme activity, a lower amount of caffeine would have been metabolized in the patients by the time of saliva sampling and a smaller salivary paraxanthine concentration should have been observed. By contrast, the paraxanthine concentration was higher in the type-2 diabetes participants, consistent with our conclusion that CYP1A2 activity was higher in the patients than in the controls.

The time-corrected paraxanthine/caffeine ratio was higher in study participants who reported higher caffeine consumption than the mean Swiss caffeine intake of 288 mg/day. Furthermore, statistical modelling revealed that high habitual caffeine intake was a significant predictor of faster CYP1A2 enzyme activity in our study sample. While type-2 diabetes status also contributed to the prediction of the paraxanthine/caffeine ratio, we suggest that out of the two predictor variables, caffeine intake was potentially the stronger mediator of faster caffeine metabolism. This is because caffeine is a known inducer of CYP1A2 activity (Chen et al. 1996; Goasduff et al. 1996; Tantcheva-Poór et al. 1999), and the diabetes patients of the present study consumed larger amounts of caffeine (Table 1). Nineteen out of 57 patients administered insulin that has also been described as an inducer of CYP1A2 activity. When only type-2 diabetes patients were assessed, however, there were no significant differences in CYP1A2 activity between insulin users and non-users. This result suggests that insulin may not be a key driver of CYP1A2 activity in our study participants. Nevertheless, larger samples are needed in future studies to corroborate the existence of higher CYP1A2 activity in the type-2 diabetes patient population, and that this higher activity is due primarily to high caffeine intake.

We found no effect of age and BMI on CYP1A2 enzyme activity (Table 2). This finding was in-line with previous research (Tantcheva-Poór et al. 1999). Furthermore, there was no significant difference between CYP1A2 enzyme activity of participants who reported taking medication over the long-term, compared to participants that were not taking medications. This finding may reflect that medication has varying effects on CYP1A2 activity (inhibition, induction, or no effect), which are drug-specific (Faber et al. 2005). Because we did not collect information regarding the specific medications of participants, it is impossible to further qualify this result.

In contrast to previous studies (Sachse et al. 1999; Tantcheva-Poór et al. 1999), female gender, contraceptive pill use, smoking, and *CYP1A2* genotype also revealed no significant effect on CYP1A2 activity. Female gender has only a small influence on the paraxanthine/caffeine ratio (Tantcheva-Poór et al. 1999), and our sample was probably not large enough to show a significant effect. In addition, the numbers of participants who reported taking oral contraceptives (n = 9) and smoking (n = 21), two fairly strong modulators of CYP1A2 activity (Tantcheva-Poór et al. 1999), were low. These low participant numbers may explain why significant effects of these covariates were not seen. The paucity of

smokers may also explain why, in the present data set, the *CYP1A2* -163 A>C genotype had no significant effect on CYP1A2 enzyme activity and speed of caffeine metabolism; since the more pronounced increase in CYP1A2 activity caused by this genetic variation is only observed in current smokers (Sachse et al. 1999; Sachse et al. 2003).

The exact mechanism that links caffeine intake to speed of caffeine clearance is not yet fully understood. Animal studies have shown increased liver microsome CYP1A2 activity and mRNA levels in rats on very high doses of caffeine (Chen et al. 1996; Goasduff et al. 1996). This observation indicates an auto-induction of caffeine on CYP1A2 (Tantcheva-Poór et al. 1999). Support also comes from epidemiological studies, where a 1.45-fold higher CYP1A2 activity was observed per daily liter of coffee intake (Faber et al. 2005; Tantcheva-Poór et al. 1999). Another suggestion is that persons with existing high CYP1A2 activity may consume more coffee because they metabolize it more quickly (Landi et al. 1996). Coffee is a complex blend of organic compounds and therefore, constituent substances, aside from caffeine, may also contribute to its inducing effect (Tantcheva-Poór et al. 1999). For example, coffee beans are roasted at high temperatures, and thus may contain compounds similar to those found in tobacco smoke or chargrilled meats - known inducers of CYP1A2 activity (Fontana et al. 1999). Moreover, coffee's diverse composition roots the existing controversy between coffee and caffeine consumption and risk of type-2 diabetes (see, Palatini et al. 2015, for review).The limitations of this study include the reliance on self-reports to determine the timing of saliva sampling and the lack of information regarding habitual consumption of some dietary components known to influence CYP1A2 activity, e.g., chargrilled meat, as well as the intake of specific medications. Also habitual caffeine intake was measured by self-report questionnaire. While the validity of this method is established (Addicott et al. 2009; Urry et al. 2016), variability exists in the amount of caffeine per serving (Bracken et al. 2002). Therefore, caffeine use may have been under- or overestimated. The correction technique applied to the paraxanthine/caffeine ratios needs further, external validation. The fitted curve explained roughly 70% of the variance in the Spigset data set (Spigset et al. 1999), leaving 30% unexplained. However, Figure 1 suggests that this proportion of unexplained variance lies at time points greater than 12 hours. We used the equation-derived paraxanthine/caffeine ratio at the 6 hour time point. Finally, despite the regression model significantly predicting the paraxanthine/ caffeine ratio, its explanatory capacity was low. This indicates that other, unknown or unmeasured predictor variables were also influencing CYP1A2 activity in our study sample. Previously, it has been noted that a large proportion of CYP1A2 activity is currently unexplained (Faber et al. 2005).

Conclusions

In conclusion, while various factors probably influence CYP1A2 activity, high caffeine intake likely plays an important role. Here, we provide evidence that a positive association between caffeine consumption and CYP1A2 activity is present in our type-2 diabetes patient sample. Future studies are warranted to establish whether higher CYP1A2 enzyme activity is indeed causally related to high caffeine intake.

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Declaration of conflicting interests

The authors declare no potential conflict of interest, financial or otherwise.

Authors' contributions

The present study has been conceived and designed by HPL, AJ, and EU. The experiments were performed by EU. Samples and data were analyzed by AJ and EU. Data were interpreted by AJ, EU, and HPL. The manuscript was written, revised and approved by EU, HPL, and AJ.

Ethics approval and consent to participate

The study was approved by the review board of the ethics committee at the ETH, Zurich (ethics number: EK 2012-N-53). Experimental protocols were conducted according to the principles of the Declaration of Helsinki. Participants were recruited via advertisements at hospitals, in magazines and at public seminars throughout German-speaking Switzerland. The data were collected after written informed consent was obtained, analyzed anonymously and individual results kept confidential.

Chapter 5:
General Discussion

Wakefulness and sleep take place periodically, and at specific times, during the 24-hour light-dark cycle. The two distinct states of wake and sleep result from interplay between the endogenous circadian clock and homeostatic processes. Cognitive performance is influenced by a person's circadian preference, degree of sleep pressure, and accordingly, the time of day that the cognitive testing takes place. Caffeine ameliorates the negative consequences of sleep deprivation on attentional cognitive processes. However, the stimulant cannot substitute for sleep, and is almost ineffective in mitigating the impact of severe sleep loss on higher-order cognitive domains (memory and executive functions). Caffeine blocks adenosine receptors. Thus, adenosine may play an important role in regulating sleep homeostasis and also attention-related cognitive processes (Chapter 2).

Using observational research methods, caffeine consumption and sleep were investigated in type 2 diabetes patients (see Table 1 for summary of key results). A case-control field study, with an age restriction of 40 to 80 years, compared type 2 diabetes (n = 134) and non-type 2 diabetes (n = 230) participants, in terms of demographic status, health, daytime sleepiness, sleep quality and sleep timing, diurnal preference, mistimed circadian rhythms and habitual caffeine intake (Chapter 3). Participants also gave saliva for *CYP1A2* genotyping and quantification of caffeine concentration. The results revealed that type 2 diabetes patients reported greater daytime sleepiness, a higher prevalence of sleep apnea and napping, and greater habitual caffeine intake. The greater caffeine intake of the patient group stemmed from the consumption of an extra cup of coffee each day, and was confirmed by higher salivary concentrations of caffeine at bedtime. Statistical modelling demonstrated that type 2 diabetes status was associated with higher self-reported caffeine consumption and higher salivary caffeine. Moreover, next to male gender, type 2 diabetes status was the strongest predictor of caffeine intake. Interestingly, subjective sleep and circadian estimates were similar between case and control groups; as was the distribution of the genotype-derived, CYP1A2 enzyme-inducibility phenotype. It was concluded that type 2 diabetes patients may self-medicate with caffeine to alleviate daytime sleepiness.

Case-control comparisons, in a non-age restricted sample, revealed that CYP1A2 enzyme activity, and thus speed of caffeine metabolism, was significantly higher in the type 2 diabetes group (Chapter 4; case: n = 57; control: n = 146). This was corroborated by higher salivary concentrations of caffeine's major metabolite, paraxanthine, at bedtime. Statistical modelling demonstrated that higher habitual caffeine intake was associated with greater

CYP1A2 enzyme activity. It was concluded that high caffeine intake in type 2 diabetes patients may raise CYP1A2 enzyme activity.

Taken together, by interfering with glucose homeostasis and liver enzyme metabolism, caffeine may undermine glycemic control, and influence the clinical efficacy of prescribed medications also metabolized by the CYP1A2 enzyme. Therefore, caffeine may reflect a lifestyle factor to be considered when promoting type 2 diabetes management.

Table 1: Summary of results in present thesis that assessed caffeine intake and sleep quality in type 2 diabetes patients using observational research methods.

Topic	Analysis	Results
Chapter 3		
Demographic characteristics	T2D case *vs*. Non-T2D control groups	○ Similar between groups: age, % home ownership, household density, level of education, % in work, work hours per week, and quality of life / well-being. ○ Higher in T2D group: % male gender, BMI, % married, and shift work.
Health behaviours	T2D *vs*. Non-T2D	○ Similar between groups: level of physical activity, and selection of water as favoured beverage. ○ Higher in T2D group: smoking, chronic medication, adherence to a calorie-controlled / low-sugar diet, and addition of artificial sweetners to hot drinks. ○ Lower in T2D group: alcohol intake, adherence to no specific dietary regime, nutritional supplements, and addition of sugar to hot drinks. ○ Within T2D group: 70 % took oral diabetes medication; 39 % took insulin. ○ < 3 % of both groups advised to avoid caffeine.
Genetic characteristics (*CYP1A2 -163C>A)*	T2D *vs*. Non-T2D	○ Similar between groups: allele (*A*, *C*) and genotype frequencies (A/A, A/C, C/C), and CYP1A2 enzyme inducibility based on *CYP1A2* genotype (AA, highly inducible; A/C, C/C, less inducible). ○ Allele and genotype frequencies in-line with published frequencies in older, non-patient, European populations.

Self-reported circadian estimates	T2D *vs*. Non-T2D	○ Similar between groups: chronotype metric and social jetlag.
Self-reported sleep estimates	T2D *vs*. Non-T2D	○ Similar between groups: average sleep duration, habitual sleep duration, habitual time in bed, sleep efficiency, sleep latency, subjective sleep quality, 'trouble' sleeping, and insomnia. ○ Higher in T2D group: sleep apnea, napping and daytime sleepiness.
Habitual caffeine intake	T2D *vs*. Non-T2D	○ Higher in T2D group: daily caffeine intake, which stemmed from higher coffee intake (+1 cup per day).
Salivary caffeine	T2D *vs*. Non-T2D	○ Higher in T2D group: salivary caffeine concentrations at bedtime.
Prediction of caffeine intake	Hierarchical multiple regression (T2D + Non-T2D groups)	○ T2D status: associated with higher habitual caffeine intake and higher salivary caffeine. ○ T2D status: the strongest predictor of caffeine intake, after male gender.
Chapter 4		
Paraxanthine/caffeine ratio (CYP1A2 enzyme activity)	T2D *vs*. Non-T2D	○ Higher in T2D group: CYP1A2 enzyme activity, and thus speed of caffeine metabolism.
Prediction of CYP1A2 enzyme activity	Multiple regression (simultaneous entry) (T2D + Non-T2D groups)	○ Higher caffeine intake: associated with higher CYP1A2 enzyme activity.

Abbreviations: %, percentage; BMI, body mass index; CYP1A2, cytochrome P450 1A2 enzyme; *CYP1A2,* cytochrome P450 1A2 gene; Non-T2D, non-type 2 diabetes; T2D, type 2 diabetes.

Daytime sleepiness and cognition in type 2 diabetes

Daytime sleepiness in type 2 diabetes

Elevated daytime sleepiness is a typical feature of type 2 diabetes (Chapter 3). This increased sleepiness has been related to patients' hyperglycemia (West et al. 2006). Obstructive sleep apnea, which typically co-exists with type 2 diabetes, in particular in the presence of autonomic neuropathy (Moon et al. 2015), is also linked to daytime sleepiness (West et al. 2006). Sleep apnea is a group of chronic, sleep-related breathing disorders, which are characterized by the occurrence of disordered breathing events during sleep. These events are classified into two main categories: obstructive and central. The classification of an event as obstructive or central depends whether, in the absence of airflow, there is ongoing respiratory effort. The more prevalent, obstructive sleep apnea, is characterized by the predominance of recurrent obstructive events that stem from partial or complete collapse of the upper airway during sleep. The resulting cessation of airflow (apneas) or decrease in airflow (hypopneas) is associated with a decrease in oxyhaemoglobin saturation, arousal from sleep, sleep fragmentation and sleep disturbance. The apnea–hypopnea index (AHI) is used to diagnose the presence and severity of sleep apnea (normal: AHI < 5 events per hour of sleep) (Moon et al. 2015). Interestingly, population-based and clinical studies using objective measures of breathing and glycemic control, have consistently demonstrated an independent association between obstructive sleep apnea and metabolic abnormalities (e.g. impaired fasting glucose, glucose intolerance, and type 2 diabetes) (see Punjabi et al. 2003, for review). Potential mediators of this association include altered adrenergic function, the direct effects of hypoxemia on glucose regulation and release of pro-inflammatory cytokines that affect metabolism (Punjabi et al. 2003). More recently, controlled laboratory studies have indicated a causal link between sleep disturbance (typical in sleep apnea) and impaired glucose homeostasis (Buxton et al. 2010; Leproult et al. 2014; Tasali et al. 2008). On the other hand, the possibility of reverse causation (i.e. metabolic abnormalities leading to obstructive sleep apnea) has also been considered. However, this area of research remains undeveloped and is limited by the difficulty of establishing the direction of causality; as a result, potential associations are speculative (Moon et al. 2015). For example, diabetic neuropathy may increase upper-airway collapsibility and increase the likelihood of obstructive, disordered breathing events; while the abnormal ventilatory responses seen in type 2 diabetes patients (Weisbrod et al. 2005) may increase the risk of central sleep apnea (Moon et al. 2015). Accordingly, a recent review by Moon and colleagues (Moon et al. 2015) describes a tentative model that links metabolic abnormalities, such as type 2 diabetes, and obstructive sleep apnea in a bi-directional manner (see Figure 1). The mechanisms through which intermittent hypoxemia

and sleep fragmentation could affect glucose metabolism are also summarised in Figure 1 and include: alterations in sympathetic nervous system activity; changes in the activity of the hypothalamic-pituitary-adrenal axis; formation of reactive oxygen species; increases in inflammatory cytokines (interleukin-6, IL-6; tumor necrosis factor - α, TNFα) and adipocyte derived factors (leptin, adiponectin, and resistin). However, the authors highlight that research confirming such causal links is still in its infancy. A key challenge to scientists is to identify the modifying effects of the shared risk factors of age and obesity on the mechanisms potentially linking the two conditions (Moon et al. 2015).

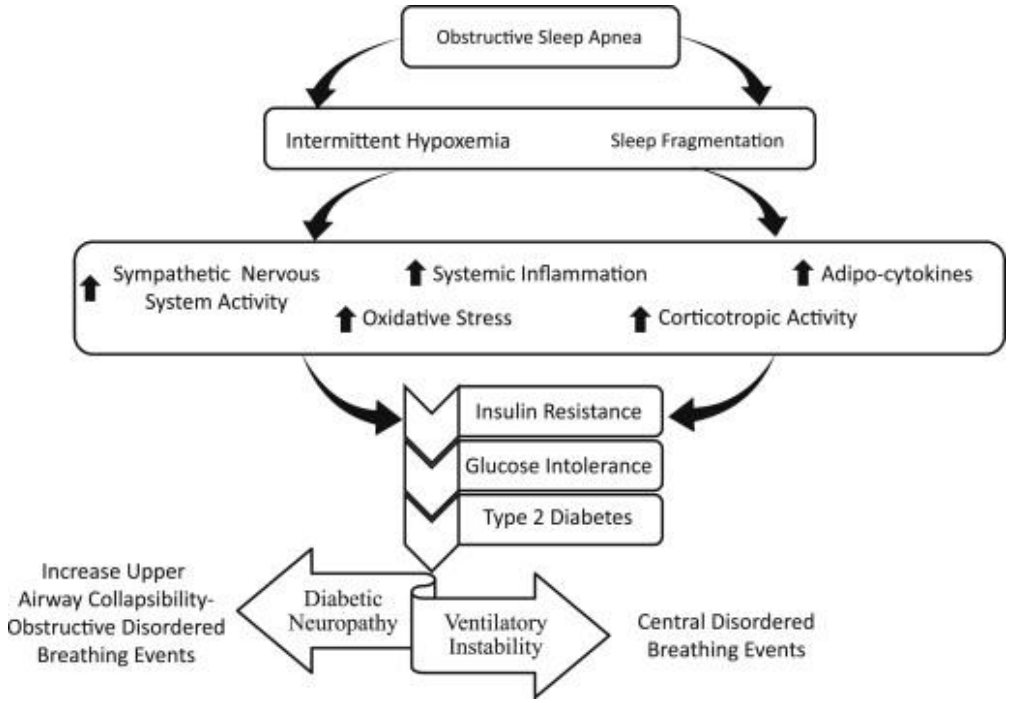

Figure 1: Associations and potential causal links between obstructive sleep apnea and metabolic abnormalities (e.g. type 2 diabetes). Source: Moon et al. 2015.

Increased subjective sleepiness also arises from sleep disruption (Chapter 2). Sleep in type 2 diabetes patients has not been extensively studied - a literature search revealed three studies (Lecube et al. 2016; Trento et al. 2008; Resnick et al. 2003) that assessed sleep in a reliable, case-control manner, using objective methods (EEG or actigraphy). Nonetheless, these preliminary data indicate that indeed, objective sleep is disturbed in type 2 diabetes, and thus may contribute to daytime sleepiness. For example, three consecutive days of

wrist-actigraphy, under free-living conditions, revealed that compared to a healthy control group (n = 23), type 2 diabetes patients (n = 47) moved significantly more in bed, and had significantly more fragmented and less efficient sleep. The authors suggested that these sleep disturbances may be caused by impaired glucose metabolism or the physical discomfort associated with the disease (Trento et al. 2008). However, these findings are limited by the small sample size and short duration of the actigraphy assessment. Resnick and colleagues (Resnick et al. 2003) used at-home polysomnography (1 night) to compare the sleep of a diabetes sample (n = 470) to that of a non-diabetes control group (n = 4,402). The results revealed poorer objective sleep in the diabetes patients: they spent significantly more time in light sleep (stages 1 and 2 of NREM sleep); significantly less time in deeper sleep (stages 3 and 4 of NREM sleep; and REM sleep); and showed greater respiratory disturbance. However, after the authors adjusted the results to account for the confounding effects of age, gender, body mass index, ethnicity and neck circumference, significant differences between case and control groups disappeared, apart from the effects on REM sleep and nocturnal breathing. Nonetheless, despite the large sample size, these findings are limited by the short-duration of the polysomnography assessment, the non-controlled home environment, and the possibility of the 'first-night' effect. A more reliable approach would be to assess sleep over several days in the controlled setting of a sleep laboratory (e.g. 1 adaption night; 2 assessment nights). The study of Lecube and colleagues (Lecube et al. 2016) revealed that type 2 diabetes may have a distinctive sleep bio-signature. That is, compared to a control group, the patient group was shown to exhibit increased sleep fragmentation through higher rates of microarousals during NREM sleep; and increased intermittent hypoxia during REM sleep. The authors highlight the importance of such findings, since both sleep fragmentation and intermittent hypoxia are thought to be key mechanisms by which sleep breathing disorders exert their negative metabolic, hemodynamic, inflammatory and vascular effects (Lecube et al. 2016). A strength of this study was the case-control approach that matched case (n = 76) and control (n = 76) participants by age, gender, BMI, and waist and neck circumference. However, a significant flaw was the short duration of the EEG assessment – only one night in the laboratory.

Much data demonstrate that pro-inflammatory cytokines (e.g. interleukin-1β, IL-1 β; TNFα) are involved in the physiological regulation of sleep (see Opp, 2005, for review). Interestingly, cytokines TNFα and interleukin-6 (IL-6) have been shown to be elevated in patients reporting excessive sleepiness (sleep apnea and narcolepsy patients; Vgontzas et al. 1997); and thus, it has been proposed that inflammatory cytokines may mediate daytime sleepiness (Vgontzas et al. 1997). A chronic low-grade inflammation and an activation of the immune system are involved in the pathogenesis of obesity-related metabolic disorders, such as type 2 diabetes (see Esser et al. 2014, for review). Accordingly, type 2 diabetes

patients may feel sleepy due to elevated circulating cytokines (Vgontzas et al. 2005). Overall, as depicted by the heuristic model in Figure 2, there is possibly a complex, feed-forward relationship between visceral fat and insulin resistance, inflammatory cytokines, stress hormones, daytime sleepiness, and sleep apnea (Vgontzas et al. 2005). That is, obesity and insulin resistance, determined by genetic, constitutional and environmental factors, progressively lead to worsening metabolic control and sleep apnea. Sleep apnea may subsequently lead to a worsening of visceral obesity and the metabolic syndrome by providing a stress stimulus, and causing nocturnal elevations of hormones (e.g. cortisol and insulin) that promote visceral adiposity, metabolic abnormalities and cardiovascular complications (Vgontzas et al. 2005).

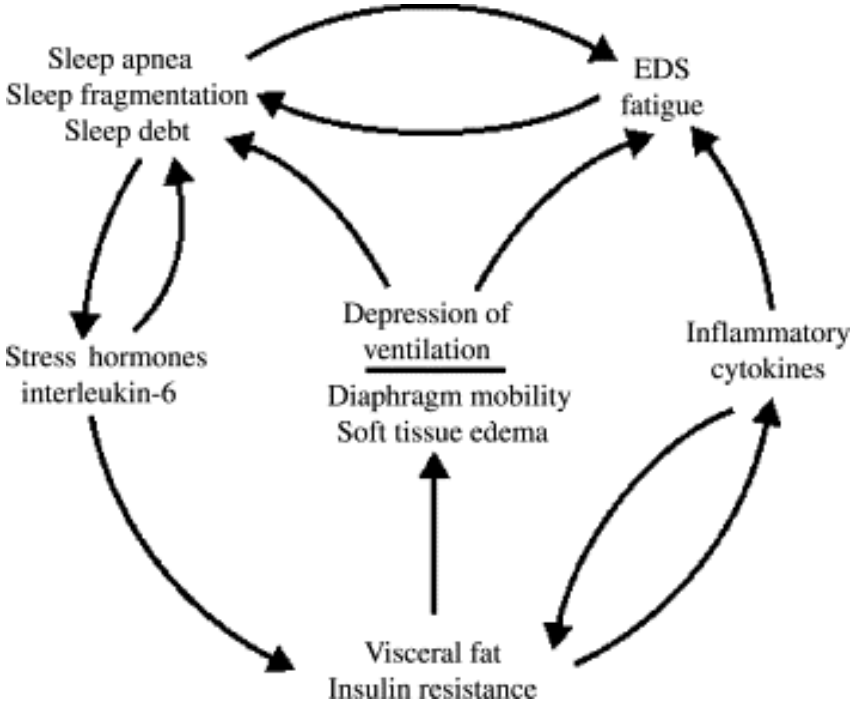

Figure 2: A heuristic model of the complex feed-forward associations between visceral fat / insulin resistance, inflammatory cytokines, stress hormones, excessive daytime sleepiness (EDS) and fatigue, and sleep apnea. Source: Vgontzas et al. 2005.

The type 2 diabetes patients assessed in the present case-control field study (Chapter 3) reported greater daytime sleepiness than the non-diabetes control group. The observational design of the study, and the lack of objective measurements of plasma hormones, sleep, and nocturnal breathing, indicates that it is impossible to discern the exact cause(s) of

sleepiness within this sample. However, the heterogeneous nature of type 2 diabetes (Inzucchi et al. 2012) suggests that, regardless, sleepiness in each patient would be likely derived from a complex blend of metabolic, sleep and nocturnal breathing abnormalities.

Cognition in type 2 diabetes

Daytime sleepiness is not necessarily an indicator of cognitive impairment. Indeed, subjective sleepiness (measured by questionnaires) and objective alertness (measured by performance on attention-focused cognitive tests; e.g. the PVT) may not be quantitatively correlated (Leproult et al. 2003; Chapter 2). Nonetheless, it is well-documented that cognitive dysfunction is seen in type 2 diabetes groups (see Awad et al. 2004; and Biessels et al. 2002; for reviews). That is, cognitive decrements in treated type 2 diabetes patients have most consistently been observed on measures of verbal memory and processing speed; while relatively preserved function has been observed on measures of visuospatial, attention, semantic and language function (Awad et al. 2004). Cross-sectional studies, which compare type 2 diabetes patients to healthy controls, report cognitive decrements across the three main cognitive domains (see Figure 1, Chapter 2); in particular, in psychomotor efficiency, learning and memory, and executive function (Awad et al. 2004). Longitudinal studies largely support this view. For example, Rawlings and colleagues (Rawlings et al. 2014) examined the association of diabetes, assessed in middle age, with subsequent 20-year cognitive decline in a population-based sample (N = 13,351). Diabetes in midlife was associated with a 19 % greater cognitive decline, compared with no diabetes. This global decline in cognitive function was driven by impairments in processing speed ('attention' domain) and executive function; and to a lesser extent, impairments in verbal learning and memory. Participants with poorly controlled diabetes had greater decline than those whose diabetes was controlled; moreover, longer-duration diabetes was linked to greater late-life cognitive decline (Rawlings et al. 2014). Regarding the potential mechanisms linking type 2 diabetes to cognitive impairment: in a recent review, Umegaki (Umegaki, 2015) highlighted that the disease seems to affect cognition in a wide range of clinical conditions, with and without neurodegeneration. That is, type 2 diabetes is an established risk factor for cognitive impairment, all-cause dementia, Alzheimer disease (AD), vascular dementia, and cognitive impairment in Parkinson disease. Thus, it is unlikely that diabetes impairs cognitive function via a single, common mechanism. Instead, as depicted in Figure 3, multiple pathogenic aspects of type 2 diabetes may directly and indirectly contribute to cognitive dysfunction, via several mediating mechanisms (Umegaki, 2015). For example, epidemiological studies have demonstrated that insulin resistance, one of the main pathophysiological features of type 2 diabetes, is associated with AD (Schrijvers et al. 2010); a Japanese cohort study revealed that the severity of AD pathology in autopsy samples correlated with pre-mortem insulin

resistance (Matsuzaki et al. 2010); and, rodent studies (mice) indicated that dysfunction of insulin signalling in neurons may accelerate tau phosphorylation, via glycogen synthase kinase (Schubert et al. 2013). Type 2 diabetes also affects the vascular system, making vascular dysfunction a potential contributor to diabetes-related, cognitive decline, especially in the presence of ischaemia. However, whether type 2 diabetes-associated cognitive dysfunction is mainly attributable to an AD-related neurodegenerative process, or a vascular contribution, is not yet understood. Finally, hyperglycemia may also contribute to cognitive dysfunction (Umegaki, 2015): in a randomised controlled trial, with a 5-year, follow-up period, comparison of measures of glycemic control (AIC) and cognitive function suggested that good management of blood glucose may delay global cognitive decline in type 2 diabetes (Luchsinger et al. 2011).

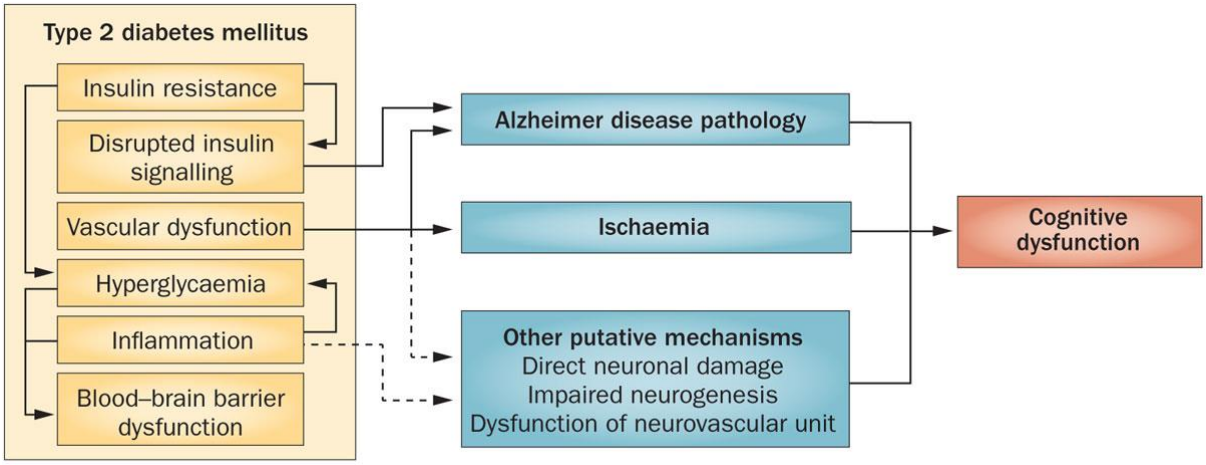

Figure 3: Potential mechanisms linking type 2 diabetes to cognitive impairment. Source: Umegaki, 2015.

Positive and negative effects of caffeine

Compared to a control group, the type 2 diabetes sample assessed in the present thesis consumed high amounts of caffeine on a daily basis. This high caffeine consumption stemmed from a high intake of coffee, equivalent to an extra cup of coffee each day (Chapter 3). The CYP1A2 enzyme activity, and thus speed of caffeine metabolism, was also higher in the diabetes group compared to a control group; this may be due to their high caffeine intake (Chapter 4).

Effects of caffeine on CYP1A2 enzyme activity

There are advantages and disadvantages to increased liver-enzyme activity (Faber et al. 2005). On one hand, greater activity is expected to shorten exposure to xenobiotics (e.g. environmental toxins), by accelerated catalysis of metabolic steps in elimination. On the other hand, if the enzyme is involved in the metabolism of prescribed drugs, induction can cause non-response to therapy, due to medications being eliminated faster than expected, resulting in lowered *in-vivo* drug concentrations (Faber et al. 2005). In the present type 2 diabetes sample, higher CYP1A2 activity would lead to faster metabolism of caffeine. This could be advantageous given caffeine's negative effects on glucose metabolism (Lane et al. 2004). However, this increased CYP1A2 activity may also reduce the therapeutic impact of patients' pharmaceutical treatment regimes. Although first- and second-line treatments for type 2 diabetes (metformin, sulfonylureas, thiazolidinediones, DPP-4 inhibitors, GLP-1 receptor agonists, insulin; Inzucchi et al. 2012) are not known substrates of CYP1A2 (Faber et al. 2005; website: http://medicine.iupui.edu/clinpharm/ddis/); elimination of concomitant medications may involve this enzyme. For example, type 2 diabetes typically co-exists with cardiovascular disorders (Inzucchi et al. 2012). The drug warfarin is the most commonly prescribed oral anticoagulant in North America, and it is partly metabolised by CYP1A2 (Holbrook et al. 2005).

Aside from caffeine's influence on enzyme CYP1A2, the stimulant has been shown to have positive and negative effects on the brain and on the body.

Effects of caffeine on cognition and mood

In both rested and sleep-restricted individuals, acute caffeine administration can improve measures of cognitive function and mood (see Haskell et al. 2005, and Ruxton, 2008, for review). Caffeine is thought to impact mood and performance via its antagonism of adenosine receptors (Fredholm et al. 1999). The most commonly reported experimental effects of caffeine are improvements to 'attentional' cognitive processes; specifically, increases in ratings of alertness (Rogers et al. 2003; Quinlan et al. 2000) and improvements in measures of reaction time and vigilance (Lieberman et al. 1987; Richardson et al. 1995; Smit & Rogers 2000). There are also reports of positive effects of caffeine on higher-order cognitive processes [information processing, memory and logical reasoning (Smith et al. 1994; Smit & Rogers 2000; Warburton et al. 2001)]; although the benefits of caffeine to memory and executive functions are less well supported (Haskell et al. 2005). For example, Haskell and colleagues (Haskell et al. 2005) used a placebo-controlled, double-blind, crossover study to investigate the acute cognitive and mood effects of caffeine in rested and healthy habitual users and habitual non-users of caffeine. In both groups, caffeine

significantly improved attentional processes (simple reaction time, digit vigilance reaction time, and reports of fatigue and alertness); as well as specific attention / memory (numeric working memory reaction time) and executive functions (sentence verification accuracy). These findings indicate that acute caffeine intake elicits absolute cognitive and mood benefits, over and above the alleviation of the negative, subjective and objective, effects of caffeine withdrawal (Haskell et al. 2005). In sleep deprived persons, caffeine typically attenuates the impairments seen to attentional process (e.g. sleepiness, alertness, vigilance, sustained attention; Chapter 2). However, caffeine has little effect at mitigating the impact of sleep loss on higher-order cognitive functions (e.g. encoding and consolidation of memory, memory retrieval, decision making, updating and monitoring of information, inhibition of automatic responses; see Chapter 2). In type 2 diabetes patients, cognitive decrements are seen across the three main cognitive domains (Awad et al. 2004). Based on research in rested, and sleep-deprived, healthy participants, it could be suggested that caffeine may improve some of the cognitive impairments seen in these patients; in particular, impairments related to 'attention' (e.g. sleepiness, alertness, vigilance). An empirical investigation into the potential short-term cognitive and mood benefits of caffeine in type 2 diabetes has not yet been reported in the literature. Also of relevance is evidence from epidemiological studies that indicates that long-term caffeine consumption is linked to reduced cognitive decline (Arab et al. 2013). In addition, a prospective study related coffee drinking at midlife to a decreased risk of dementia and Alzheimer's disease in later life (Eskelinen et al. 2009). Given that type 2 diabetes has been linked to greater late-life cognitive decline (Rawlings et al. 2014), and to Alzheimer's disease (Umegaki, 2015), habitual caffeine consumption may be beneficial for the long-term cognitive health of these patients.

Effects of caffeine on sleep

On the other hand, it is well-documented that caffeine has negative effects on sleep (Chapter 2 and 3), via a disruption of sleep homeostasis. A recent systematic review explored evidence from epidemiological studies and randomised controlled trials (Clark & Landolt, 2016). It was established that caffeine typically prolongs sleep latency, reduces total sleep time and sleep efficiency, and worsens perceived sleep quality; moreover, SWS and slow wave activity were generally reduced; while stage 1, wakefulness and sleep arousals were increased (Clark & Landolt, 2016). Such findings are noteworthy given that sleep restriction and suppression of SWS have been linked to reduced glycemic control (Buxton et al. 2010; Leproult et al. 2014; Tasali et al. 2008). For example, a rigorously controlled, laboratory experiment showed that 100 mg of oral caffeine at bedtime significantly prolonged sleep latency, reduced SWS in the first sleep cycle and impaired sleep efficiency (Landolt et al. 1995a). Moreover, the equivalent of caffeine contained in one or two double-expressos

(200 mg), ingested up to 16 hours before sleep, also induced reliable changes in the sleep EEG, indicative of more superficial sleep. Specifically, total sleep time and sleep efficiency were significantly reduced; and SWS was lower, although not to a significant extent (Landolt et al. 1995b). Such findings were despite evidence that salivary caffeine levels were close to zero at the beginning of the sleep period (Landolt et al. 1995b; Landolt et al. 2004).

Effects of caffeine on skeletal muscle

In the body, or 'periphery', both pure caffeine (Greer et al. 2001; Lane et al. 2004) and caffeinated coffee (Moisey et al. 2008) have been shown to significantly impair glucose homeostasis. That is, glucose disposal is reduced by approximately 22%, due to an acute reduction in insulin sensitivity (Greer et al. 2001; Lane et al. 2004) (Chapter 1; Chapter 3). Consequently, it has been suggested that caffeine abstinence in type 2 diabetes may have beneficial effects, which are comparable to oral medications used to control postprandial glucose (Lane et al. 2004). However, it is noteworthy that these detrimental effects have only been shown in sedentary participants (Greer et al. 2001; Lane et al. 2004; Moisey et al. 2008). Conversely, in physically active participants, acute caffeine administration improves athletic performance (see Shearer & Graham, 2014, for review). Indeed, the positive effects of caffeine on athletic performance can be seen in various physical activities and across a range of exercise intensities (from 1 minute, up to more than 90 minutes). These opposing effects of caffeine - the positive impact on physical performance (in active persons), but the negative impact on glycemic control (in resting persons) - are thought to be mediated by skeletal muscle; although the adenosine-related mechanisms and molecular pathways are incompletely understood (Shearer & Graham, 2014). During physical activity, muscle power, and thus performance, is thought to be enhanced by caffeine due to sparing of muscle glycogen, enhanced glucose and fatty acid uptake, and reduced lactate accumulation (Graham, 2001). Yet, in resting muscle, caffeine impedes glucose uptake, potentially via an interaction between insulin-stimulated mechanisms (Derave et al. 1999; Han et al. 1998; Vergauwen et al. 1994). While it is surprising that this effect is not seen in moving muscle, in this active state, plasma insulin has declined to very low levels (Shearer & Graham, 2014). In sedentary persons, adenosine antagonism may alter the body's use of glucose via changes to extracellular delivery, cell membrane signalling, and / or intracellular signalling and processing (Halseth et al. 2000; Wasserman et al. 1998; Wasserman et al. 2005).

The present type 2 diabetes group

The type 2 diabetes group assessed in the present thesis reported high habitual caffeine intake (Chapter 3). Perhaps surprisingly, this high caffeine consumption did not appear to impede their (subjective) sleep quality. Indeed, self-reported sleep duration and sleep quality

were similar to the non-diabetes control group, and also were typical of a healthy, aging population (Bliwise, 2005; Buysse et al. 1989; Roenneberg et al. 2012). However, warranting concern is objective evidence that our diabetes group had high salivary caffeine concentrations at bedtime (mean: 14 µmol/l; n = 123). This concentration is close to that reached one hour after the intake of 200 mg caffeine (equivalent to a 'strong' double expresso), following two weeks of caffeine abstinence (16 – 20 µmol/l; n = 30; Landolt et al. 2012). During the sleep period, participants would naturally be physically inactive, and thus their skeletal muscle, in a largely resting state. The literature demonstrates that caffeine impedes glucose homeostasis in sedentary participants; this is reflected by reduced glucose disposal, mediated by increased insulin resistance of skeletal muscle (Greer et al. 2001; Lane et al. 2004; Moisey et al. 2008). Importantly, this impediment has been shown in habitual caffeine users (Lane et al. 2004; Moisey et al. 2008), as well as non-users (Greer et al. 2001); this suggests that regular caffeine consumption, and the potential development of caffeine tolerance, does not remove the negative impact of caffeine on resting muscle. Thus, it can rationally be suggested that the nocturnal glycemic control of the present type 2 diabetes patients may be compromised by the high levels of caffeine in their body. The duration of this impediment is not yet clear, since published studies (e.g. Greer et al. 2001; Lane et al. 2004; Moisey et al. 2008) only assess glucose homeostasis for 2 to 3 hours post caffeine dose. Moreover, the speed of caffeine metabolism, and thus half-life of caffeine, shows substantial inter-individual variability (Blanchard & Sawers, 1983), largely due to differences in CYP1A2 enzyme activity (see Chapter 4, and Faber et al. 2005, for review). Nonetheless, the detrimental effect of caffeine on glucose disposal may continue well into the sleep episode.

Glycemic control and cognition in type 2 diabetes: caffeine intake and sleep quality considerations

Despite the negative effects of caffeine on glycemic control and sleep, a recommendation of total caffeine abstinence in type 2 diabetes may be short-sighted. The acute and chronic benefits of caffeine to cognition cannot be ignored; moreover, epidemiological studies consistently link habitual, high coffee consumption with reduced risk of type 2 diabetes (Ding et al. 2014). An alternative approach would be to attempt to harness the benefits of caffeine, while avoiding, or at least ameliorating, the negatives. This could potentially be achieved by restricting caffeine intake to healthy / safe levels, and importantly, monitoring the timing of caffeine consumption across the day.

The habitual caffeine intake of patients can be assessed by self-report questionnaire. If a detailed record of dietary sources of caffeine is required, the questionnaire shown in Appendix III can reliably be used. Statistical analyses showed that self-reported caffeine intake, measured using this complex questionnaire, was significantly correlated with salivary caffeine concentrations (r = 0.360; $p < 0.001$; n = 393) (see Supplementary Figure 1, Appendix III). However, if time is limited, a simpler questionnaire can also be utilized. This short questionnaire would ask subjects to report their daily consumption of coffee, tea, cola, energy drinks, chocolate and caffeine pills. Self-reported caffeine intake, recalculated to assume this simple set of questions, was also significantly correlated with salivary caffeine concentrations (r = 0.321; $p < 0.001$; n = 393) (see Supplementary Figure 2, Appendix III). Moreover, the estimates of habitual caffeine intake, assessed by the simple and complex questionnaires, were significantly correlated with each other (r = 0.842; $p < 0.001$; n = 435) (see Supplementary Figure 3, Appendix III).

Regarding recommendations for appropriate daily caffeine intake: A recent report by the European Food Safety Authority (EFSA NDA Panel, 2015) concluded that single doses of caffeine up to 200 mg, from any caffeine source, have no safety concerns for the general adult population; total caffeine intake should not exceed 400 mg per day (EFSA NDA Panel, 2015). These recommendations are in line with previous research that investigated the dose-dependent, health-related effects of caffeine (see Nehlig, 2015, for review).

Regarding appropriate timing of caffeine intake: After oral ingestion of caffeine, absorption to the blood stream is rapid, with plasma concentrations peaking after approximately 30 minutes (Blanchard & Sawers, 1983). The subsequent speed of caffeine metabolism, and thus half-life of caffeine, shows high inter-individual variability (2.7 to 9.9 hours) (Blanchard & Sawers, 1983). The half-life of caffeine can be determined by administering an oral caffeine dose to participants that have fasted from caffeine, alcohol and tobacco for 3 days previously. Post caffeine dose, blood is sampled at regular time intervals for 24 hours. Plasma caffeine concentrations can then be measured using HPLC methods (Blanchard & Sawers, 1983). However, while this protocol is highly accurate, it is invasive, expensive and time-consuming. An alternative and simpler method can also be used (Fuhr & Rost, 1994; Tantcheva-Poór et al. 1999). This method approximates the caffeine half-life by estimating the CYP1A2-mediated, half-life of caffeine. Here, 12-hour, caffeine-fasted participants are administered a known dose of oral caffeine. Prior to the caffeine dose, saliva samples are taken. A second saliva sample is taken 6 hours after the caffeine dose. Subsequently, salivary caffeine and paraxanthine concentrations are measured using HPLC. The following equation can then be used to estimate caffeine clearance (Tantcheva-Poór et al. 1999):

$$Cl = [(C_{para\text{-}post} - C_{para\text{-}pre}) \times 11.681] \ / \ [(C_{caff\text{-}post} - C_{caff\text{-}pre}) \times (t_{\text{-}post} - t_{\text{-}pre})]$$

Such that: Cl = caffeine clearance (ml/minute per kilogram body weight); C = concentration (nmol/l); *para* = paraxanthine; *caff* = caffeine; *post* = post caffeine dose; *pre* = before caffeine dose; t = time between saliva samples (hours).

Having estimated the caffeine clearance, the following two equations can be used, sequentially, to calculate the approximate half-life of caffeine (Ritter et al. 2008):

1) $kel = Cl / Vd$

2) $T^{half\text{-}life} = \ln (2) \ / \ kel$

Such that: kel = elimination rate constant; Cl = caffeine clearance (ml/minute per kilogram body weight); Vd = volume of distribution for caffeine (l/kg; in humans, Vd = 0.61 l/kg body weight; Benet et al. 1996); T = time; ln = log.

Finally, having determined the caffeine half-life of a patient, the following equation, adapted from Nova and colleagues (Nova et al. 2012), could be used to estimate circulating caffeine concentrations at bedtime. Thereafter, caffeine intake across the day could be adapted as necessary:

$$C_b = (C_1 \times 0.5^{(T_b - T_1) / half\text{-}life}) + (C_2 \times 0.5^{(T_b - T_2) / half\text{-}life}) + (C_3 \times 0.5^{(T_b - T_3) / half\text{-}life}) + \text{etc.}$$

Such that: C_b = caffeine at bedtime (mg); C_1 = caffeine content of first caffeine portion (mg); T_b = bedtime (decimal hours); T_1 = time of first caffeine portion (decimal hours); *half-life* = half-life of caffeine (decimal hours); C_2 = caffeine content of second caffeine portion (mg); T_2 = time of second caffeine portion (decimal hours); etc. For further details and examples see Appendix III.

For example, assuming a caffeine half-life of 4.8 hours, and a bedtime of 23:00, consuming a double expresso (c.154 mg caffeine) at breakfast (08:00) and lunchtime (13:00) leads to an approximate bedtime, plasma caffeine of 54 mg. If the double expresso is substituted for a single expresso (c.77 mg caffeine), the approximate bedtime, plasma caffeine reduces to 27 mg. It should be noted, however, that before concise recommendations regarding the

appropriate timing of caffeine intake can be made, more research is required to clarify the dose-dependent effects of caffeine on glycemic control and sleep in resting persons.

Regarding sleep quality: sleep restriction and suppression of SWS impede glycemic control via an acute reduction in insulin sensitivity (Buxton et al. 2010; Leproult et al. 2014; Tasali et al. 2008). There is a dose-response effect of caffeine on sleep (Clark & Landolt, 2016): objective sleep quality progressively worsens with increasing doses of caffeine, as quantified by sleep latency, wake-after-sleep-onset, sleep duration and sleep efficiency (Hindmarch et al. 2000; Karacan et al. 1976; Rosenthal et al. 1991). Moreover, the time spent in SWS tends to decrease with progressive increase in caffeine dose (Karacan et al. 1976). Overall, these data indicate that restricting caffeine intake to earlier in the day in order to promote nocturnal glycemic control, may also ameliorate the adverse impact of caffeine on sleep quality.

Closing words

The American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) do not provide recommendations related to caffeine and coffee consumption in type 2 diabetes patients. Existing research, as well as the novel data presented in this thesis, indicate that caffeine assessment is warranted. Caffeine influences the body and the brain in ways that are relevant to type 2 diabetes sufferers. That is, it has divergent effects on skeletal muscle that impact glucose homeostasis. In addition, caffeine modulates attentional cognitive processes and sleep quality. Restricting total daily caffeine intake, and monitoring the timing of intake, may help patients to harness the positive effects of caffeine, but ameliorate the negative effects.

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Abbreviations

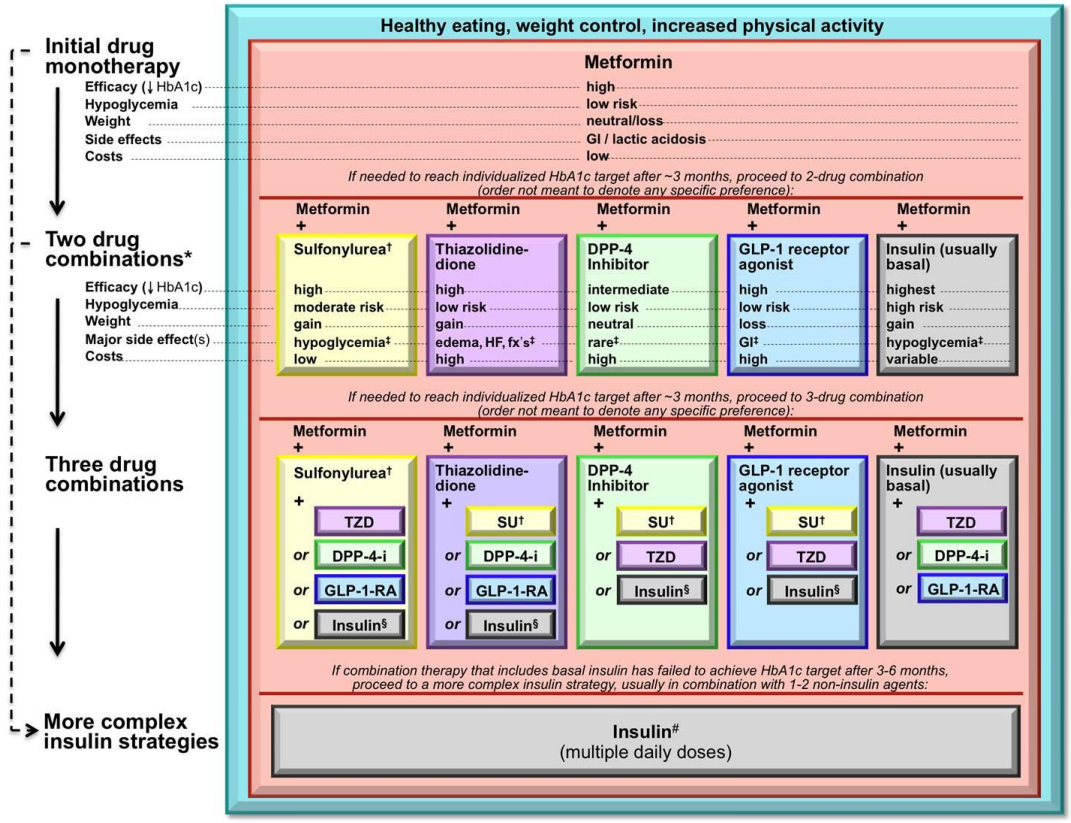
5'-N	5'-nucleotidase
A	A allele
A_1	Adenosine receptor 1
A_{2A}	Adenosine receptor 2A
A_{2B}	Adenosine receptor 2B
A_3	Adenosine receptor 3
A1C	Glycated haemoglobin
AD	Alzheimer disease
ADA	Adenosine deaminase
ADA	American Diabetes Association
ADORA2A	Adenosine receptor 2A gene
ADP	Adenosine-di-phosphate
AHI	Apnea–hypopnea index
AK	Adenosine kinase
AMP	Adenosine-mono-phosphate
ATP	Adenosine-tri-phosphate
B	Unstandardized coefficient
BDNF	Brain-derived neurotrophic factor
Beta	Standardized coefficient
BF	Basal forebrain
BMI	Body mass index
BQL	Below quantification limit
C	C allele
Ca	Calcium
CBT	Core body temperature
CNT	Sodium-driven concentrative transporters
CPAP	Continuous positive air-way pressure
CYP1A2	Cytochrome P450 1A2 enzyme
CYP1A2	Cytochrome P450 1A2 gene
DMH	Dorsal medial hypothalamus
DNA	Deoxyribonucleic acid
DPP-4	Dipeptidyl peptidase-4
EASD	European Association for the Study of Diabetes
ECG	Electrocardiogram

EDS	Excessive daytime sleepiness
EEG	Electroencephalogram
EFSA	European Food Safety Authority
EMG	Electromyogram
ENT	Equilibrative nucleoside transporters
EOG	Electrooculogram
ER	Endoplasmic reticulum
ESS	Epworth Sleepiness Scale
fMRI	Functional magnetic resonance imaging
FPG	Fasting plasma glucose
G	G allele
GABA	*Gamma*-aminobutyric acid
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
HDL	High-density lipoprotein
HPLC	High performance liquid chromatography
HT	Haplotype
IGT	Iowa Gambling Task
IGTT	Intravenous glucose tolerance test
IL-1 β	Interleukin-1β
IL-6	Interleukin-6
K	Potassium
KSS	Karolinska Sleepiness Scale
LC	Locus coeruleus
LH	Lateral hypothalamus
MCTQ	Munich Chronotype Questionnaire
MEQ	Morningness-Eveningness Questionnaire
mGluR5	Metabotropic glutamate receptors of subtype 5
MMTT	Mixed-meal tolerance test
NMDA	N-Methyl-Daspartic acid
Non-T2D	Non-type 2 diabetes
NREM	Non-rapid-eye movement (sleep)
OGTT	Oral glucose tolerance test
P	P-value
PET	Positron emission tomography
PSQI	Pittsburgh Sleep Quality Index
PVT	Psychomotor vigilance task

Ref	Reference
rPVT	Rat version of psychomotor vigilance task
REM	Rapid-eye movement (sleep)
SAH	S-adenosyl-homocysteine
SAHH	S-adenosyl-homocysteine hydrolase
SCALES	Visual analog scales
SCN	Suprachiasmatic nucleus
SNARE	Soluble NEF (N-ethylmaleimide-sensitive factor) attachment protein receptor
SNP	Single nucleotide polymorphism
STROBE	Strengthening the Reporting of Observational Studies in Epidemiology
SWA	Slow-wave activity
SWS	Slow-wave sleep
T	T allele
T2D (M)	Type 2 diabetes (mellitus)
TCF7L2	Transcription factor 7-like 2 gene
TMN	Tuberomammillary nucleus
TNFα	Tumor necrosis factor - α
UV	Ultraviolet
VLPO	Ventro-lateral-preoptic area
vmPFC	Ventromedial prefrontal cortex
WHO-5	World Health Organization Well-Being Index

Appendix I:

Supplementary information to chapter 1



Supplementary Figure 1: Anti-hyperglycemic therapy in type 2 diabetes: general recommendations. Moving from the top to the bottom of the figure, potential sequences of anti-hyperglycemic therapy. In most patients: begin with lifestyle changes; metformin monotherapy is added at, or soon after, diagnosis (unless there are explicit contraindications). If the HbA1c target is not achieved after ~3 months, consider one of the five treatment options combined with metformin: a sulfonylurea, TZD, DPP-4 inhibitor, GLP-1 receptor agonist or basal insulin. Choice is based on patient and drug characteristics, with the overriding goal of improving glycemic control while minimising side effects. Shared decision-making with the patient may help in the selection of therapeutic options. The figure displays drugs commonly used both in the USA and / or Europe. Insulin is likely to be more effective than most other agents as a third-line therapy, especially when glycated haemoglobin (AIC) is very high (e.g. ≥ 9.0%). The therapeutic regimen should include some basal insulin before moving to more complex insulin strategies. Dashed arrow line on the left-hand side of the figure denotes the option of a more rapid progression from a two-drug combination directly to multiple daily insulin doses, in those patients with severe hyperglycemia (e.g. AIC ≥ 10.0–12.0%).

[*] Consider beginning at this stage in patients with very high AIC (e.g. ≥ 9%). [†] Consider rapid-acting, non-sulfonylurea secretagogues (meglitinides) in patients with irregular meal schedules or who develop late postprandial hypoglycemia on sulfonylureas. [‡] In Inzucchi et al. (2012; p. 1584 - 1585), see text box 'Properties of currently available glucose-lowering agents that may guide treatment choice in individual patients with type 2 diabetes mellitus' for additional potential adverse effects and risks, under 'Disadvantages'. [§] Usually a basal insulin in combination with non-insulin agents. [#] Certain non-insulin agents may be continued with insulin [see text and Figure 3 of Inzucchi et al. (2012) for details]. Consider beginning at this stage if patient presents with severe hyperglycemia (AIC ≥ 10.0 – 12.0%) with or without catabolic features (weight loss, ketosis, etc). DPP-4-i, DPP-4 inhibitor; Fx, bone fracture; GI, gastrointestinal; GLP-1-RA, GLP-1 receptor agonist; HF, heart failure; SU, sulfonylurea. Source: Inzucchi et al. 2012.

STROBE Statement: checklist of items that should be included in reports of observational studies.

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported
Objectives	3	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4	Present key elements of study design early in the paper
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed

		Case-control study—For matched studies, give matching criteria and the number of controls per case
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias	9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy
		(*e*) Describe any sensitivity analyses
Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(b) Indicate number of participants with missing data for each variable of interest
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time
		Case-control study—Report numbers in each exposure category, or summary measures of exposure
		Cross-sectional study—Report numbers of outcome events or summary measures
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included

		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
Key results	18	Summarise key results with reference to study objectives
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

**Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.*

Source: Vandenbroucke JP, von Elm E, Altman DG, Gøtzsche PC, Mulrow CD, Pocock SJ, Poole C, Schlesselman JJ, Egger M, for the STROBE Initiative. (2007). Strengthening the Reporting of Observational Studies in Epidemiology (STROBE): explanation and elaboration. PLoS Med; 4: e297.

Appendix. Pittsburgh Sleep Quality Index (PSQI)

Name _____ ID # _____ Date _____ Age _____

Instructions:

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.

1. During the past month, when have you usually gone to bed at night?

   USUAL BED TIME _____

2. During the past month, how long (in minutes) has it usually take you to fall asleep each night?

   NUMBER OF MINUTES _____

3. During the past month, when have you usually gotten up in the morning?

   USUAL GETTING UP TIME _____

4. During the past month, how many hours of *actual sleep* did you get at night? (This may be different than the number of hours you spend in bed.)

   HOURS OF SLEEP PER NIGHT _____

For each of the remaining questions, check the one best response. Please answer *all* questions.

5. During the past month, how often have you had trouble sleeping because you...

   (a) Cannot get to sleep within 30 minutes

Not during the past month _____	Less than once a week _____	Once or twice a week _____	Three or more times a week _____

   (b) Wake up in the middle of the night or early morning

Not during the past month _____	Less than once a week _____	Once or twice a week _____	Three or more times a week _____

   (c) Have to get up to use the bathroom

Not during the past month _____	Less than once a week _____	Once or twice a week _____	Three or more times a week _____

   (d) Cannot breathe comfortably

Not during the past month _____	Less than once a week _____	Once or twice a week _____	Three or more times a week _____

   (e) Cough or snore loudly

Not during the past month _____	Less than once a week _____	Once or twice a week _____	Three or more times a week _____

   (f) Feel too cold

Not during the past month _____	Less than once a week _____	Once or twice a week _____	Three or more times a week _____

   (g) Feel too hot

Not during the past month _____	Less than once a week _____	Once or twice a week _____	Three or more times a week _____

   (h) Had bad dreams

Not during the past month _____	Less than once a week _____	Once or twice a week _____	Three or more times a week _____

   (i) Have pain

Not during the past month _____	Less than once a week _____	Once or twice a week _____	Three or more times a week _____

(j) Other reason(s), please describe _____

_____

How often during the past month have you had trouble sleeping because of this?

Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
_____	_____	_____	_____

6. During the past month, how would you rate your sleep quality overall?

   Very good _____
   Fairly good _____
   Fairly bad _____
   Very bad _____

7. During the past month, how often have you taken medicine (prescribed or "over the counter") to help you sleep?

Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
_____	_____	_____	_____

8. During the past month, how often have you had trouble staying awake while driving, eating meals, or engaging in social activity?

Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
_____	_____	_____	_____

9. During the past month, how much of a problem has it been for you to keep up enough enthusiasm to get things done?

   No problem at all _____
   Only a very slight problem _____
   Somewhat of a problem _____
   A very big problem _____

10. Do you have a bed partner or roommate?

    No bed partner or roommate _____
    Partner/roommate in other room _____
    Partner in same room, but not same bed _____
    Partner in same bed _____

If you have a roommate or bed partner, ask him/her how often in the past month you have had...

(a) Loud snoring

Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
_____	_____	_____	_____

(b) Long pauses between breaths while asleep

Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
_____	_____	_____	_____

(c) Legs twitching or jerking while you sleep

Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
_____	_____	_____	_____

(d) Episodes of disorientation or confusion during sleep

Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
_____	_____	_____	_____

(e) Other restlessness while you sleep; please describe _____

_____

Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
_____	_____	_____	_____

Source: Buysse DJ, Reynolds CF, 3rd, Monk TH, Berman SR, Kupfer DJ. (1989). The Pittsburgh Sleep Quality Index: a new instrument for psychiatric practice and research. Psychiatry Res; 28: 193-213.

Munich ChronoType Questionnaire (MCTQ)

Please enter your age, gender, etc.. This information is important for our evaluations

Age: _____ **female** **male** **Height** _____ **Weight** _____

On work days ...

I have to get up at... _____ o'clock
I need... _____ min to wake up
I regularly wake up... before the alarm with the alarm
From... _____ o'clock I am fully awake
At around... _____ o'clock, I have an energy dip
On nights before workdays, I go to bed at _____ o'clock...
... and it then takes me... _____ min to fall asleep
If I get the chance, I like to take a siesta/nap ...
correct I then sleep for..._____ min
not correct I would feel terrible afterwards

On free days (please only judge normal free days, i.e., without parties etc.) ...

My dream would be to sleep until... _____ o'clock
I normally wake up at... _____ o'clock
If I wake up at around the normal (workday) alarm time, I try to get back to sleep...
correct not correct
if I get back to sleep, I sleep for another... _____ min
I need ... _____ min to wake up
From... _____ o'clock I am fully awake
At around... _____ o'clock, I have an energy dip
On nights before free days, I go to bed at... _____ o'clock...
... and it then takes me... _____ min to fall asleep
If I get the chance, I like to take a siesta/nap ...
correct I then sleep for..._____ min
not correct I would feel terrible afterwards

once I am in bed, I would like to read for ... _____ min, ...
... but generally fall asleep after no more than ... _____ min.

I prefer to sleep in a completely dark room correct not correct
I wake up more easily when morning light shines into my room correct not correct

How long per day do you spend on average outside (really outside) exposed to day light?
On work days: ___ hrs. ___min. On free days: ___ hrs. ___min.

Self assessment

After you have answered the preceding questions, you should have a feeling to which chronotype (time-of-day-type) you belong to. If for example, you like (and manage) to sleep quite a bit longer on free days than on workdays, or if you cannot get out of bed on Monday mornings, even without a Sunday-night-party, then you are more a late type. If, however, you regularly wake up and feel perky once you jump out of bed, and if you would rather go to bed early than to an evening concert then you are an early type. In the following questions, you should categorise yourself and your family members.

Please tick only one possibility!

Description of categories:

extreme early type = 0
moderate early type = 1
slight early type = 2
normal type = 3
slight late type = 4
moderate late type = 5
extreme late type = 6

I am...	0	1	2	3	4	5	6
as a child, I was ...	0	1	2	3	4	5	6
as a teenager, I was ...	0	1	2	3	4	5	6
In case you are older than 65: in the middle of my life, I was ...	0	1	2	3	4	5	6
My parents are/were...							
Mother ...	0	1	2	3	4	5	6
Father ...	0	1	2	3	4	5	6
My siblings are/were ... (please underline Brother or Sister)							
Brother/Sister	0	1	2	3	4	5	6
Brother/Sister	0	1	2	3	4	5	6
Brother/Sister	0	1	2	3	4	5	6
Brother/Sister	0	1	2	3	4	5	6
Brother/Sister	0	1	2	3	4	5	6
Brother/Sister	0	1	2	3	4	5	6
Brother/Sister	0	1	2	3	4	5	6
My partner (girl/boy friend, spouse, significant other) is/was ...	0	1	2	3	4	5	6

Source: Roenneberg T, Wirz-Justice A, Merrow M. (2003). Life between clocks: daily temporal patterns of human chronotypes. J Biol Rhythms; 18: 80–90.

THE EPWORTH SLEEPINESS SCALE

Name: _____

Today's date: _____ Your age (years): _____

Your sex (male = M; female = F): _____

How likely are you to doze off or fall asleep in the following situations, in contrast to feeling just tired? This refers to your usual way of life in recent times. Even if you have not done some of these things recently try to work out how they would have affected you. Use the following scale to choose the *most appropriate number* for each situation:

0 = would *never* doze
1 = *slight* chance of dozing
2 = *moderate* change of dozing
3 = *high* chance of dozing

Situation	Chance of dozing
Sitting and reading	_____
Watching TV	_____
Sitting, inactive in a public place (e.g. a theater or a meeting)	_____
As a passenger in a car for an hour without a break	_____
Lying down to rest in the afternoon when circumstances permit	_____
Sitting and talking to someone	_____
Sitting quietly after a lunch without alcohol	_____
In a car, while stopped for a few minutes in the traffic	_____

Source: Johns MW. (1991). A new method for measuring daytime sleepiness: the Epworth Sleepiness Scale. Sleep; 14: 540-545.

Appendix II:
Supplementary information to chapter 3 and chapter 4

Supplementary Table 1: Caffeine content of products available for consumption in German-speaking Switzerland.

Caffeine product	Size of serving (ml)	Total caffeine per serving (mg)	Information source (website)
COFFEE			
Expresso-based coffee. Single shot (e.g. expresso, latte, cappuccino, mocha.)	44	77	Caffeine Informer [a]
Instant coffee	240	57	Caffeine Informer
Brewed/filter coffee	240	107.5	Caffeine Informer
Decaffeinated coffee	240	4.5	Caffeine Informer
COLD COFFEE ('Emmi cafe latte')			
Cappuccino	230	80	Manufacturer
Caramel	230	60	Manufacturer
Expresso	230	120	Manufacturer
Light	230	80	Manufacturer
Macchiato	230	80	Manufacturer
Tahiti	230	60	Manufacturer
Zero	230	110	Manufacturer
TEA			
Brewed/loose-leaf black tea	240	48	Caffeine Informer
Green tea/white tea	240	25	Caffeine Informer
ENERGY DRINKS			
Redbull	355	114	Manufacturer

Redbull	250	80	Manufacturer
Redbull (sugar free)	250	80	Manufacturer
Migros own brand	250	80	Manufacturer
Migros own brand (sugar free)	250	80	Manufacturer
OK energy drink	355	114	Manufacturer
OK energy drink (light)	250	80	Manufacturer
Coop own brand	250	75	Manufacturer
Coop own brain (sugar free)	250	75	Manufacturer
Monster Energy	500	160	Manufacturer
Rockstar Energy Drink	500	160	Manufacturer
Lucozade	380	46	Manufacturer
Relentless Energy drink	500	160	Manufacturer
SOFT DRINKS			
Coca cola, Pepsi, flavoured cola, shop-branded cola	330	38	Manufacturer
Coca cola, Pepsi, flavoured cola, shop-branded cola	500	58	Manufacturer
Diet coca cola, diet Pepsi, Coke Zero, Pepsi Max, shop-branded, diet flavoured cola	330	38	Manufacturer
Diet coca cola, diet Pepsi, Coke Zero, Pepsi Max, shop-branded, diet flavoured cola	500	58	Manufacturer
Caffeine-free Diet Coca Cola	330 or 500	0	Manufacturer
Dr Pepper	330	38	Manufacturer
Dr Pepper	500	58	Manufacturer
Iced tea (e.g. Lipton, Nestea)	330	22	Manufacturer
Iced tea (e.g. Lipton, Nestea)	500	33	Manufacturer
Iced tea (light/zero)	330	22	Manufacturer
Iced tea (light/zero)	500	33	Manufacturer

DRINKING CHOCOLATE			
Hot chocolate (e.g. Suchard Express, Caotina)	240	7	Manufacturer
Cold chocolate (e.g. Nesquik, Micao, Comella)	240	7	Manufacturer
SOLID CHOCOLATE			
Milk	25g	6.25	Manufacturer
Dark	25g	17	Manufacturer
ICE CREAM			
Coffee-flavoured ice cream (e.g. Haagen-Dazs, Ben & Jerry)	240	59	Manufacturer
CAFFEINE PILLS / SUPPLEMENTS			
Caffeine pills (e.g. ProPlus)	2 tablets	100	Manufacturer
Supplements for general fatigue / lack of well-being (e.g. Tonikum D Flussig)	10	0.9	Manufacturer
MEDICATION			
Cold and Flu (e.g. Rhinitin retard, Rhin-X)	1 capsule	25	Manufacturer
Painkillers (e.g. Contra-Schmerz, Migrane-Kranit, Panadol Extra)	500 mg	65	Manufacturer
Anti-nausea / motion sickness (e.g. Itinerol B6)	2 capsules	40	Manufacturer

Note: If there were several brands available for a given caffeine-containing product (e.g. Lipton and Nestea Iced Tea), the estimated caffeine content was averaged across the brands.

[a] Caffeine informer website: http://www.caffeineinformer.com/the-caffeine-database

Supplementary Table 2: Type 2 diabetes duration and sociodemographic characteristics.

Variable	Type 2 Diabetes (n=134)	Non-Type 2 Diabetes (n=230)	*P*-value
TYPE 2 DIABETES DURATION			
○ < 1 year (%)	8.3	-	N/A
○ 1-3 years (%)	15.0	-	N/A
○ 4-6 years (%)	20.3	-	N/A
○ 7-9 years (%)	12.8	-	N/A
○ ≥ 10 years (%)	43.6	-	N/A
SOCIODEMOGRAPHIC DATA			
Relationship status (%)			0.159
○ Never married	11.9	17.0	0.226
○ Married	67.9	55.7	**0.026**
○ Separated or divorced	17.2	23.0	0.229
○ Widowed	3.0	4.3	0.585
Home ownership (% owner)	51.5	55.7	0.448
Household density (no. persons/no. rooms)[a]	0.4 (±0.2)	0.5 (±0.2)	0.640
Education (continued after high school; % yes)	98.5	97.0	0.495
Work (% yes)[b]	50.0	49.3	0.913
Work (hours per week)[c]	16.7 (±20.2)	15.6 (±19.1)	0.807

Abbreviations: T2D, type 2 diabetes; Non-T2D, non-type 2 diabetes; N/A, not applicable. Data for continuous variables are means (± standard deviation) of raw data. *P*-values (2-tailed) were calculated using independent samples t-tests, comparing T2D and Non-T2D groups, on raw data. If raw data was abnormally distributed, the data was transformed to achieve a normal distribution before the t-test was applied. Data for categorical variables are %. *P*-values (exact; 2-tailed) were calculated using Fisher's exact test.

[a] Raw data transformation: Reciprocal; [b] % working >0 hours per week; T2D: n=132; NonT2D: n=223; [c] T2D: n=133; NonT2D: n=223. Raw data transformation: Log10.

Experimental Procedures

Calculations on data derived from Pittsburgh Sleep Quality Index (PSQI)

Habitual time in bed = typical bedtime vs. typical get-up time
Habitual sleep duration = habitual time in bed – typical sleep latency*
**sleep latency = time taken to fall asleep*
Sleep efficiency = (habitual sleep duration / habitual time in bed) x 100

Calculations on data derived from Munich Chronotype Questionnaire (MCTQ)

Note: workers were assumed to have 5 work days and 2 free days each week; non-workers and retired participants were assumed to have 7 free days each week. However, if a non-worker or retired person reported separate work and free day timings, then these timings were used in the analysis.

Sleep duration = sleep onset (bedtime + sleep latency) vs. wake time
Average sleep duration = [(work day sleep duration x 5) + (free day sleep duration x 2) / 7]
Chronotype metric = midpoint of sleep on free days – (0.5 x sleep debt*)
**sleep debt = free day sleep duration – average sleep duration*
Social jetlag = midpoint of sleep on work days vs. midpoint of sleep on free days

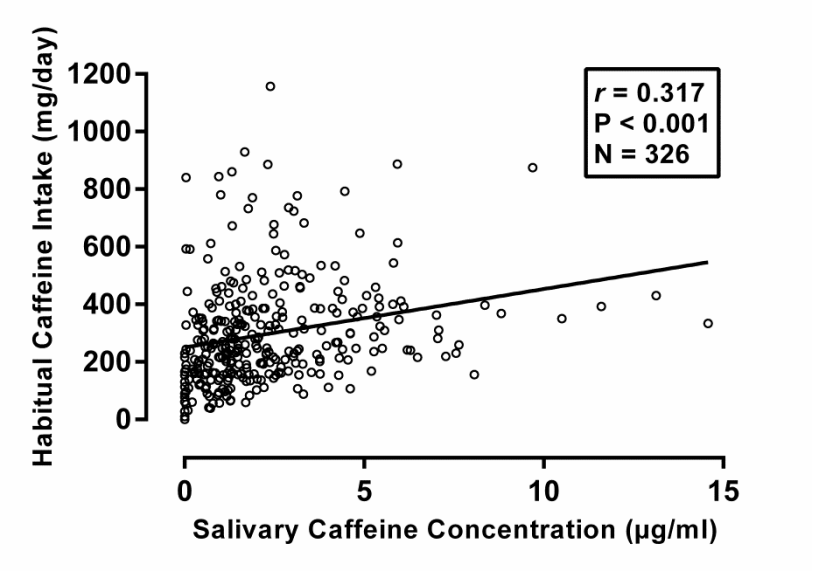
Genomic assessment with salivary DNA

Genomic DNA was extracted from saliva according to DNA Genotek's instructions. Participants were genotyped for the cytochrome P450-1A2 gene (*CYP1A2*. -163C>A. SNP ID: rs762551. Assay ID: C_8881221_40) with TaqMan SNP Genotyping Assays (Applied Biosystems, Rotkreuz, Switzerland). Allele-specific polymerase chain reaction (PCR) was performed on a TaqMan thermal cycler (ABI PRISM®7900HT system; Life Technologies, Zug, Switzerland). The reaction volume contained 20 ng genomic DNA, 4 µl TaqMan Universal Master Mix (Applied Biosystems, Rotkreuz, Switzerland), 4 µl 20X SNP Genotyping Assay Mix, and 1.6 µl distilled H_2O. Annealing temperature was set to 60°C. After running the PCR, an end‑point fluorescence measurement with the SDS 2.2 software package (Applied Biosystems, Rotkreuz, Switzerland) was obtained, to examine the samples and discriminate between the specific alleles. All genetic analyses were replicated at least once for independent confirmation of the results.

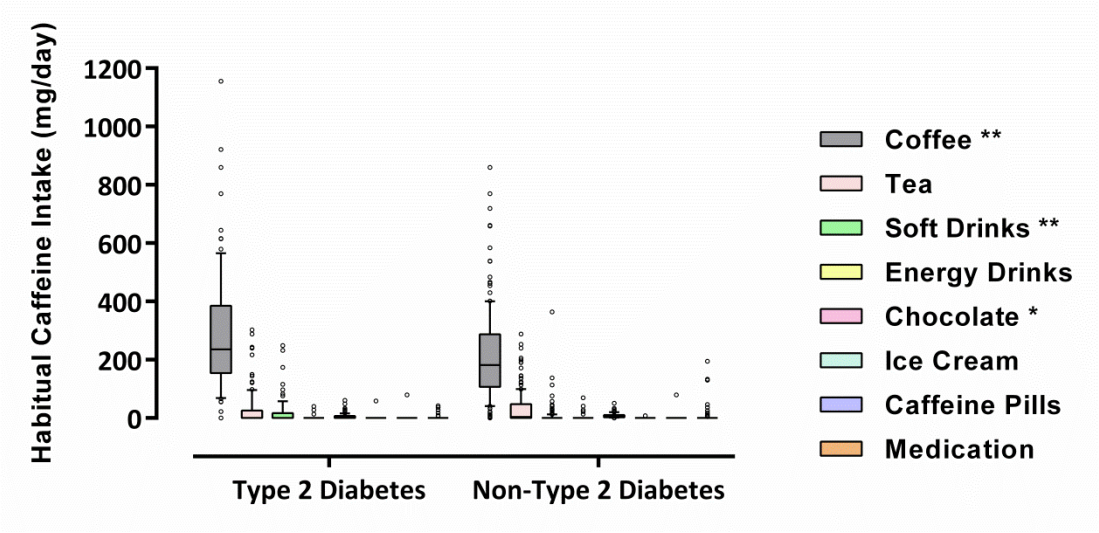
HPLC assessment of salivary caffeine and paraxanthine

After thawing, saliva was extracted from the Salivette® according to the manufacturer's instructions (centrifugation for 2 minutes at 1,000 g). Salivary caffeine and paraxanthine (caffeine's major metabolite) concentrations were quantified by high performance liquid chromatography (HPLC) coupled to a UV detector, essentially as described by Fuhr & Rost (1994) with minor modifications as follows. The HPLC system consisted of a separations module equipped with a temperature-controlled autosampler (Alliance e2695 XC Separations Module, Waters, Dättwil, Switzerland) and a photodiode array UV detector (2998 PDA Detector, Waters). To summarize, a 225 µl aliquot of saliva was prepared by addition of 75 µl of trichloroacetic acid 20 % containing the internal standard (100 mg/l hydroxyethyltheophylline). After vortex mixing and centrifugation (2000 g for 10 minutes at +4 °C), 20 µl of the supernatant were injected onto a Nucleosil 100 C18 reverse phase column (column dimensions 125 x 4 mm; 5 µm particle size; Macherey-Nagel, Oensingen, Switzerland) and eluted using a 4 mmol/l acetic buffer (pH 4.0) containing 1 % of acetonitrile, 1 % of methanol, and 1.6 % of tetrahydrofurane (v/v). The initial flow was increased from 0.8 ml/min to 1.0 ml/min within 2 minutes, and then kept stable at 1.0 ml/min for 17 min, before initial conditions were restored after 2 additional minutes. The samples were usually analyzed in sets of twenty-five, with a calibration row before each set of participants' samples, and a blank sample every ten unknown. Calibration was based on peak area ratios of paraxanthine and caffeine, respectively, over internal standard for ultraviolet absorption at 273 nm and data point weighting by the inverse of concentrations. The lower limit of quantification (LLOQ) was 0.077 µg/ml for caffeine and 0.024 ug/ml for paraxanthine. Precision and accuracy were not more than 14 % and 10 %, respectively, for the entire concentration range (0.077 – 15.462 µg/ml for caffeine and 0.024 – 12.624 µg/ml for paraxanthine).

In chapter 3, results below the quantifiable limit (BQL) for both caffeine and paraxanthine were assumed to reflect zero caffeine (0 µg/ml). Results that were BQL for caffeine, but quantifiable for paraxanthine, were assumed to reflect half the LLOQ for caffeine (0.077/2 = 0.039 µg/ml).



Supplementary Figure 1: Comparison of HPLC-determined salivary concentrations of caffeine and survey estimates of habitual caffeine intake. Pearson's product-moment correlation: r = 0.317. Significance (2-tailed): $p < 0.001$. N=326.



Supplementary Figure 2: Habitual caffeine intake of type 2 diabetes and non-type 2 diabetes groups by dietary category. Boxplots represent self-reported caffeine intake split into separate dietary categories (box: 25th percentile, median and 75th percentile; whiskers: 10th to 90th percentiles; dots: individual data points outside of the whisker range). The estimates of caffeine consumption were based on the caffeine content reported by manufacturers of Swiss products or the website 'Caffeine Informer.' (See Supplementary Table 1). Statistics compared type 2 diabetes (n=134) and Non-type 2 diabetes (n=230) groups. If normally distributed data was available, independent samples t-test was used; if data was not normally distributed, Mann-Whitney U test was used. [**$p < 0.001$; *$p = 0.001$. Coffee: independent samples t-test on square-root data (2-tailed). Soft drinks: Mann-Whitney U test on raw data (exact; 2-tailed). Chocolate: independent samples t-test on Log10 data (2-tailed)].

Appendix III:

Supplementary information to chapter 5

Consumption of Caffeinated Drinks and Foods Questionnaire

Instructions:

- Please indicate how frequently you *usually* consume the following foods and drinks.
- Please specify the number of times you consume each item by indicating a number 'per day' **OR** 'per week'. If you never consume the item then please tick 'never'.
- Please do not leave anything blank.
- Note: For soft drinks, please differentiate between 'normal' and 'diet' versions and also serving size (can or bottle).

DRINKS

COFFEE	Number of cups / mugs per day	OR	Number of cups / mugs per week	OR	Never
Instant coffee					
Brewed or filter coffee					
Expresso-based coffee, SINGLE shot (e.g. expresso, latte, cappuccino, mocha, either hot or iced)					
Cold coffee (e.g. Emmi Caffe Latte)					
Decaffeinated coffee					

Other coffee (Please specify) _____					

- *Please specify your usual brand (s) of coffee:*

TEA	**Number of cups / mugs per day**	**OR**	**Number of cups / mugs per week**	**OR**	**Never**
Black tea (brewed or loose leaf)					
Green tea					
Herbal / Fruit tea					
Decaffeinated tea					
Other tea (Please specify) _____					

- *Please specify your usual brand (s) of tea:*

SOFT DRINKS	**Number of servings per day**	**OR**	**Number of servings per week**	**OR**	**Never**
Can (330ml) of ***ordinary* cola** (e.g. Coca Cola, Pepsi, flavoured colas, shop-branded cola)					
Bottle (500ml) of ***ordinary* cola** (e.g. Coca Cola, Pepsi, flavoured colas, shop-branded cola)					

Can (330ml) of ***diet / light / sugar free*** **cola** (e.g. diet Coca Cola, Coke Zero, Diet Pepsi, Pepsi Max, diet flavoured cola, diet shop-branded cola)					
Bottle (500ml) of ***diet / light / sugar free*** **cola** (e.g. diet Coca Cola, Coke Zero, diet Pepsi, Pepsi Max, diet flavoured cola, diet shop-branded cola)					
Can (330ml) of **diet, caffeine free, Coca Cola**					
Bottle (500ml) of **diet, caffeine free, Coca Cola**					
Can (330ml) of **Mezzo Mix (cola & orange)**					
Bottle (500ml) **of Mezzo Mix (cola & orange)**					
Can (330ml) of **Dr Pepper**					
Bottle (500ml) of **Dr Pepper**					
Can (330ml) of ***ordinary*** **iced tea** (e.g. Lipton, Nestea)					
Bottle (500ml) of ***ordinary*** **iced tea** (e.g. Lipton, Nestea)					
Can (330ml) of ***diet*** **iced tea** (e.g. Lipton, Nestea)					

Bottle (500ml) of ***diet* iced tea** (e.g. Lipton, Nestea)					

- *Please specify your usual brand (s) of soft drinks:*

STIMULANT / ENERGY DRINKS	**Number of servings per day**	**OR**	**Number of servings per week**	**OR**	**Never**
Small can (250-355ml) of ***ordinary* energy drink** (e.g. Redbull, OK, shop-branded)					
Large can (500ml) of ***ordinary* energy drink** (e.g. Monster, Relentless, Rockstar, shop-branded)					
Small can (250-355ml) of ***diet / light / sugar free* energy drink** (e.g. sugar free versions of Redbull, OK, shop-branded)					
Large can (500ml) of ***diet / light / sugar free* energy drink** (e.g. sugar free versions of Monster, Relentless, Rockstar, shop-branded)					
Lucozade (380ml bottle)					
Other energy / stimulant drink (Please specify)					

- *Please specify your usual brand (s) of energy/stimulant drink:*

DRINKING CHOCOLATE	Number of cups / mugs per day	OR	Number of cups / mugs per week	OR	Never
Hot drinking chocolate (e.g. Suchard Express, Caotina)					
Cold drinking chocolate (e.g. Nesquik, Micao, Comella)					

- *Please specify your usual brand (s) of drinking chocolate:*

FOODS

SOLID CHOCOLATE	Number of servings per day	OR	Number of servings per week	OR	Never
Note: 25mg is a quarter of a standard-sized, 100mg, chocolate bar					
25mg of **milk** chocolate					
25mg of **dark** chocolate					

- *Please specify your usual brand (s) of chocolate:*

ICE CREAM	Number of servings per day	OR	Number of servings per week	OR	Never
Cup (240ml) of **coffee-flavoured ice cream** (e.g. Haagen-Dazs, Ben & Jerry)					

- *Please specify your usual brand (s) of ice cream:*

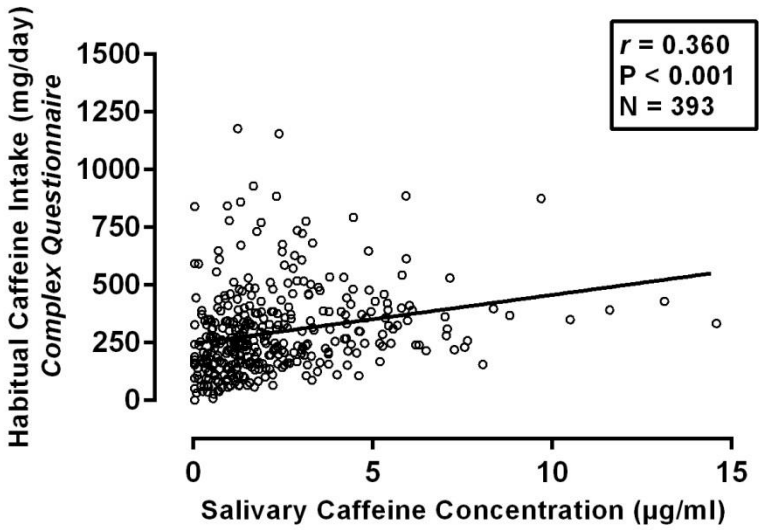
OTHER

STIMULANT / ENERGY PILLS	**Number of doses per day**	**OR**	**Number of doses per week**	**OR**	**Never**
Caffeine pills (e.g. ProPlus)					
Anti-fatigue supplements (e.g. Tonikum D Flussig)					

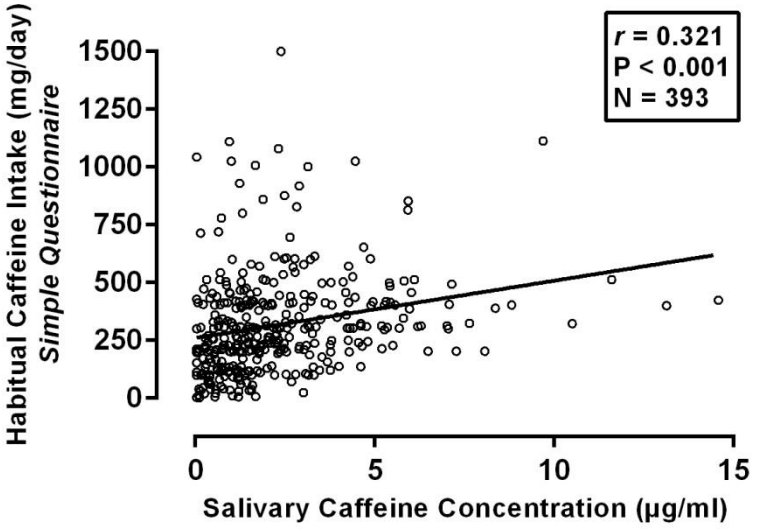
- *Please specify your usual brand (s) of energy pills:*

MEDICATION *(only if it contains caffeine)*	**Number of doses per day**	**OR**	**Number of doses per week**	**OR**	**Never**
Anti-nausea / motion sickness (e.g. Itinerol B 6)					
Cold / Flu (e.g. Rhinitin retard, Rhin-X)					
Hayfever / Antihistamine					
Painkillers (e.g. Contra-Schmerz, Migrane-Kranit, Panadol Extra)					

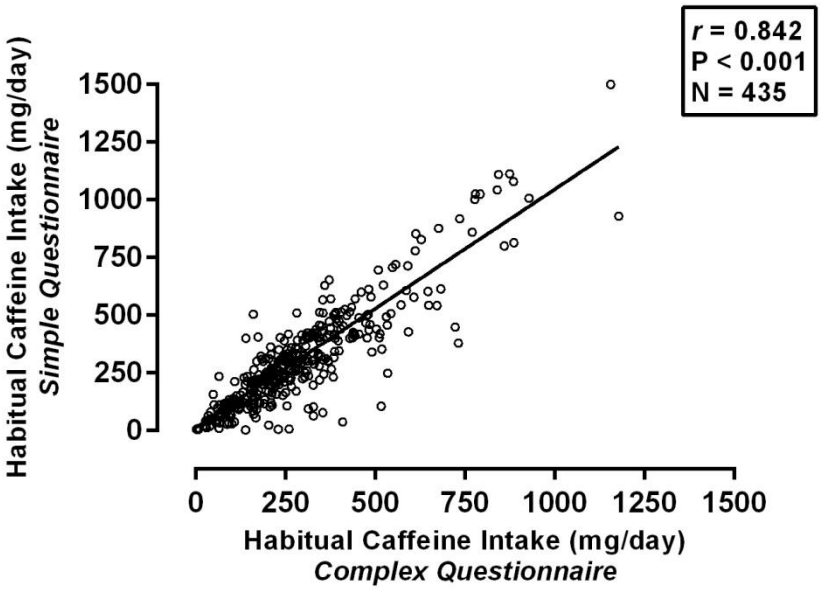
- *Please specify your usual brand (s) of caffeine-containing medication:*



Supplementary Figure 1: Comparison of HPLC-determined salivary concentrations of caffeine and 'complex' questionnaire estimates of habitual caffeine intake. Pearson's product-moment correlation: *r* = 0.360. Significance (2-tailed): p < 0.001. N = 393. Daily habitual caffeine intake calculated using caffeine-content data from Supplementary Table 1 in Appendix II.



Supplementary Figure 2: Comparison of HPLC-determined salivary concentrations of caffeine and 'simple' questionnaire estimates of habitual caffeine intake. Pearson's product-moment correlation: *r* = 0.321. Significance (2-tailed): p < 0.001. N = 393. Daily habitual caffeine intake calculated using the following caffeine-content criteria: coffee = 100 mg / serving; black and green tea = 30 mg; cola drink = 40 mg; energy drink = 80 mg; chocolate = 50 mg (Bachmann et al. 2012). In addition, the caffeine content of caffeine pills was included in the calculations (caffeine pills = 100 mg / serving of 2 pills).



Supplementary Figure 3: Comparison of 'complex' questionnaire estimates of daily habitual caffeine intake and 'simple' questionnaire estimates of habitual caffeine intake. Pearson's product-moment correlation: $r = 0.842$. Significance (2-tailed): $p < 0.001$. N = 435.

Estimating plasma caffeine at bedtime

Note: equation adapted from Nova and colleagues (Nova et al. 2012). It assumes that caffeine is metabolized with an exponential decay; and that caffeine portions are consumed instantaneously at the reported time.

$$C_b = (C_1 \times 0.5^{(T_b - T_1) / \textit{half-life}}) + (C_2 \times 0.5^{(T_b - T_2) / \textit{half-life}}) + (C_3 \times 0.5^{(T_b - T_3) / \textit{half-life}}) + \text{etc.}$$

C_b = caffeine at bedtime (mg); C_1 = caffeine content of first caffeine portion (mg); T_b = bedtime (decimal hours); T_1 = time of first caffeine portion (decimal hours); *half-life* = half-life of caffeine (decimal hours); C_2 = caffeine content of second caffeine portion (mg); T_2 = time of second caffeine portion (decimal hours); etc.

Examples:

1) A person with a caffeine half-life of 4.8 hours drinks a double expresso (154 mg) at 08:00 and a double expresso (154 mg) at 13:00. Bedtime: 23:00
Equation-derived caffeine at bedtime = 54.0 mg

2) A person with a caffeine half-life of 4.8 hours drinks a double expresso (154 mg) at 13:00 and a double expresso (154 mg) at 20:00. Bedtime: 23:00
Equation-derived caffeine at bedtime = 136.2 mg

3) A person with a caffeine half-life of 4.8 hours drinks a single expresso (77 mg) at 08:00 and a single expresso (77 mg) at 13:00. Bedtime: 23:00
Equation-derived caffeine at bedtime = 27.0 mg

Curriculum vitae

Emily Urry

Date of birth: 30th July, 1982
Place of birth: Guildford, Great Britain
Citizenship: Great Britain

Education and training

2011 - 2016 Doctoral student at: a) the department of Health Sciences and Technology (D-HEST) at the Swiss Federal Institute of Technology (ETH), Zürich; b) the Chronobiology & Sleep Research Laboratory at the University of Zürich, Zürich, Switzerland.

2009 - 2010 Master of Science in Nutrition & Psychological Sciences at the University of Northumbria, Newcastle upon Tyne, Great Britain.

2009 Internship at the Brain, Performance and Nutrition Research Centre, Northumbria University, Newcastle upon Tyne, Great Britain (3 months).

2007 Chartered Financial Analyst (CFA; Level 1) at 7 City Professional Education, London, Great Britain (6 months).

2005 Certificate in Investments (Financial Regulation & Securities) at BPP Professional Education, London, Great Britain (1 month).

2001 - 2004 Bachelor of Science in Experimental Psychology at the University of Bristol, Bristol, Great Britain.

2001 Diploma in Advanced Spanish at the University of Cuernavaca, Cuernavaca, Mexico (4 months).

1993 - 2000 Schooling (GCSEs and A Levels) at Benenden School, Cranbrook, Great Britain.

Membership of professional organisations

Impact Hub Zurich; Zurich Center for interdisciplinary Sleep Research (ZiS)

List of publications

Urry E, Jetter A, Landolt HP. (2016). Assessment of CYP1A2 enzyme activity in relation to type 2 diabetes and habitual caffeine intake. Nutrition & Metabolism, in press.

Urry E, Jetter A, Holst SC, Berger W, Spinas GA, Langhans W, Landolt HP. (2016). A case-control field study on the relationships among type 2 diabetes, sleepiness and habitual caffeine intake. J Psychopharmacol, in press.

Urry E, Landolt HP. (2015). Adenosine, caffeine, and performance: from cognitive neuroscience of sleep to sleep pharmacogenetics. Current Topics in Behavioural Neurosciences; 25: 331-366.

Landolt HP, Urry E. (2013). Functional neuroimaging: sedating medication effects. In: E Nofzinger et al., eds. Neuroimaging of Sleep and Sleep Disorders. Cambridge: Cambridge University Press, pp. 396-405.

Leonards U, Urry E, Scott-Samuel N E. (2004). Saccadic responses to glow: differential latencies for light-emitting and light-reflecting objects. Perception; 33: 113 (Suppl.).

Oral presentations at national meetings

December 2015:
Chronobiology & Sleep Research Laboratory Seminar, University of Zürich
'Relationships among type 2 diabetes, sleepiness, and habitual caffeine intake: a case-control field study'.

February 2014:
Progress Report Seminar, Institute of Pharmacology and Toxicology, University of Zürich
'Caffeine, sleep quality and health'.

February 2014:
Clinical Research Priority Program (CRPP), Sleep and Health Seminar, University Hospital Zürich
'Caffeine, sleep quality and health'.

June 2013:
Chronobiology & Sleep Research Laboratory Seminar, University of Zürich
'Causal roles for caffeine and disturbed sleep in type 2 diabetes risk'

Poster presentations at national and international meetings

June 2015:
Annual Congress of the Swiss Society of Pediatrics (SSP) and the Schweizerische Gesellschaft für Schlafforschung, Schlafmedizin und Chronobiologie (SSSSC), Interlaken, Switzerland
Urry E, Spinas GA, Langhans W, Dürr R, Landolt HP.
'Lifestyle considerations in type 2 diabetes risk: sleep quality, sleep timing and habitual caffeine consumption'

January 2015:
Annual Symposium of the Clinical Research Priority Program (CRPP) 'Sleep and Health' and the Zurich Center for interdisciplinary Sleep Research (ZiS), University Hospital Zürich, Switzerland
Urry E, Spinas GA, Langhans W, Dürr R, Landolt HP.
'Lifestyle considerations in type 2 diabetes risk: sleep quality and habitual caffeine consumption'

September 2014:
22nd Congress of the European Sleep Research Society (ESRS) 2014 Tallinn, Estonia
Urry E, Spinas GA, Langhans W, Dürr R, Landolt HP.
'Lifestyle considerations in type 2 diabetes risk: sleep quality and habitual caffeine consumption'

August 2014:
Poster Day of the Institute of Pharmacology & Toxicology, University of Zürich, Switzerland

Urry E, Spinas GA, Langhans W, Dürr R, Landolt HP.
'Lifestyle considerations in type 2 diabetes risk: sleep quality and habitual caffeine consumption'

August 2014:
Symposium of the Zurich Center for Integrative Human Physiology (ZIHP), University of Zürich, Switzerland
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