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regulating energy metabolism and satiety“

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Für meine Familie

*„Das Leben ist wert, gelebt zu werden, sagt die Kunst, die schönste Verführerin;
das Leben ist wert, erkannt zu werden, sagt die Wissenschaft.“*

Friedrich Nietzsche (1844-1900)

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AFMU	5-Acetylamino-6-formylamino-3-methyluracil
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
BMI	Body mass index
C.	Coffea
cAMP	Cyclic adenosine monophosphate
CCCP	Carbonylcyanide m-chlorophenylhydrazone
CCK	Cholecystokinin
CNS	Central nervous system
FCCP	Carbonylcyanide p-trifluoromethoxyphenylhydrazone
GIP	Glucose-dependent insulintropic polypeptide
GLP-1	Glucagon-like peptide 1
HOMA-IR	Homeostatic model assessment of insulin resistance
MB	Market blend
NAD ⁺	Nicotinamide adenine dinucleotide
NMP	N-methylpyridinium
OGTT	Oral glucose tolerance test
POMC	Pro-opiomelanocortin
SB	Study blend
TAN	Total adenosine nucleotides
UCP	Uncoupling protein

1 Introduction

1.1 Coffee - botanical aspects and processing

The coffee plant belongs botanically to the genus *Coffea* (C.) of the family *Rubiaceae*. About 100 species are discriminated within the genus, but only two of them are commercially important: *Coffea arabica*, commonly termed Arabica coffee and *Coffea canephora* var. *robusta*, commonly Robusta coffee [1]. For the marketing year 2012/2013, the world coffee production is forecast to reach about 9.1 million tons, whereupon Arabica coffee accounts for 5.3 million tons and Robusta coffee for about 3.7 million tons [2]. Other species such as *Coffea liberica* or *Coffea excelsa* are of minor importance and represent only 1% of the world production [3].

The origin of *C. arabica* is found in the Ethiopian highlands at altitudes between 1300 m and 2000 m, whereas *C. canephora* is more widely spread in tropical Africa at altitudes lower than 1000 m. In the meantime, cultivation of coffee plants occurs in subtropical, tropical and equatorial regions of Central and South America, South East Asia and Africa [3]. However, based on their origin, Arabica and Robusta coffees are adapted to different ecological environments and, therefore, restrict potential producing regions to a given species or variety. While Arabica coffee grows at subtropical climate at altitudes from 500 m up to 2500 m and prefers well-defined rainy and dry seasons with high light intensity, Robusta coffee tolerates warmer temperatures and higher humidity levels in the lowlands from sea level up to 1000 m [4, 5]. In general, optimal climatic conditions for coffee cultivation are stable spring or summer temperatures between 15°C and 25°C, average rainfall between 1400 and 2500 mm as well as a balanced proportion of sun and shade. Field studies have shown that the coffee quality, e.g. size, sensory properties and constituent profile, depends on the environmental factors temperature [6, 7], rainfall [8], shading [9, 10] and cultivation altitude [11].

Wild grown coffee trees may reach a height of about 6 – 10 m. To facilitate harvesting on plantations, the cultured coffee plants are kept as shrubs of 2 – 2.5 m. The evergreen coffee shrubs are characterized by leathery short-stemmed leaves and white fragrant blossoms [12]. After fertilization of the flower, coffee cherry maturation lasts 6 to 8 months and 9 to 11 months for *C. arabica* and *C. canephora* var. *robusta*, respectively [1]. The ripened deep red fruits have a diameter of 1 – 2 cm and enclose two stone-fruit seeds, called coffee beans, in the sweet pulp [12, 13]. A horny endocarp, the parchment, surrounds both hemispheres and separates them from each other. Coffee beans pos-

sess an elliptical form, with flattened adjacent sides, and are covered by the yellowish transparent silverskin [12].

There are two ways of harvesting ripe fruits. The method applied depends on the climatic conditions of the coffee region. In areas with seasonal rainfalls, that promote homogeneous blossoming and ripening, coffee cherries are periodically harvested by the stripping technique. Thereby cherries and leaves are stripped from the branches either by hand or mechanically. In contrast, in regions with continuous rainfall, ripe dark-red cherries are found throughout the entire year and have to be precisely selected by hand from immature fruits. This manual picking of ripe coffee cherries is work intensive, but yields in high coffee quality. However, the implementation of modern post-harvesting techniques contributes to an improvement of ripe cherries separation from unwanted products [4].

In order to obtain the seeds of the mature coffee cherries the pulp, the mucilage and the silverskin have to be removed. Therefore, two basic post-harvesting treatments are distinguished: the dry method and the wet method. During dry processing, coffee cherries are spread out on yards and naturally dried by solar radiation. To assure constant drying and avoid fermentation, coffee cherries need to be turned several times a day. The final moisture content of sun-dried coffee cherries amounts to 11% after two to three weeks of drying process. In the final hulling step, the dried pulp is removed mechanically and yields in natural green coffee beans [13]. Although the natural dry processing is ecologically friendly, it is not applicable in regions with high humidity. In those areas, a wet process is conducted that is more pollutant due to the high water usage and the resulting liquid and solid residues that require safe disposal [4]. During the wet process, the pulp is removed mechanically by a depulping machine in the presence of water. However, coffee beans are still covered with mucilage and the parchment that are enzymatically removed during the following fermentation step, either by naturally existing microorganisms or addition of specific enzymes. Afterwards, the redundant fermentation products are washed away and the remaining coffee beans are dried to a moisture content of 11% within a few days [13]. The semidry method presents a combination of dry and wet processing and is characterized by mechanical pulp removal followed by direct drying of the parchment coffee without fermentation [3].

During post-harvest processing, the chemical composition of coffee beans changes and, according to the method applied, differences in the constituent profile have been monitored. Brazilian Arabica coffee processed by the wet method contained higher amounts of chlorogenic acid and trigonelline, while sucrose was reduced and caffeine contents

remained unchanged in comparison to a semidry method [14]. The aroma profile of coffee beans was also modified differently according to the post-harvest treatment [15].

Moreover, post-harvest processing displays a crucial step for microbial contamination and fermentation. During post-harvest treatment, especially infections with ochratoxin A producing species of the genus *Aspergillus* reduce coffee quality and present a risk for product safety. Batista *et al.* [16] observed that dry processing on ground yards offers a higher risk of infection with ochratoxigenic fungi than dry processing on asphalt yards, presumably based on the direct contact with the soil, while the depulping during wet processing reduced bean contamination. However, ochratoxin A is decomposed upon coffee roasting and after artificial contamination only about 5% ochratoxin A remained in dark roast coffee beans [17].

Coffee producing countries provide green coffee beans after post-harvest processing, which are packed in jute or sisal bags that may be stored under optimal conditions several years. In general, green coffee is shipped to the consuming countries where roasting is performed as the storage time of roasted coffee is limited. Moreover, the coffee industry is able to more precisely adapt coffee blending and roasting to the prevailing consumer's preference.

1.2 Roasting-induced compositional changes of coffee

Roasting is the most important step within coffee production and causes profound changes in the morphology and chemical composition, thereby favoring the desirable aroma, taste and brown color of the roasted coffee beans. The temperatures and times applied during the process of roasting strongly depend on the roasting technique and the desired roasting degree of the coffee beans. For instance, a dark roast coffee is obtained after oven roasting of coffee beans for either 120 minutes at 200°C or 11 minutes at 300°C [18]. In contrast, roasting of coffee beans at 190 °C for 10 minutes in a rotary laboratory roaster also generates a dark roast [19]. However, detailed roasting profiles are often confidential.

Coffee roasting is based on a heat energy transfer from the roaster to the bean. Commonly used roasting temperatures range between 180°C and 300°C and roasting times vary between 3 and 15 minutes. The roasting process starts with a drying step at 100°C that is characterized by bean color changes from green to yellow, water evaporation and a release of volatile compounds. The further temperature rise above 190°C induces py-

rolysis reactions that drastically change the physical properties and chemical composition of the coffee beans. The volume of the bean increases by 70%, while a mass loss up to 20% is observed that is primarily based on dehydration and carbon dioxide release [4, 20]. With increased temperature and gas formation, the internal pressure of the bean raises until they crack at the end of the roasting process leading to a boost of volatile aroma compound release. Upon roasting, bean density has been demonstrated to be diminished from 1.3 g cm^{-3} of green beans to 0.65 g cm^{-3} of roasted beans [20]. Additionally, the initial moisture of 12% in green beans has been shown to decrease resulting in a final moisture of 1% to 3% [21, 22]. Moreover, the textural and mechanical properties of coffee beans change during the roasting process. Due to heat-induced transformation of the tissue composition, beans become brittle and fragile [23]. At the end of the roasting process, the coffee beans show a cinnamon brown coloring resulting from non-enzymatic browning reactions like Maillard reactions and caramelisation. The final roasting step involves the addition of cold air or water to the beans to rapidly stop the process. Cooling down the coffee beans avoids burning and maintains the desired roasting degree with the specific flavor and coloring of the beans [24].

The final roasting degree is traditionally classified on luminosity as light, medium or dark, and results of ground coffee color measurements are mostly expressed with the chromaticity coordinates $L^*a^*b^*$ [25, 26]. Luminosity (L^*) decreases progressively with increasing roasting degree. While light roasted ground coffees possess L^* color values of 29 – 31, medium roasted ones show L^* color values of 26 – 27 and dark roasts hold L^* values of approximately 23 – 24 [18, 27]. Increased weight loss is also associated with enhanced roasting degree. According to Anese and Nicoli [27], light roasts hold weight loss values of approximately 14.5%, medium roasts of about 16.2% and dark roasts of about 18.9%. However, the magnitude strongly depends on the roasting temperature and time applied, and therefore limits sample comparison [18]. Mostly, multiple parameters are regarded to more precisely characterize the roasting degree. Alessandrini *et al.* [28] were able to link near infrared spectra of differently roasted coffee samples to the roasting attributes weight loss, density and moisture, and provide a new analytical method to estimate and predict the roasting degree. Likewise, the application of an electronic nose off-line system coupled to an artificial neuronal network has also been proposed to evaluate the roasting degree [29].

Assessment of the coffee roasting degree is an important tool to control the roasting process and ensure coffee quality. Depending on the roasting conditions, individual coffee constituents are generated or decomposed differently, although the roasted coffees

might present a similar final roasting degree. Therefore, it is essential to determine the chemical composition of a roasted coffee especially with regard to an association with its potential health beneficial effect. Beside the modulation of the ingredient profiles through specified roasting conditions, coffee species and varieties play an important role as these naturally determine the composition of certain compounds, e.g. caffeine, polyphenols, chlorogenic acids, fatty acids, tocopherols, trigonelline and free amino acids [30-33]. Table 1 summarizes the average chemical composition of green and roasted Arabica and Robusta coffee beans.

Table 1. Chemical composition of green and roasted *C. arabica* and *C. canephora* beans in g per 100 g (adapted from Farah [34] and Stadler *et al.* [35]). This material is reproduced with the permission of John Wiley & Sons, Inc..

Component [g per 100 g]	<i>C. arabica</i>		<i>C. canephora</i>	
	Green	Roasted	Green	Roasted
Carbohydrates/ fiber				
Sucrose	6.0 – 9.0	4.2	0.9 – 4.0	1.6
Reducing sugars	0.1	0.3	0.4	0.3
Polysaccharides	34 – 44	31 – 33	48 – 55	37
Lignin	3.0	3.0	3.0	3.0
Pectin	2.0	2.0	2.0	2.0
Nitrogenous compounds				
Protein/ peptides	10 – 11	7.5 – 10	11 – 15	7.5 – 10
Free amino acids	0.5		0.8 – 1.0	
Caffeine	0.9 – 1.3	1.1 – 1.3	1.5 – 2.5	2.4 – 2.5
Trigonelline	0.6 – 2.0	0.2 – 1.2	0.6 – 0.7	0.3 – 0.7
Nicotinic acid		0.02 – 0.03		0.01 – 0.03
N-methylpyridinium		0.06 – 0.25		
Lipids				
Coffee oil	15 – 17	17	7.0 – 10	11
Diterpenes	0.5 – 1.2	0.9	0.2 – 0.8	0.2
Minerals	3.0 – 4.2	4.5	4.4 – 4.5	4.7
Acids and esters				
Chlorogenic acids	4.1 – 7.9	1.9 – 2.5	6.1 – 11.3	3.3 – 3.8
Aliphatic acids	1.0	1.6	1.0	1.6
Quinic acid	0.4	0.8	0.4	1.0
Melanoidins		25		25

Many chemical reactions occur simultaneously during the roasting process. The content of carbohydrates, proteins and amino acids decreases during roasting, giving rise to the formation of Maillard reaction products. While chlorogenic acids and trigonelline are strongly degraded, the lipid fraction and caffeine appears to be very stable upon heat treatment (Table 1).

In green coffee beans, soluble and insoluble high molecular weight carbohydrates prevail as they function as structure building elements of the cell, while monosaccharides contribute only in minor proportions to the total carbohydrate content [36, 37]. During the roasting process, depolymerisation and fragmentation of the polysaccharides takes place, resulting in an increase of water-soluble carbohydrates. Depending on the roasting conditions, about 12 – 40% of the polysaccharides are decomposed [38]. Moreover, oligomers and monomers are converted to sugar degradation products, thereby augmenting the availability of reducing sugars for their participation in the Maillard reaction [39].

The major protein in green coffee is the coffee storage protein (11S), representing about 45% of the endospermic protein and thereby accounting for about 5 – 7% of green coffee dry matter [40]. The main amino acids are glutamic acid, phenylalanine, serine, alanine, aspartic acid and lysine [41]. Differences in the amino acid profile between Robusta and Arabica green beans could also be ascertained [36]. Roasting induced only slight variations in crude protein levels [20], though fragmentation of proteins [42] as well as a shift in the protein composition due to pyrolysis and polymerization reactions have been observed [41]. In contrast, free amino acids are rapidly decomposed during roasting so that only trace amounts are detectable in roasted coffee [41]. Free amino acids present essential nitrogenous flavor precursors. Their reaction with sugar degradation products may lead to the formation of Maillard reaction products.

The heat treatment during coffee roasting induces a complex set of chemical reactions, e.g. Maillard reactions, involving cyclisation, retroaldolisation, Amadori rearrangement, pyrolysis, isomerisation, dehydration and condensation [43]. Within different stages, a myriad of volatile aromatic compounds and brown-colored melanoidins are formed. Melanoidins are defined as nitrogenous, high molecular and brownish compounds originating in the late stages of the Maillard reaction. Several coffee constituents have been proven to participate in their generation such as free amino acids, proteins with reactive amino acid side chains, reducing mono- and disaccharides as well as polysaccharides with reducing ends and chlorogenic acids [44-47].

In green coffee, triacylglycerides are the dominant lipid fraction and account for about 75%. The second most abundant group displays terpene esters, e.g. cafestol, methylcafestol and kawheol, representing about 15% of total lipids [48, 49]. Moreover, 16-O-methylcafestol is only found in Robusta coffee and is, therefore, a reliable indicator for the discrimination of Arabica and Robusta coffee [48]. Minor proportions of partial acylglycerols, free sterols, sterol esters and free fatty acids have also been detected in the coffee oil. The most prevalent fatty acids are palmitic acid, linoleic acid and stearic acid [48, 49]. Tocopherols (α , β , γ) are also present in green coffee [50]. Alves *et al.* [32] found green Arabica coffee to contain higher amounts of α - and β -tocopherol than Robusta coffee and further showed that contents were only little affected by roasting. In general, the lipid content of coffee beans is hardly changed during the roasting process [12, 20].

The content of caffeine (1,3,7-trimethylxanthine), the most prevalent alkaloid in coffee, is conspicuously higher in green Robusta coffee than in Arabica coffee [5, 30, 51]. Caffeine is considered to be relatively stable against heat treatment [5, 12, 52]. The calculated slope for the time-dependent degradation of caffeine at 240°C was steeper during roasting of Arabica coffee (-0.231 g kg^{-1} per minute) than during roasting of Robusta coffee (-0.161 g kg^{-1} per minute). After 15 minutes of roasting Arabica and Robusta coffees lost about 11.3% and 7.63%, respectively, of the initial amount of caffeine [51]. However, Franca *et al.* [20] reported a loss of 29% – 36% after roasting of Arabica coffee at 200°C for 1 h in a convective oven.

Trigonelline (1-methylpyridinium-3-carboxylate) is another important alkaloid in raw coffee beans. Analysis of trigonelline contents of unroasted *C. arabica* beans including 114 samples showed average concentrations of $6 - 13 \text{ g kg}^{-1}$ whereas in *C. canephora* var. *robusta* (90 samples) concentrations of about $3 - 11 \text{ g kg}^{-1}$ were found [53]. Differences in the mean trigonelline content of green coffee were also determined by Casal *et al.* [51] who detected about 8.91 g kg^{-1} and 6.32 g kg^{-1} in Arabica coffee from Brazil and Robusta coffee from the Ivory Coast, respectively. Mazzafera [54] determined the trigonelline contents in the seeds of several rare coffee species and varieties and found the lowest concentration with 7.7 g kg^{-1} dry matter in *C. dewevrei* cv. Excelsa. In contrast, *C. canephora* cv. Guarini possessed the highest concentration with 31 g kg^{-1} dry matter [54]. However, upon thermal treatment trigonelline is largely degraded and contents in roasted coffee vary depending on the roasting times and temperatures applied. In contrast, the concentration of nicotinic acid increases [51]. This observation is based on the decomposition of trigonelline via a non-decarboxylative pathway (Figure 1). Here, the N-methyl group of

trigonelline migrates leading to methylnicotinate. In the presence of water, the methyl ester is hydrolyzed, subsequently generating nicotinic acid [55]. Light roasted Arabica coffee (2 min, 260°C) contained about 55 $\mu\text{mol g}^{-1}$ dry matter, but concentrations gradually diminished to 5 $\mu\text{mol g}^{-1}$ dry matter in darker roasts (7 min, 260°C). At the same time, the amount of nicotinic acid raised from 0.4 $\mu\text{mol g}^{-1}$ dry matter in light roasted to 3 $\mu\text{mol g}^{-1}$ dry matter in dark roasted coffee [56]. Hence, decomposition of trigonelline is not exclusively occurring *via* the non-decarboxylating pathway. A second mechanism for the degradation of trigonelline has been proposed that involves a decarboxylation, leading to a zwitterionic intermediate (Figure 1) that can be further converted to 3-methylpyridine or hydrolyzed to 1-methylpyridinium hydroxide. Pyridine is generated upon thermally induced methanol loss of 1-methylpyridinium hydroxide [55]. Stadler *et al.* [57] were able to prove the formation of the suggested zwitterionic intermediate 1-methylpyridinium (N-methylpyridinium, NMP) in their model studies by employing trigonelline hydrogen chloride instead of trigonelline hydrate as precursor. Furthermore, the authors postulate that NMP might react with nucleophiles leading to the formation of dialkylpyridinium [35, 57]. Quantification of NMP was accomplished in coffees of various roasting degrees. Here, a strong positive correlation between the NMP concentration and the roasting degree could be ascertained [35]. On a weight basis, a very dark roast coffee contained up to 0.25% NMP [35].

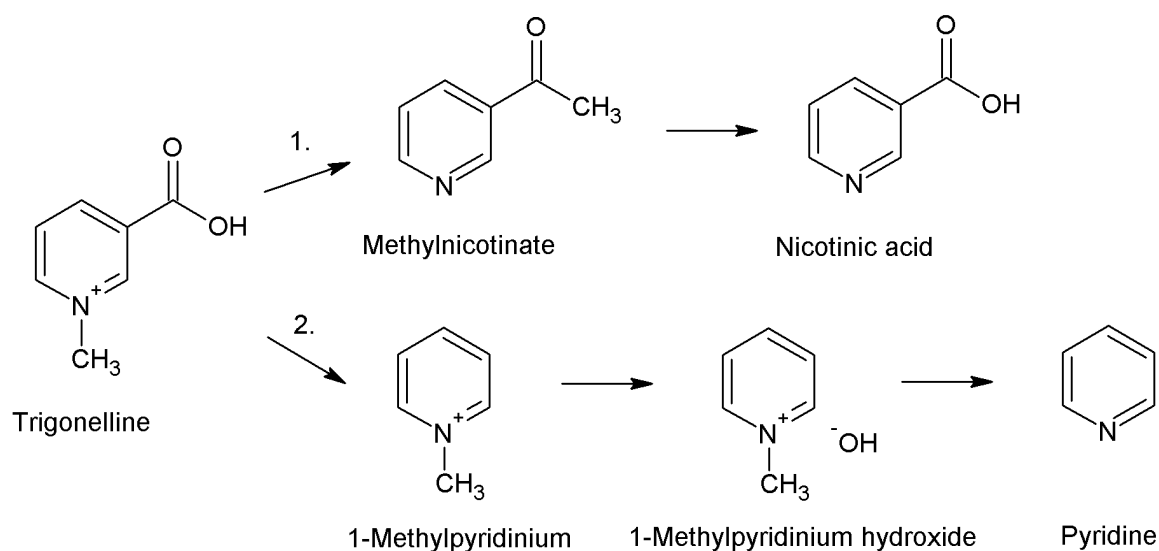


Figure 1. Degradation of trigonelline upon coffee roasting (adapted from Rizzi and Sanders [55] and Stadler *et al.* [57]). This material is reproduced with the permission of John Wiley & Sons, Inc..

Green coffee contains a large variety of phenolic compounds among those chlorogenic acids are the most abundant, representing about 4 – 11% of green coffee dry matter [58-61]. Basically, chlorogenic acids are esters of one molecule of quinic acid and up to three molecules of *trans*-hydroxycinnamic acids, mostly caffeic acid, *p*-coumaric acid or ferulic acid (Figure 2) [62]. Caffeoylquinic acids are the dominant chlorogenic acids and higher concentrations are found in Robusta varieties compared to Arabica varieties [30, 58]. Minor concentrations of other phenolic compounds, e.g. phenolic glycosides, tannins, lignans, proanthocyanidins and anthocyanidins, have also been determined in green coffee beans [5, 61]. Phenols are thermally instable and the roasting process causes a progressive destruction and transformation that is intensified with increasing roasting degree. While the application of mild roasting conditions induced losses of about 60%, almost 100% of the initial chlorogenic acids were degraded in very dark roasts [20, 58, 63]. Chlorogenic acid decomposition is based on several chemical reactions that step-wise lead to isomerisation of chlorogenic acids, release of cinnamic acids and quinic acid. Upon decarboxylation and further degradation, a number of phenolic compounds such as catechol, 4-ethylcatechol, 4-vinylguaiacol, guaiacol and pyrogallol are generated [64, 65]. Moreover, chlorogenic acids may be transformed to chlorogenic acid lactones [66] or incorporated to melanoidins [46].

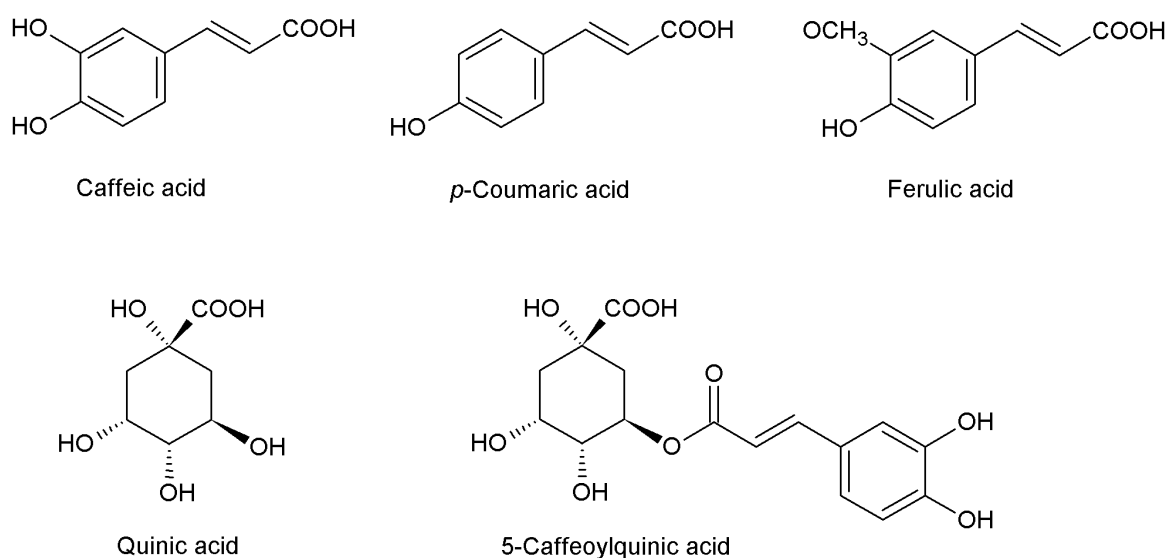


Figure 2. Chemical structures of the phenolic compounds caffeic acid, *p*-coumaric acid and ferulic acid, quinic acid and 5-caffeoylquinic acid.

About 850 volatile compounds have been identified in roasted coffee, each contributing to the aroma profile of coffee. However, key odorants for specific flavor attributes have been assigned. Methylpropanol, 2-methylbutanal and 3-methylbutanal display the main volatiles for the sweetish/ caramel attribute of coffee, while acetaldehyde and propanol are characteristic compounds of the attribute fruity. The key odorant of the sulphorus/ roasty group is 2-furfurylthiol, and for the smoky/phenolic group it is 4-vinylguaiacol. Alkylpyrazines and furanones have the greatest impact on the coffee aroma attributes earthy and spicy, respectively [67]. The concentrations of these aroma compounds and the final aroma profile of roasted coffee largely depends on the raw bean variety, quality and the thereof resulting concentration of potential aroma precursors as well as the roasting process and the final roasting degree [67]. In order to estimate flavor evolution during the roasting process, the online analysis of volatile formation in the roaster off-gas by proton transfer reaction time-of-flight mass spectrometry has emerged [68]. More recently, a new approach for online monitoring of evolving volatiles and semi-volatiles during the roasting process was published, focusing on the analysis of volatiles evoked by roasting of individual coffee beans [69]. For the development of a functional coffee beverage that gains consumer acceptance, it is relevant to understand the aroma formation during the roasting process and link the pleasurable sensory properties with a high availability of health beneficial coffee components.

1.3 Potentially bioactive coffee constituents: daily intake and metabolism in humans

Several beverages, e.g. coffee, black and green tea, yerba mate, cola, and several energy drinks, contribute to the daily caffeine uptake [70, 71]. In the US, the caffeine intake was estimated to reach about 193 mg per day, whereupon coffee displays the major source and accounts for about 71% of the total caffeine intake [72]. Rudolph *et al.* [71] estimated the caffeine intake of the Austrian population by means of a food-frequency questionnaire. The average daily caffeine uptake amounted to 264 ± 129 mg [71]. However, the daily caffeine intake is globally varying, being low in China with about 16 mg per day and high in Denmark with about 390 mg per day [70]. After oral ingestion, caffeine is rapidly absorbed and highly bioavailable. The consumption of 300 mL regular coffee leads to plasma peak caffeine concentrations of 27 – 34 μ M [73] that are reached 15 to 120 min post load [73-75]. Following eight week consumption of 3 cups regular coffee per day, fasting baseline caffeine plasma concentrations amounted to 0.3 μ M [76]. The plasma half-life of caffeine averages 2.5 – 4.5 hours [75, 77]. Caffeine is predomi-

nately metabolized in the liver by the enzyme cytochrome-P450 1A2, which facilitates demethylation leading to the formation of paraxanthine (80%), theobromine (11%) and theophylline (4%) (Figure 3) [78, 79]. Thereby, the conversion to paraxanthine and its further degradation are considered as a rate-limiting process in caffeine clearance [79, 80]. Further demethylation promotes the formation of monomethylated xanthines that are subsequently oxidized by the enzyme xanthine oxidase to methyluric acids [81]. Moreover, trimethyluric acids and dimethyluric acids may be directly generated from caffeine, paraxanthine, theobromine or theophylline [81, 82]. Additionally, paraxanthine is partially converted in the liver by a N-acetyltransferase-mediated ring opening to 5-acetylamino-6-formylamino-3-methyluracil (AFMU) [83], which, in turn, might undergo spontaneous deformylation to S-acetyl-6-amino-3-methyluracil in the urine [81]. In 24-hour urine samples, 11 different metabolites could be detected after administration of 300 mg caffeine [84]. The main metabolites of paraxanthine such as AFMU, 1-methyluric acid and 1-methylxanthine accounted for about 60% of all caffeine-derived metabolites [81]. In contrast, only 1 – 3% of the ingested caffeine were found unchanged in the urine within 48 hours after caffeine administration [85]. Caffeine pharmacokinetics in healthy volunteers have been observed to depend on the ethnic origin, the physiological state, cigarette smoking and the intake of oral contraceptive steroids. The differences are mainly based on cytochrome-P450 1A2 activity variations [84, 86, 87].

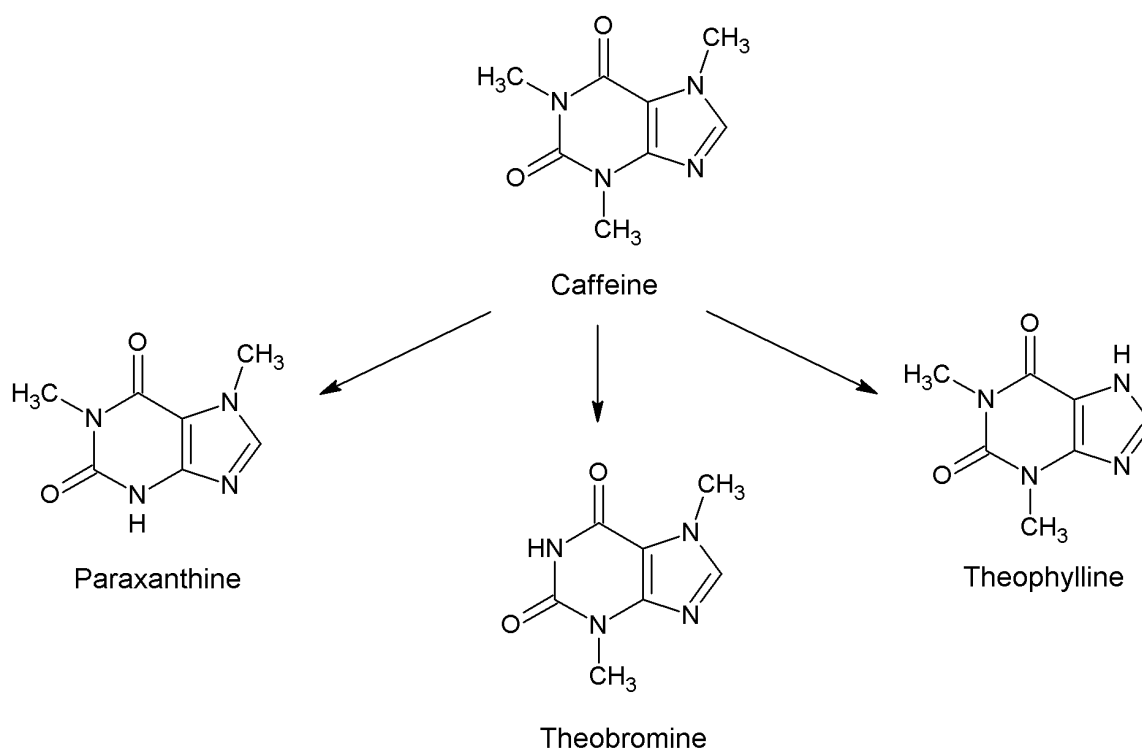


Figure 3. Metabolism of caffeine by the liver enzyme cytochrome-P450 1A2

Coffee is also a major dietary source for trigonelline. However, several foods, e.g. alfalfa sprouts, chick peas, lentils, peas and pumpkin also contain trigonelline [88, 89], resulting in a basal plasma concentration of about 160 nM in humans after a coffee-free diet for 10 days [90]. The ingestion of 350 mL medium-dark Arabica coffee containing 809 μmol trigonelline raised plasma concentrations to 6.5 μM ($\pm 17.3\%$) after 3.2 hours and 5.5 μM ($\pm 13.6\%$) after 2.3 hours in female and male volunteers, respectively. After reaching maximum plasma concentrations, trigonelline levels only slowly decreased. Eight hours after intake, urinary excretion in female and male volunteers amounted to 46.2% and 57.4%, respectively, of the ingested dose [90]. While 48 hours after coffee consumption, urinary excretion of trigonelline was still significantly higher than in non-coffee drinkers, after 72 hours, trigonelline concentrations returned to basal urinary levels [91].

N-methylpyridinium was identified as a degradation product upon heat treatment of trigonelline [35, 57]. Although, trigonelline is found in a couple of foods, coffee presents the primary dietary source for NMP due to its roasting-induced formation [92]. After 10 days of coffee abstinence, no NMP was detectible ($< 10 \text{ pmol mL}^{-1}$) in the plasma of the volunteers [90]. In order to investigate the pharmacokinetic behavior of NMP, healthy subjects consumed about 350 mL of freshly prepared coffee brew, corresponding to a total intake of about 172 μmol or 16.2 mg NMP. Plasma peak concentrations of NMP reached about 777 nM ($\pm 13.9\%$) in females and 804 nM ($\pm 28.7\%$) in males after 1.67 hours and 1.05 hours, respectively. Shortly after ingestion, excretion of NMP *via* the urine started. Within the first 2 hours, females and males already excreted 39.6 μM and 49.9 μM , respectively, representing 23.0% and 29.0% of the ingested dose. After 8 hours, urinary excretion of NMP amounted to 61.9% and 69.1% of the ingested dose in females and males, respectively. Therefore, the authors concluded that, after fast absorption, NMP effectively enters the vascular system that allows systematic distribution, followed by rapid urinary excretion [90]. These results indicate that NMP as well as trigonelline are highly bioavailable, hardly metabolized and may reach peripheral tissues in their original structure. Moreover, NMP was proposed as a reliable dietary biomarker of coffee consumption in humans that is clearly verifiable in urinary samples up to 72 hours after coffee ingestion, hence presenting a suitable constituent for compliance control of coffee intake in human intervention studies [91].

The term niacin combines both nicotinic acid and nicotinamide, found among others in meat, dairy products and whole wheat [93]. Estimation of nicotinic acid and nicotinamide in selected foods revealed remarkably higher nicotinamide than nicotinic acid concentrations in breakfast cereals, pickled herring and beef liver, whereas coffee presented high-

er amounts of nicotinic acid than nicotinamide, due to its generation from trigonelline during the roasting process. Hence, coffee consumption effectively contributes to the dietary intake of nicotinic acid [56]. However, nicotinic acid was not detectable in traces in the plasma samples of volunteers after consumption of 350 mL coffee brew containing 3 mg nicotinic acid [90]. Only after bolus administration of 1000 mg to volunteers within a pharmacokinetic study performed by Pfuhl *et al.* [94], nicotinic acid was recovered in plasma samples but concentrations were only slightly above the limit of detection (50 ng mL⁻¹). The difficulty of nicotinic acid detection in human plasma might be explained by the rapid conversion to nicotinic acid mononucleotide and nicotinic acid adenine dinucleotide to generate nicotinamide adenine dinucleotide (NAD⁺), an essential coenzyme involved in several redox reactions in energy metabolism [93].

1.4 Principles of energy homeostasis

Energy metabolism is subjected to a precise regulation to maintain whole-body energy homeostasis. In order to ensure the balance between energy intake and energy expenditure, the central nervous system (CNS) plays a crucial role. Moreover, individual cells recognize and respond to energy requirements, hence, adjusting intracellular metabolic activity. It is well known that disruption of this tightly regulated system results in elevated incidence of obesity, type 2 diabetes, dyslipidaemia or hypertension. Sensing of the energy status as well as intact regulation of food intake, fuel metabolism and energy expenditure is essential to maintain energy balance and to prevent associated human diseases.

Several processes contribute to energy expenditure that can be assigned either to the obligatory or the facultative energy expenditure. Obligatory energy expenditure merges the basal metabolic rate, including maintenance of body temperature, ion gradients across the cell membranes as well as basal synthetic reactions and the energy expenditure associated with nutrient digestion, absorption and metabolism. Facultative energy expenditure involves energetic costs upon pregnancy, lactation or growth, diet-induced or cold-induced heat production and exercise that shows high inter-individual variability [95]. The generation of heat is commonly termed as thermogenesis and based on exothermic reactions in energy metabolism. The energy expenditure that is due to thermogenesis can be measured by direct calorimetry. In contrast, indirect calorimetry determines the amount of oxygen consumed and carbon dioxide produced allowing the discrimination between adenosine triphosphate (ATP) generation and heat production [96].

On the cellular level, mitochondria play a fundamental role in energy metabolism by coupling oxygen consumption to the synthesis of ATP. After ingestion, dietary nutrients, such as glucose and fatty acids, are dissipated *via* glycolysis and β -oxidation, respectively, and the citric acid cycle. The resulting reducing agents NADH/H^+ and FADH_2 are further subjected to the mitochondrial respiratory chain. A scheme of the mitochondrial electron transport chain is depicted in Figure 4.

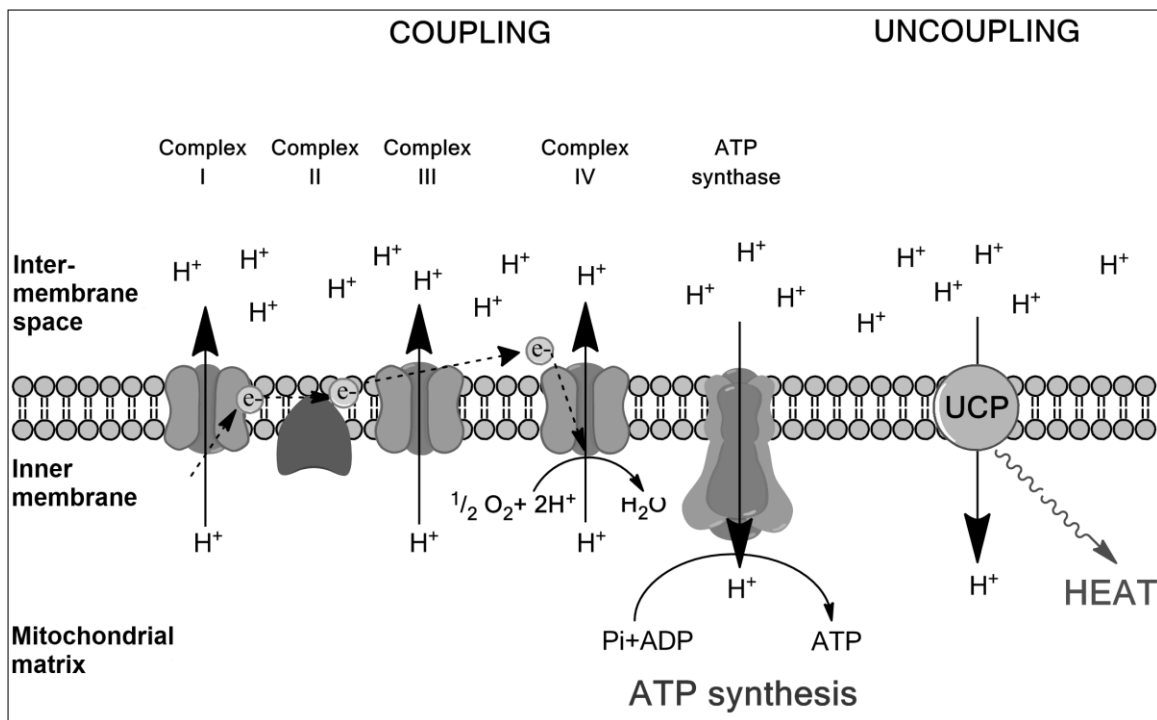


Figure 4. Mitochondrial electron transport chain. Upon coupling, the energy of the proton gradient established by the respiratory complexes is used by the ATP synthase for ATP production while upon uncoupling, e.g. by uncoupling proteins, the energy is dissipated as heat (adapted from Riedel *et al.* [97]).

Oxidation of the reducing agents delivers electrons to the electron transport chain and allows the respiratory complexes, which are located in the inner mitochondrial membrane, to pump protons from the mitochondrial matrix to the intermembrane space. Hence, they generate a proton electrochemical potential gradient. At complex IV, the cytochrome-c oxidase, molecular oxygen acts as the final electron acceptor and reacts with hydrogen ions to produce water. The energy provided by the proton reflux is used by the ATP synthase to phosphorylate ADP to ATP. In a coupling state, oxygen consumption rates are directly proportional to ATP synthesis [96]. In aerobic organisms, mitochondrial oxidative phosphorylation displays the primary source of ATP. However, oxygen consumption can be uncoupled from ATP production, leading to thermogenesis. Here, the energy of the established proton gradient is released as heat and not available

for ATP synthesis. During uncoupling the proton permeability of the inner mitochondrial membrane is increased either by naturally occurring proton leaks or uncoupling agents. Carbonylcyanide *m*-chlorophenylhydrazone (CCCP), carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and dinitrophenol belong to the group of artificial uncoupling substances. In contrast, fatty acids and uncoupling proteins (UCPs) are uncouplers that are naturally present in the organism [98, 99]. Uncoupling proteins are mitochondrial anion carrier proteins that are located in the inner mitochondrial membrane, having their C- and N-terminus on the cytosolic side [100]. Several uncoupling proteins have been identified that are differently expressed in various tissues. UCP-1 is only expressed in brown adipose tissue, while UCP-3 is also highly present in skeletal muscle, white adipose tissue and to a lower extent in heart tissue. In contrast, UCP-2 is expressed ubiquitously [101]. Since uncoupling proteins promote thermogenesis, their induction might be a target to increase energy expenditure.

In order to take up energy, food displays the only source for humans. The regulation of food intake, hence hunger and satiety sensations, occurs primarily in the central nervous system. Several biochemical parameters are involved for which hormonal and metabolic signals play a crucial role [102]. In response to nutrient ingestion, gastrointestinal peptides like peptide YY, cholecystokinin (CCK), glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are released and evoke a satiation signal [103]. However, due to their short half-lives, these gastrointestinal peptides acutely reduce meal size and exhibit only little effects on subsequent meal intake and body weight [104]. Meal anticipation promotes the secretion of ghrelin in the stomach, an orexigenic peptide that stimulates growth hormone secretion and neuropeptide Y release in the hypothalamus, thus inducing hunger and increasing meal size [104]. In addition, Cummings *et al.* [105] provide evidence that ghrelin is also involved in long-term regulation of body weight. Food intake and subsequent digestion result in an increase of plasma free fatty acids and glucose as well as insulin concentrations, whereby the elevated metabolic status also mediates an eating-inhibitory effect. Apart from these gastrointestinal and metabolic signals, several hormones affect energy homeostasis by acting on hypothalamic neuronal pathways. In the arcuate nucleus, two neuronal populations mediate opposed effects in the regulation of energy homeostasis. One population expresses pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript. Cleavage of the precursor POMC leads to several active peptides that initiate anorexigenic pathways and promote a negative energy balance by increasing energy expenditure and decreasing food intake. In contrast, the second population synthesizes the orexigenic peptides neuropeptide Y and agouti-related peptide that exhibit anabolic effects and

promote a positive energy balance [102, 106]. The adipocyte-derived hormones leptin and adiponectin regulate appetite and energy balance by acting on hypothalamic neurons and peripheral tissue, thereby contributing to long-term body weight maintenance. Leptin is secreted from the adipose tissue and concentrations correlate with the amount of energy stored in fat. Activation of the leptin receptor in the hypothalamus, especially in the arcuate nucleus, mediates the repression of orexigenic pathways by inhibition of neuropeptide Y release [107]. Adiponectin levels, in contrast to leptin, are inversely related to adiposity. In peripheral tissues, adiponectin exhibits profound effects on fatty acid oxidation, improves insulin sensitivity and promotes energy expenditure [102]. Additionally, adiponectin acts in the central nervous system, presumably on the melanocortin pathway, and contributes to decreased body weight by enhancing energy expenditure [108]. The release of insulin also serves as an adiposity signal in the CNS by stimulating POMC synthesis and, consequently, initiating catabolic reactions resulting in body weight reduction in the long term [104, 106, 109]. Furthermore, serotonin, a neurotransmitter in the CNS, is involved in appetite regulation. Endogenous serotonin is released within the hypothalamus in response to raising peripheral CCK and tryptophan concentrations as well as certain macronutrients. Consequently, the serotonin-mediated inhibition of neuropeptide Y and cleavage of POMC reduce food intake [110, 111].

Monitoring the body's energy status is essential for the precise control of energy metabolism. Here, the AMP-activated protein kinase (AMPK) plays a key role not only in capturing the energy status of individual cells but also in regulating whole-body energy homeostasis. On the cellular level, activation of AMPK occurs upon energy deficiency when the intracellular AMP:ATP ratio rises. AMPK is allosterically activated by 5'AMP, but phosphorylation of the complex by upstream kinases, e.g. LKB1 and Ca^{2+} -calmodulin-dependent protein kinase, is required for functioning [112-114]. As a consequence, ATP-producing pathways are switched on and ATP-consuming pathways are switched off. In peripheral tissues, such as skeletal muscle, liver and adipose tissue, the AMPK-induced stimulation of catabolism includes induction of fatty acid oxidation and concomitant suppression of lipid biosynthesis by inactivation of acetyl-CoA carboxylase, fatty acid synthase and glycerol phosphate acyl transferase. Moreover, AMPK activation promotes glucose uptake and glycolysis by increasing the expression of glucose transporters and phosphorylation of 6-phosphofructo-2-kinase, respectively. Inhibition of protein synthesis was also demonstrated [112]. In addition, evidence exists for the role of AMPK in the regulation of whole-body energy metabolism. The hormones leptin and adiponectin are capable to activate AMPK in skeletal muscle and liver cells [113]. Furthermore, Minokoshi *et al.* [115] demonstrated hypothalamic AMPK to regulate food intake and

body weight. It was shown that overexpression of dominant negative AMPK reduced the expression of neuropeptide Y and agouti-related peptide in the arcuate nucleus and consequently decreased food intake and body weight in mice. The inhibition of AMPK as essential target for the transmission of leptin's action on body weight and food ingestion was suggested, since leptin was shown to suppress AMPK activity in the arcuate and paraventricular hypothalamus [115]. In contrast, ghrelin stimulated hypothalamic AMPK activity and inhibited AMPK in adipose tissue and liver of rats, thus resulting in increased appetite and lipid deposition [116]. Hence, sensing of the energy status is essential for the regulation of energy homeostasis and the balance of energy intake and energy expenditure, thereby promoting a healthy body weight.

1.5 Effects of coffee consumption on body weight maintenance in humans

In 1977, a study by Bauer *et al.* [117] was published investigating the effect of a life-long replacement of water by coffee in adult mice. Coffee-fed mice showed lower body weight than the water-fed control mice, although they consumed as much or more food and fluid. However, they also presented markedly impaired physical conditions, loss of fur and consistently reduced life time. Considering the daily coffee uptake by a mouse (4 mL per 30 g body weight), this would correspond to a consumption of about 8 L of coffee per day for a 60 kg human. Emanating from this observation, the question arose whether habitual coffee consumption also promotes body weight loss without adverse side effects and if so, what the active principle in coffee and its underlying mode of action is. Epidemiological evidence exists for an association between moderate coffee consumption and body weight maintenance. Greenberg *et al.* [118] ascertained a significant association between the daily intake of ≥ 2 cups of caffeinated or decaffeinated coffee and reduced weight gain among the < 60 year old subjects in their 8.4 year prospective cohort study including a total of 7,006 volunteers. In a 12-year prospective study by Lopez-Garcia *et al.* [119] that included 58,157 participants, an increased coffee consumption was associated with decreased weight gain. This effect was stronger in women than in man, and among women even more pronounced for those possessing a body mass index (BMI) $> 25 \text{ kg m}^{-2}$. Moreover, results from controlled human intervention trials support the hypothesis of coffee as body weight regulating beverage. Overweight subjects that consumed Coffee Slender®, a green coffee extract enriched coffee, showed a significant body weight and body fat reduction by $5.4 \pm 0.6 \text{ kg}$ and by $3.6 \pm 0.3 \text{ kg}$, respectively, after 12 weeks of intervention with 11 g coffee per day containing about 500 mg chlorogenic acids [120]. These reductions were significantly different from the regular

coffee group consuming the same amount of conventional instant coffee. The author therefore postulates that chlorogenic acid might play an important role presumably by modifying glucose metabolism and promoting the usage of fat deposits [120]. Furthermore, Vinson *et al.* [121], investigating the body weight modulating effect of a 6-week intake of the chlorogenic acid-rich green coffee extract GCA™, also detected a reduction in body weight, BMI and body fat after administration of 1050 mg green coffee extract to 16 overweight subjects. These findings strengthen the body weight modulating effect of coffee, although the coffee product in this study was administered as encapsulated dietary supplement and not as beverage from roasted coffee beans. However, a contradictory result was observed in an intervention study published by Kotyczka *et al.* [122]. The obese subjects lost about 2.5 ± 1.0 kg after consumption of daily 500 mL of a dark roast coffee for 4 weeks, while no changes were monitored after consumption of the same amount of light roast coffee, possessing higher contents of chlorogenic acids [122]. Besides these findings, supplementation of a weight-maintaining diet with a beverage containing coffee-extracted mannoooligosaccharides (4 g per day) for 12 weeks also resulted in a body weight loss and a reduction in total body volume in overweight men, though not in women [123]. Although the results of the presented controlled clinical studies point to a beneficial effect of coffee on body weight maintenance, it is challenging to ascribe the salutary properties to a specific coffee constituent. Coffee contains a multitude of potential health beneficial compounds such as caffeine, chlorogenic acids, trigonelline, nicotinic acid, N-methylpyridinium, catechols, pyrazines and melanoidins. In order to identify key active components, individual substances have to be regarded, but also their possible interaction in the organism has to be considered.

1.6 Impact of coffee and coffee constituents on energy metabolism

Since body weight loss occurs if energy expenditure exceeds energy intake, the potential of coffee and coffee constituents to stimulate energy metabolism, thereby promoting energy expenditure and reducing energy intake by inducing satiety as potential mechanisms, should be discussed.

Caffeine is by far the most widely investigated coffee constituent. In the CNS, caffeine acts as a psychostimulant. It increases alertness and cognitive function by interacting adenosine receptor-associated neurotransmission. There are four types of adenosine receptors known that exhibit opposed effects. Upon activation, the A_{2A} and A_{2B} adenosine receptors stimulate adenylyl cyclase, while A₁ and A₃ adenosine receptor activation

causes an inhibition. In general, stimulation of adenosine receptors leads to repression of neuronal activity. It is well established that caffeine and its metabolites act as adenosine receptor antagonists with high binding affinity to the A_1 and A_{2A} type. Promotion of behavioral function by caffeine, therefore, is attributed to the blockage of these receptors and thereof associated changes in intracellular signaling cascades and neurotransmitter release [124, 125]. Moreover, caffeine affects the cardiovascular system. Acute caffeine ingestion increases systolic and diastolic blood pressure, but in the long term the effect attenuates. Enhanced norepinephrine release and direct antagonism to adenosine, both resulting in vasoconstriction, have been proposed to explain alterations in blood pressure and heart rate [126]. Caffeine also seems to have an impact on renal function. Ingestion of approximately 300 mg caffeine may enhance diuresis. However, this effect is unlikely evoked by adenosine receptor inhibition. Osswald and Schnermann [127] suggested a solute diuresis.

A beneficial effect of caffeine on energy metabolism, especially energy expenditure, is also postulated. Already in 1936, Horst *et al.* [128] compared the effect of caffeinated (4 mg kg^{-1}) and decaffeinated coffee on oxygen consumption rates in healthy humans by applying a closed circuit helmet, and found an increase in the average basal oxygen consumption after 30 min and 90 min of regular coffee ingestion. Several controlled clinical studies applied indirect calorimetry, based on the measurement of oxygen consumption rates, to investigate the impact of coffee and caffeine on energy expenditure and to estimate their thermogenic effect in humans. A stimulation of energy expenditure over various time periods was demonstrated in lean and obese subjects after administration of regular coffee, but not so after administration of decaffeinated coffee [129-131]. A similar result was obtained after administration of equal amounts of a regular coffee (200 mg caffeine) and a high caffeine containing coffee (400 mg caffeine) whereupon the latter enhanced resting energy expenditure by 14.4% in healthy male and female subjects within 3 hours post-ingestion, while the regular coffee had no effect [132]. Ingestion of 100 mg anhydrous caffeine also augmented the resting metabolic rate within the following 12 hours in lean and post-obese (normal-weight, but previously obese) volunteers. In the post-obese group caffeine consumption additionally enhanced diet-induced thermogenesis [133]. Moreover, consumption of caffeine (8 mg kg^{-1} body weight) and caffeinated coffee (4 mg kg^{-1} body weight) was reported to especially promote the utilization of fat as energy substrate in normal weight volunteers [130]. Astrup *et al.* [134], administering different amounts of caffeine (100 – 400 mg) to healthy normal weight subjects, further demonstrated a positive correlation between the caffeine dose and the 3-hour integrated responses of thermogenesis, glucose, lactate and triglycerides. In contrast, plasma re-

sponses of the caffeine metabolites paraxanthine, theobromine and theophylline showed no significant correlation with the analyzed variables of energy metabolism [134]. These data clearly support the assumption of caffeine, being a key coffee compound, to enhance energy expenditure in humans.

It might be deduced that the increase in resting energy expenditure observed in the human intervention studies presented above results from a caffeine-induced stimulation of oxygen consumption in the electron transport chain in mitochondria. Several studies, employing isolated mitochondria or intact cells from various human or animal tissues, aimed to explore the impact of pure caffeine administration on mitochondrial oxygen consumption and oxidative phosphorylation. However, the results of the performed cell model studies are partly opposed and seem to strongly depend on the caffeine dose and the cell type applied. In isolated human muscle fibers, a stimulating effect on mitochondrial oxygen consumption was monitored after direct addition of 15 mM caffeine. According to the authors, this result was caused by an activation of myosin ATPase as consequence of a Ca^{2+} release from the sarcoplasmic reticulum [135, 136]. Direct respiratory stimulation by caffeine in concentrations higher than 2 mM was also found in rat interscapular brown adipose tissue [137]. However, when applying lower caffeine concentrations up to 250 μM , no effect on respiration activity in this cell type was observed [138]. In contrast, a dose-dependent inhibition of mitochondrial oxygen consumption was demonstrated in isolated rat liver mitochondria and half maximum inhibition was calculated to be reached by 60 mM caffeine [139]. Here, the authors explained their findings by a methylxanthines-induced inhibition of calcium uptake into mitochondria and suggest alterations in membrane proteins.

Caffeine-induced upregulation of uncoupling proteins might also partly contribute to its thermogenic effect. It was shown, that subcutaneous injection of high-dose caffeine (60 mg kg^{-1}) upregulated UCP-1 expression in brown adipose tissue as well as UCP-2 and UCP-3 expression in brown adipose tissue and skeletal muscles of obese yellow KK mice [140]. Upon uncoupling of the electron transport chain, heat release is enhanced, while ATP production is reduced. ATP deficiency and an increase in the ADP/ATP ratio also stimulate mitochondrial energy production.

Antagonism to adenosine may provide a further mechanism to explain the stimulating effect of caffeine energy metabolism. Activation of the adenosine A_1 receptor prevents lipolysis in the adipose tissue [141]. In contrast, inhibition of the A_1 adenosine receptor would increase adenylyl cyclase activity and, in turn, enhance the formation of cAMP. Moreover, the inhibition of 3',5'-cyclic nucleotide phosphodiesterase by methylxanthines

in concentrations higher than 0.1 mM, resulting in elevated cAMP levels, might also be of relevance to explain caffeine's mode of action [142]. Increased cAMP mediates an energy demand that subsequently promotes energy generating pathways, e.g. lipolysis.

Promotion of lipolysis in adipocytes and the provision of fatty acids as energy substrates for ATP production is a characteristic feature of energy deficiency to maintain the biological function of the organism. Consequently, the energy storages are dissipated, resulting in reduced body fat and body weight. Nakabayashi *et al.* [143] found an inhibitory effect of caffeine ($\geq 250 \mu\text{M}$) on intracellular lipid accumulation in 3T3-L1 adipocytes, when added during differentiation and subsequently up to 10 days. A similar result in 3T3-L1 cells was obtained by Murosaki *et al.* [144], showing an inhibition of lipid accumulation after 8 days of caffeine treatment and an induction of lipolysis after 24 hours. Though, the authors could not proof these observations in their mouse model feeding experiment. In contrast, feeding a diluted regular coffee (1:2 with water) to spontaneously diabetic KK-A^y mice for 5 weeks was shown to, not only decrease subcutaneous and retroperitoneal fat storages, but also to improve liver lipids by reducing total liver lipids and triglycerides. The effect was recovered after giving a caffeine solution (250 mg L^{-1}) only. Hence, these results pointed to caffeine as an effective coffee component [145]. In healthy men, intravenous injection of caffeine (4 mg kg^{-1}) evoked free fatty acid mobilization within 3 hours post-administration. Though, the authors conclude that the caffeine metabolite paraxanthine may at least in part play a role in lipolysis as a strong positive correlation was found between plasma paraxanthine and free fatty acids concentrations [146].

Apart from the augmentation of energy expenditure and induction of lipolysis, caffeine and coffee are supposed to affect glucose metabolism. It was shown that caffeine administered intravenously to humans and regular coffee consumption not only increased plasma free fatty acids but also total glucose response and relative insulin response [147, 148]. During an oral glucose tolerance test (OGTT), ingestion of low caffeine concentrations of 1 mg kg^{-1} body weight decreased insulin sensitivity in male and female volunteers in the short-term. This effect was intensified in a dose-dependent manner with increasing caffeine concentrations up to 5 mg kg^{-1} [149]. Therefore, consumption of regular coffee was demonstrated to impair insulin sensitivity in the short-term as estimated by means of glucose tolerance tests [150]. Lowered insulin sensitivity was also monitored after 4-week administration of 1 L regular coffee per day to healthy subjects, leading to the assumption that tolerance development to the adverse effects of high coffee consumption is of longer duration [151]. In contrast, no alterations in parameters of glucose

metabolism were found in overweight volunteers after consumption of 5 cups of regular coffee per day for 8 weeks [152]. Data on the impact of decaffeinated coffee consumption suggest no impact on insulin and glucose response in humans [148, 152-154]. While acute ingestion of decaffeinated coffee resulted in increased plasma glucose and insulin levels in young male volunteers within the first hour of an OGTT in a study by Greenberg *et al.* [155], Battram *et al.* [156] reported the opposed effect in their human intervention study and demonstrated a reduction in the glucose response within 2 hours after administration of decaffeinated coffee to male volunteers.

Although caffeine plays a crucial role in the evaluation of health beneficial effects provoked by coffee intake, other coffee constituents have been proposed to have an influence on energy metabolism. Chlorogenic acids, the most prevalent polyphenols in coffee, are also hypothesized to facilitate body weight reduction [120, 121]. Investigations to clarify the molecular mechanisms were predominately focussed on parameters of glucose and lipid metabolism. In overweight subjects, administration of 1 g chlorogenic acid was shown to effectively attenuate the OGTT-induced glucose and insulin boost after 15 min compared to the placebo [154]. Recently, a reduction in the total glucose response during an OGTT was also shown in *Lep^{db/db}* mice after acute or 14 days intraperitoneal injection of 250 mg kg⁻¹ chlorogenic acid [157]. Enhanced glucose uptake into peripheral tissues has been proposed as potential mechanism. In cell culture experiments, one hour treatment of 3T3 adipocytes with 10 – 100 µM chlorogenic acid augmented cellular glucose uptake [158]. Incubation of L6 skeletal muscle cells with 2 mM chlorogenic acid also increased glucose uptake after one hour, while after 24 hours of exposure a concentration of 1 mM was effective [159]. The plasma glucose lowering effect of chlorogenic acid was ascribed to the activation of AMPK and subsequent translocation of glucose transporter type 4 [157, 159]. Inhibition of the hepatic glucose-6-phosphatase, resulting in reduced glucose release, has also been proposed as potential underlying mechanisms [160]. Moreover, supplementation of a high-fat diet with chlorogenic acid improved plasma and liver lipid profile and diminished body weight gain in animal models [161, 162]. Alterations in plasma adipokine levels, stimulation of β-oxidation and peroxisome proliferator-activated receptor alpha expression in the liver, as well as AMPK activation, resulting in fatty acid synthesis inhibition, have been observed after chlorogenic acid treatment and might explain the beneficial effects on lipid metabolism [157, 161, 162]. However, it has to be considered that chlorogenic acid is substantially metabolised in humans and plasma concentrations reached after coffee consumption are in the nanomolar range [163, 164], hence far beyond doses employed in the above presented

studies. Therefore, research on the effectiveness of physiological doses, obtained by coffee ingestion, is required.

The alkaloid trigonelline is also proposed to exert hypoglycaemic effects [165]. Administration of 500 mg trigonelline to overweight subjects decreased the plasma glucose and insulin response at the 15 min time point during an OGTT [154], though not mediated by an action on the incretin hormones GLP-1 and GIP [166]. Studies on the long-term effect of trigonelline feeding (50 mg kg⁻¹ body weight per day) of diabetes-induced rats showed reduced blood glucose, triglyceride and total cholesterol levels as well as improved insulin sensitivity when compared to the diabetes group after 4 weeks. Moreover, values of these parameters were similar to the non-diabetes control [167]. Further analyses provide similar results, pointing to a protective effect of high dose trigonelline on diabetes development [89, 168]. Additionally, trigonelline feeding of diabetic KK-A^y mice for three weeks was effective in reducing triglycerides in liver and adipose tissue [169]. These animal studies present some hints to a beneficial effect of trigonelline to ameliorate diabetic symptoms, but doses applied here are far above the physiological intake of humans. Moreover, evidence from controlled clinical human intervention studies is weak, and currently the mode of action is poorly explainable.

N-methylpyridinium, the degradation product of trigonelline is highly present in dark roast coffee and recent investigations dedicated to its chemopreventive capacity [170, 171], antioxidant activity [172] and inhibition of gastric acid secretion [173]. Only one study published by Kotyczka *et al.* [122] presented a first hint to a body weight modulating effect in obese subjects after 4 weeks intake of daily 500 mL NMP-rich dark roast coffee. This effect is hardly explainable as the study was primarily focused on the effect of coffee consumption on the antioxidant defense and parameters of energy metabolism have not been investigated. Therefore, there is no knowledge about the potential of NMP to modify cellular energy metabolism.

Nicotinic acid is an essential nutrient for pellagra prevention and acts as precursor for the electron carrier NAD⁺ that plays an important role in mitochondrial energy production. Gram doses of nicotinic acid are effectively applied to treat hyperlipidaemia since it lowers plasma cholesterol, total blood lipids, plasma free fatty acids and concomitantly increases high density lipoprotein cholesterol [174-177]. With the deorphanization of the nicotinic acid receptor, appearing in two subtypes (HM74A and HM74), nicotinic acid's mode of action became explainable [178-180]. Nicotinic acid suppresses lipolysis in adipocytes through a nicotinic acid receptor-mediated inhibition of the adenylyl cyclase, resulting in reduced cAMP levels. Moreover, Westphal *et al.* [181] demonstrated an in-

crease in plasma adiponectin and leptin in male subjects with metabolic syndrome after 6 weeks of extended-release niacin (1500 mg per day) treatment, but no improvement of atherosclerosis-associated inflammatory parameters could be ascertained. However, pharmacological doses up to 3 gram have to be administered to observe these effects, since nicotinic acid is rapidly metabolized. In contrast, after ingestion of 350 mL freshly prepared coffee brew the total nicotinic acid uptake amounts to about 3 mg [90]. Hence, it might be supposed that in the intestine already lower concentrations of nicotinic acid are sufficient to modify lipid metabolism.

Taken together, the literature provides evidence for the stimulating effect of coffee on energy metabolism in humans. Increased energy expenditure may, at least partly, be attributed to caffeine. However, coffee contains a multitude of constituents, from those some compounds, e.g. chlorogenic acids, trigonelline and nicotinic acid, have already been hypothesized to affect glucose and/or lipid metabolism. Moreover, a wide range of alterations associated with energy metabolism after coffee consumption has been monitored in humans that, at present, are incompletely elucidated. Verification of the mechanisms of action is ongoing. Additionally, coffee still provides a broad spectrum of new and poorly investigated compounds that might also contribute to the overall effect of coffee consumption on energy metabolism.

1.7 Impact of coffee and coffee constituents on satiety

A reduction in food intake also evokes a decrease in body weight at remaining energy expenditure. Evaluation of the satiating effects of a special food or compound mostly involves estimation of food intake and subjective feelings of hunger and satiety in combination with measurements of satiety-associated hormones and peptides. The visual analogue scale is a widely spread method to determine subjective appetite sensation and is presented to the volunteers as a composed line with the strongest opposed statements at each end. According to the appetite feelings, volunteers put a mark on the line and the distance is measured to assess individual ratings [182].

Several studies have been conducted to investigate the impact of coffee consumption on food intake and satiety, but results are inconsistent so far. A study by Gavrieli *et al.* [183] found high-caffeinated coffee (6 mg caffeine kg⁻¹ body weight) to be more effective in reducing the total energy intake of one day in comparison to low-caffeinated coffee (3 mg caffeine kg⁻¹ body weight) and the water control after bolus administered to overweight

subjects, but could neither verify this effect in normal weight volunteers nor demonstrate changes in appetite feelings. Another hint to caffeine as a potentially active compound was provided by Tremblay *et al.* [184], presenting a reduction in spontaneous energy intake in normal-weight men after consumption of 300 mg caffeine, but not so in women and hence, suggesting a gender-specific sensitivity to caffeine. Food intake was also assessed after intraperitoneal injection of caffeine (50 mg kg⁻¹) in food-deprived and fed rats [185]. In both cases, administration of caffeine decreased the food intake of the animals. Caffeine, therefore, is considered as a potential coffee constituent that reduces short-term food intake although a precise mode of action is not yet clarified.

A recent study by Greenberg and Geliebter [186] provided contradictory results and found significantly lower hunger feelings accompanied by higher plasma peptide YY levels in healthy male volunteers after consumption of decaffeinated coffee. Consumption of regular coffee showed less pronounced effects and administration of caffeine in water did not differ from the water control. The authors of this study therefore concluded, that coffee constituents, other than caffeine, account for the observed effects. Moreover, no changes in acute energy intake, hunger feelings or biochemical indicators of hunger and satiety, e.g. plasma ghrelin, peptide YY or GLP-1, after consumption of either caffeinated or decaffeinated coffee were observed in healthy male volunteers in another study by Gavrieli *et al.* [187].

The above mentioned investigations were focused on the short-term effects of coffee or caffeine on food intake and satiety. Controlled studies, proving a reduction in energy intake and increased satiation after prolonged coffee consumption, are rare. Indication for reduced food intake after 4-weeks of daily 500 mL coffee consumption was provided in a study by Kotyczka *et al.* [122] showing that the total daily energy intake was decreased by about 12% in the obese subjects of the NMP-rich dark roast coffee group also demonstrating a mean body weight loss of about 2.5 kg. However, no biochemical parameters, associated with hunger and satiety, have been determined in this study.

Taken together, evidence from the literature for a satiety-promoting effect of coffee exists, but the outcome of the available studies is partly opposed. It still remains unclear whether coffee consumption causes profound changes in the biochemical parameters involved in the regulation of hunger and satiety, and thereby contributes to prolonged body weight management. Moreover, the active compounds still need to be identified.

1.8 Statement of objectives

Maintaining the energy balance of the organism is a complex network involving the regulation of energy metabolism from a single cell and a specific tissue to the whole body. The literature provides some evidence for coffee to beneficially modulate body weight by altering parameters of energy metabolism, e.g. by increasing energy expenditure or reducing food intake. Some coffee constituents such as caffeine and chlorogenic acid have already been proposed as potentially active compounds, but results are partly contradictory. Moreover, the multitude of compounds present in coffee makes it difficult to define a key active compound and a primary mechanism that explains the observations from epidemiological studies and controlled clinical intervention trials. Although there is some indication for a weight modulating effect of coffee, it is still unclear (1) what are the mechanisms of action, (2) which are the most active compounds and (3) if there are synergistic or antagonistic effects.

Therefore, this dissertation aimed to characterize the effect of coffee and coffee components on mechanisms regulating energy metabolism and satiety on the basis of cell culture studies and a human intervention trial. Cell culture studies were carried out to evaluate the potential of selected coffee constituents to stimulate mitochondrial energy expenditure by inducing substrate utilization and increasing thermogenesis. The ability of caffeine, a widely studied compound in coffee, to alter energy production pathways was studied first. Since currently very little is known about the impact of trigonelline on energy metabolism, and no knowledge about the effect of N-methylpyridinium in this research area exists, further investigations were focused on these structurally related substances. In addition, the impact of pyridine derivatives such as nicotinic acid, trigonelline and NMP that are also present in coffee, on intestinal lipid metabolism was studied. The aim was to elucidate their impact on fatty acid uptake of intestinal cells, identify their key structural determinants and clarify the underlying mode of action.

In the human intervention study, the weight-reducing effect of an optimized coffee beverage (study blend, SB) in comparison to a reference coffee (market blend, MB) was studied. Both coffees contained equal amounts of caffeine. However, the SB coffee was characterized by higher amounts of NMP, but lower amounts of chlorogenic acid and trigonelline than the MB coffee. This dissertation, as part of the human intervention study, concentrated on effects of coffee on parameters of energy metabolism and thermogenesis as well as on the mechanisms regulating satiety.

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2 Results

This cumulative dissertation is based on the following research articles and manuscripts.

(1) “Caffeine dose-dependently induces thermogenesis but restores ATP in HepG2 cells in culture“

Annett Riedel, Marc Pignitter, Christina M. Hochkogler, Barbara Rohm, Jessica Walker, Gerhard Bytof, Ingo Lantz and Veronika Somoza

Food and Function 2012, 3, 955-964

The impact of caffeine, the most abundant alkaloid in coffee, on mechanisms of energy production and thermogenesis was investigated in the liver carcinoma cell line HepG2 and in mouse 3T3-L1 adipocytes.

I analyzed caffeine's cytotoxic effects and carried out cellular oxygen consumption and mitochondrial membrane potential measurements. I further examined the UCP-2 and carnitine-palmitoyl transferase protein expression. Moreover, I determined fatty acid uptake and lipid accumulation, contributed to the determination of the adenosine nucleotide contents as well as lactate release measurements. In addition, I performed the statistical analysis of the results. I prepared the manuscript and wrote most of the introduction, material and methods, results and discussion section.

(2) “N-methylpyridinium, a degradation product of trigonelline upon coffee roasting, stimulates respiratory activity and promotes glucose utilization in HepG2 cells“

Annett Riedel, Christina M. Hochkogler, Roman Lang, Gerhard Bytof, Ingo Lantz, Thomas Hofmann and Veronika Somoza

Food and Function 2014, DOI: 10.1039/C3FO60320B

The pyridine derivatives N-methylpyridinium and trigonelline were studied for their ability to modify cellular energy production pathways and thermogenesis in HepG2 cells and their effectiveness as bioactive coffee constituents was compared.

I performed metabolic activity studies simultaneously evaluating cellular respiration, extracellular acidification and cellular impedance. I further contributed to this research article by quantifying the cellular adenosine nucleotides and assessing glucose and fatty acid uptake. Moreover, I determined the mitochondrial membrane potential and the uncoupling-protein-2 expression as parameters of thermogenesis. Beside the performance of most of the cell culture studies, I did the statistical analyses and wrote the manuscript.

(3) “Structure-dependent effects of pyridine derivatives on intestinal fatty acid uptake: Regulation of nicotinic acid receptor and fatty acid transporter expression”

Annett Riedel, Roman Lang, Barbara Rohm, Malte Rubach, Thomas Hofmann, and Veronika Somoza

The Journal of Nutritional Biochemistry 2014, in revision

The impact of pyridine derivatives, a widely distributed compound class found in several foods and commodities (e.g. coffee), on the intestinal fatty acid uptake was investigated to identify their structural determinants. Moreover, the mechanism of action of the selected compounds nicotinic acid and N-methyl-4-phenylpyridinium was elucidated.

I measured the fatty acid uptake in differentiated Caco-2 cells and performed multiple regression analyses to identify the most important structural characteristics of the test compounds. Furthermore, I conducted cytotoxicity experiments, 2-NDBG uptake and intracellular calcium mobilization studies. I also carried out gene expression analyses and determined the trans-epithelial electrical resistance. Moreover, I performed the statistical analyses and prepared the manuscript.

(4) “A 4-week consumption of medium roast and dark roast coffee affects parameters of energy status in healthy subjects”

Annett Riedel, Natalie Dieminger, Tamara Bakuradze, Roman Lang, Gina Montoya, Christina M. Hochkogler, Swantje Winkler, Gerhard Bytof, Ingo Lantz, Herbert Stiebitz, Thomas Hofmann, Elke Richling, Doris Marko, Dorothea Schipp, Jochen Raedle and Veronika Somoza

Food Research International 2014, in revision

The aim of this human intervention study was to assess the long-term effect of two regular coffees on energy metabolism regarding changes in the plasma lipid profiles and alterations in glucose homeostasis. Moreover, the impact of coffee consumption on energy status and thermogenesis was determined.

Within this study, I did blood sample preparations and determined plasma leptin, adiponectin, osteopontin, glucose, free fatty acid and serum insulin concentrations. I isolated lymphocytes from whole blood and therein analyzed the UCP-2 content and contributed to the quantification of adenosine nucleotides. Furthermore, I participated in the statistical analysis of all included parameters. As first author, I also prepared the manuscript.

(5) “Influence of a four week coffee consumption on nutrient intake, body composition, biomarkers associated with satiety and DNA integrity in humans”

Tamara Bakuradze, Gina Alejandra Montoya Parra, **Annett Riedel**, Veronika Somoza, Roman Lang, Natalie Dieminger, Thomas Hofmann, Swantje Winkler, Ute Hassmann, Doris Marko, Dorothea Schipp, Jochen Raedle, Gerhard Bytof, Ingo Lantz, Herbert Stiebitz, and Elke Richling

Food Research International, manuscript submitted December 13th, 2013

This research article investigated and compared the effect of a 4-week consumption of two differently processed coffees on body composition, nutrient intake and satiety. Additionally the impact of coffee consumption on the antioxidant status in healthy humans was analyzed.

I contributed to this article by conducting studies on the satiety parameters ghrelin and serotonin. Furthermore, I was involved in the discussion of the data and the evaluation of the manuscript.

2.1 “Caffeine dose-dependently induces thermogenesis but restores ATP in HepG2 cells in culture”

Annett Riedel^a, Marc Pignitter^a, Christina M. Hochkogler^a, Barbara Rohm^a, Jessica Walker^a, Gerhard Bytof^b, Ingo Lantz^b and Veronika Somoza^a

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PAPER

Caffeine dose-dependently induces thermogenesis but restores ATP in HepG2 cells in culture

Annett Riedel,^a Marc Pignitter,^a Christina M. Hochkogler,^a Barbara Rohm,^a Jessica Walker,^a Gerhard Bytof,^b Ingo Lantz^b and Veronika Somoza^{*a}

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Caffeine has been hypothesised as a thermogenic agent that might help to maintain a healthy body weight. Since very little is known about its actions on cellular energy metabolism, we investigated the effect of caffeine on mitochondrial oxidative phosphorylation, cellular energy supply and thermogenesis in HepG2 cells, and studied its action on fatty acid uptake and lipid accumulation in 3T3-L1 adipocytes at concentrations ranging from 30–1500 μM . In HepG2 cells, caffeine induced a depolarisation of the inner mitochondrial membrane, a feature of mitochondrial thermogenesis, both directly and after 24 h incubation. Increased concentrations of uncoupling protein-2 (UCP-2) also indicated a thermogenic activity of caffeine. Energy generating pathways, such as mitochondrial respiration, fatty acid oxidation and anaerobic lactate production, were attenuated by caffeine treatment. Nevertheless, HepG2 cells demonstrated a higher energy charge potential after exposure to caffeine that might result from energy restoration through attenuation of energy consuming pathways, as typically found in hibernating animals. In 3T3-L1 cells, in contrast, caffeine increased fatty acid uptake, but did not affect lipid accumulation. We provide evidence that caffeine stimulates thermogenesis but concomitantly causes energy restoration that may compensate enhanced energy expenditure.

Introduction

Overweight and adiposity are among the most prevalent health problems that contribute to the development of type II diabetes mellitus and the metabolic syndrome. Maintaining a healthy body weight, therefore, is relevant to health promotion.

A reduction in body weight is the consequence of a negative energy balance whereupon energy expenditure exceeds energy intake. Food is the only energy source for the human organism to maintain its biological functions. After ingestion, nutrients are metabolized and the resulting reducing agents, NADH/H⁺ and FADH₂, are used by the mitochondrial respiratory chain to produce ATP. During oxidative phosphorylation, the amounts of NADH/H⁺, FADH₂ and oxygen consumed correlate directly with the synthesized amounts of ATP.¹ In contrast, during thermogenesis, mitochondrial respiration is uncoupled from oxidative phosphorylation; hence, energy is lost as heat instead of energy conservation through ATP formation.

Natural uncoupling is provoked by uncoupling proteins (UCPs) and fatty acids. Synthetic uncoupling agents like

carbonylcyanide *m*-chlorophenylhydrazone (CCCP), carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) or dinitrophenol are commonly used to mimic thermogenesis *in vitro*.²

Several foods, such as red peppers,³ green tea⁴ or coffee,⁵ are supposed to affect human energy metabolism by inducing thermogenesis and/or increasing the metabolic rate. Recent human intervention studies showed a reduction in body weight and/or body fat after long-term coffee consumption.^{6,7} Acheson *et al.* presented a stimulating effect of caffeinated coffee (4 mg kg⁻¹) on the base-line metabolic rate over a time span of 150 min in normal weight and obese subjects that was stronger than that of decaffeinated coffee. The authors also postulated an increased postprandial thermogenesis for a meal when taken with caffeinated coffee.⁵ *In vitro*, administration of 15 mM caffeine was demonstrated to exhibit a direct effect on the mitochondrial activity of isolated human muscle fibers by increasing the respiration rate and concomitantly decreasing the mitochondrial membrane potential.⁸ Furthermore, Kogure *et al.* showed that subcutaneous caffeine injection (60 mg kg⁻¹) to obese mice upregulated UCP gene expression in skeletal muscles and brown adipose tissue and, therefore, may contribute to thermogenesis.⁹ Thus, caffeine is proposed to be the active thermogenic agent of coffee in the short-term. Considering plasma peak concentrations of 54 μM in humans after consumption of about 4 cups of coffee,¹⁰ the applied concentrations in previous *in vitro* studies

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are hardly transferable to humans since they are far from concentrations that can be found in plasma after coffee consumption or caffeine administration. Nevertheless, the long-term effect of caffeine on body weight regulation in humans by increase of energy expenditure remains doubtful as adaptation to caffeine occurs.¹¹

In the energy deficient state, lipolysis and fatty acid oxidation in adipose tissue are stimulated to provide substrates for ATP formation through the mitochondrial respiratory chain. Induction of lipolysis and inhibition of lipid accumulation are important targets for the reduction of body fat and body weight. *In vitro* studies on caffeine already demonstrated an inhibitory effect on intracellular lipid accumulation in mouse 3T3-L1 adipocytes.^{12,13}

Since caffeine has been suggested to affect energy metabolism, this study aimed to characterize the effect of moderate caffeine concentrations on mechanisms of mitochondrial energy generation, thermogenesis and substrate supplying pathways combined in HepG2 cells and 3T3-L1 cells as well as the comparison of the short-term and long-term effect in one model system to elucidate the complex mechanisms of mitochondrial energy supply by caffeine. We hypothesised that caffeine stimulates mitochondrial thermogenesis in the short-term and in the long-term, but does not necessarily increase substrate oxidation rather than induce energy restoration.

Results

Cellular oxygen consumption

Cellular respiration was assessed in HepG2 cells after direct addition and 24 h pre-treatment with caffeine. The basal respiration was significantly reduced in HepG2 cells after direct addition of caffeine, although not in a dose-dependent manner (Fig. 1A). There was no direct effect of caffeine on the non-phosphorylation respiration after inhibition of ATP-synthase by oligomycin (OM). To test for maximum respiratory capacity, the respiratory chain uncoupler FCCP was added at a final concentration of 1 μ M, a concentration that was ascertained to induce maximal respiration in controls (data not shown).

FCCP-stimulation caused a 1.9-fold increase in oxygen consumption of control cells compared to basal respiration. After administration of 30 μ M caffeine, no respiratory stimulation could be observed by the addition of FCCP. The respiration rate was solely 63% $\pm$ 13% in comparison to the initial respiration prior to caffeine addition (100%) and hardly reached the value of basal respiration during caffeine addition (73% $\pm$ 8%) (Fig. 1A). Maximum respiration was not affected by caffeine at 300 μ M and 1500 μ M, and reached values similar to those of control cells.

In addition to the direct effects of caffeine on mitochondrial respiration, oxygen consumption was also determined after 24 h of exposure of HepG2 cells with caffeine. A dose-dependent decrease in basal respiration was noted after treatment with caffeine (Fig. 1B). Incubation with caffeine reduced the basal oxygen consumption rate by 33% (300 μ M) and 36% (1500 μ M), respectively. A 24 h treatment with caffeine affected neither oxygen consumption rates during ATP-synthase inhibition by oligomycin nor FCCP-uncoupled respiration rates.

The respiratory control ratio (RCR) calculated for untreated control cells was in accordance to values previously established for HepG2 cells by Desquiere *et al.*¹⁴ Calculation of the RCR after treatment with caffeine revealed a significant reduction of the ratio of FCCP-stimulated and ATP-synthase-inhibited respiration after direct administration of caffeine at 30 μ M. Direct addition of higher caffeine concentrations as well as 24 h pre-treatment did not have an impact on the RCR of HepG2 cells (Table 1).

Mitochondrial membrane potential ($\Delta\psi_m$)

The results displayed in Fig. 2A and Fig. 2B represent the amount of depolarised cells based on the decrease of the

Table 1 Respiratory control ratio of HepG2 cells after direct addition and 24 h incubation with caffeine^a

	Control	30 μ M	300 μ M	1500 μ M
Direct	4.65 $\pm$ 0.52 ^a	1.80 $\pm$ 0.19 ^b	4.24 $\pm$ 0.54 ^a	4.57 $\pm$ 0.55 ^a
24 h	4.33 $\pm$ 0.45	3.08 $\pm$ 0.37	2.82 $\pm$ 0.29	3.96 $\pm$ 0.65

^a Results are expressed as mean $\pm$ SEM, $n \geq 5$, significant differences are indicated with the letters a and b ($p < 0.05$).

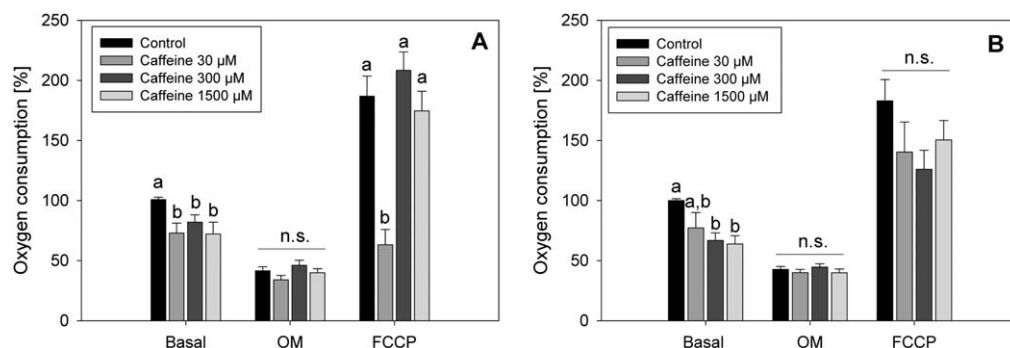


Fig. 1 Cellular oxygen consumption of HepG2 cells after direct addition of caffeine (A) and 24 h pre-treatment with caffeine (B) measured with a Clark-type electrode. The effects of caffeine on basal respiration, non-phosphorylating respiration after oligomycin (OM) addition and the maximum respiratory capacity induced by FCCP are shown for both time points. Results were normalized to basal respiration of controls (100%) (A) or initial respiration (100%) of each measurement (B), respectively. Data were expressed as mean $\pm$ SEM of at least 5 individual experiments. Significances were tested within one respiration phase by one-way ANOVA with SNK post hoc test ($p < 0.05$) and indicated with the letters a and b; n.s. = not significant.

mitochondrial membrane potential. Here, a decreased formation of JC-1 aggregates results in the reduction of yellow fluorescence.

Direct administration of caffeine led to a statistically significant increase of depolarisation induced by all tested caffeine concentrations. Although the observed effect of caffeine was not dose-dependent, an increased depolarisation up to 11% in the presence of caffeine at 300 μM could be reached, pointing to a mild uncoupling effect exhibited by caffeine directly (Fig. 2A). A more pronounced effect was observed after 24 h treatment of HepG2 cells with caffeine, leading to a significant and dose-dependent linear increase of depolarised cells. The rising amount of depolarisation induced by caffeine exposure ranged from +10% (30 μM) to +23% (1500 μM) in comparison to control cells. In contrast, treatment of cells with CCCP, a well-known respiratory chain uncoupler, led to strong induction of depolarisation of approximately 80% in total and a collapsed $\Delta\psi_{\text{m}}$.

Uncoupling protein-2 (UCP-2) protein expression

The determination of UCP-2 in HepG2 cells demonstrated a dose-dependent increase in the UCP-2 content due to caffeine exposure for 24 h, as shown in Fig. 3. A treatment with caffeine at 1500 μM resulted in a significant increase ($p < 0.05$) of UCP-2 up to 40% compared to non-treated controls.

Cellular content of AMP, ADP and ATP

To establish the effect of caffeine on ATP production, cellular contents of AMP, ADP and ATP were quantified and the energy charge potential as a marker for cellular energy supply was calculated. Fig. 4A shows the energy charge potential (ECP) values after 24 h exposure of HepG2 cells in the absence or presence of caffeine. Untreated controls presented a high energy potential that surprisingly could be further increased by treatment with caffeine at 30 μM and 300 μM , respectively. The strongest effect on the ECP was achieved after incubation with caffeine at 30 μM , showing a dose-dependent reduction of the ECP with increasing caffeine concentrations, hence, obtaining values similar to controls after treatment with caffeine at 1500 μM . Quantitative data of AMP, ADP and ATP normalized to total adenosine nucleotides (TAN) demonstrated a significantly lower percentage of AMP with concomitant higher

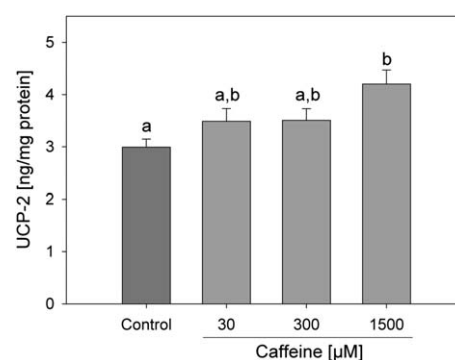


Fig. 3 UCP-2 protein expression of HepG2 cells after 24 h incubation with caffeine determined by ELISA. Significant differences ($p < 0.05$) between treatments were tested by one-way ANOVA with SNK post hoc test and are indicated with the letters a and b. Data represent mean $\pm$ SEM for four individual experiments.

percentage of ATP after 24 h treatment of HepG2 cells with 30 μM caffeine pointing to higher cellular ATP provision than in control cells (Fig. 4B). This effect could also be observed after exposure to 300 μM caffeine, although less pronounced. Data suggest a high cellular energy supply in HepG2 cells after 24 h exposure to caffeine (Fig. 4A) and an augmentation of depolarisation (Fig. 2B). The latter is associated with a decreased mitochondrial membrane potential that is typically monitored during uncoupling of the mitochondrial respiratory chain and related to reduced mitochondrial oxidative phosphorylation.

Lactate release

Glucose is the most important energy substrate that is degraded to pyruvate during glycolysis, then decarboxylated to acetyl-CoA and used for production of NADH/ H^+ through the citric acid cycle. NADH/ H^+ is a reducing agent that serves as an energy source for ATP production through mitochondrial oxidative phosphorylation.

As the mitochondrial oxygen consumption seems to be reduced in HepG2 cells due to caffeine exposure without impairment of the intracellular ATP content, lactate production as an alternative way for ATP formation was examined. Caffeine

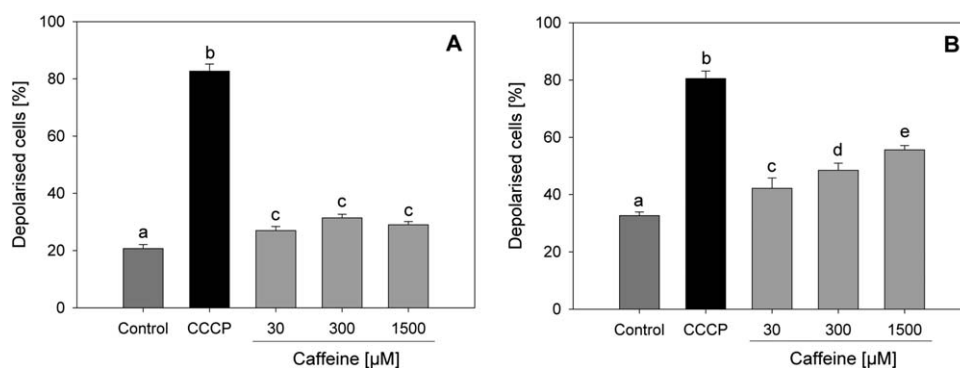


Fig. 2 Mitochondrial membrane depolarisation of HepG2 cells induced by caffeine directly (A) and after 24 h incubation (B) measured by flow cytometry with the mitochondrial membrane potential sensitive fluorescent dye JC-1. The uncoupler CCCP (50 μM) was used as a positive control. Results are shown as mean $\pm$ SEM ($n \geq 3$) and dose-dependency was tested by one-way ANOVA with SNK post hoc test. Significant differences ($p < 0.05$) between treatments are indicated with the letters a–e.

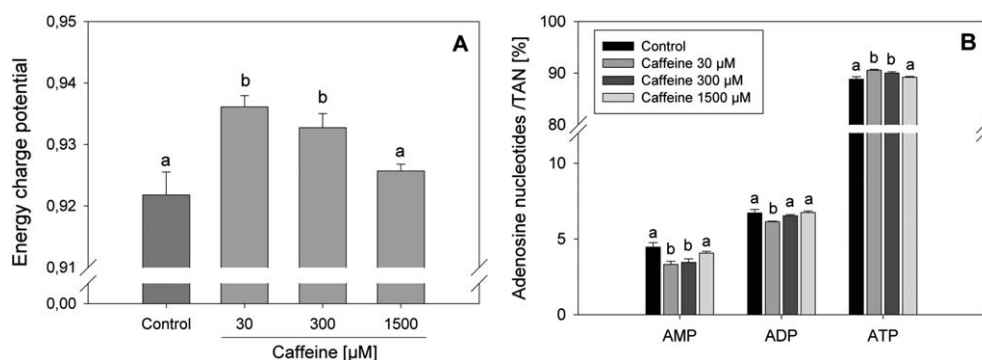


Fig. 4 Energy charge potential (ECP) (A) and cellular AMP, ADP and ATP content quantified by HPLC-DAD in HepG2 cells after 24 h treatment with caffeine. Cellular AMP, ADP and ATP content was normalized to total adenosine nucleotides (TAN). $ECP = [ATP + 0.5(ADP)]/TAN$ was calculated according to the formula by Pradet and Raymond.³¹ Results ($n = 4$) are shown as mean $\pm$ SEM (A) and percentage distribution of TAN (B). Significances were tested by one-way ANOVA with SNK post hoc test for pair-wise comparison ($p < 0.05$) and indicated with the letters a and b.

reduced lactate concentrations in the cell culture medium (Fig. 5). This decrease was statistically significant for treatment with caffeine at 1500 μM, showing a reduction of 13% in lactate production compared to control cells.

Western blot analysis of carnitine palmitoyl transferase 1 (CPT1)

CPT1 is located in the outer mitochondrial membrane and is involved in fatty acid metabolism by regulating the transport of long-chain fatty acids into the mitochondria for β -oxidation. Western blot analyses revealed no effect of caffeine on CPT1 expression in HepG2 cells (Fig. 6A and 6B) after 24 h incubation.

Fatty acid uptake

The impact of caffeine on the uptake of long-chain fatty acids *via* fatty acid transport proteins (FATPs) was studied in 3T3-L1 adipocytes expressing FATP1 and FATP4¹⁵ and HepG2 cells expressing FATP2 and FATP5¹⁶ respectively. As positive control, insulin (100 nM) was used in both cell lines.

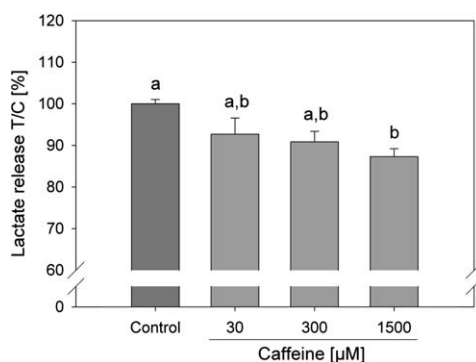


Fig. 5 Lactate release of HepG2 cells determined by LC-MS in incubation medium after 24 h exposure to caffeine. Results were normalized to untreated controls (100%) and are displayed as mean $\pm$ SEM of four individual experiments. Statistical analysis was performed by one-way ANOVA with SNK post hoc test and considered to be significant at $p < 0.05$ indicated with the letters a and b.

Administration of insulin to 3T3-L1 adipocytes resulted in a significantly increased fatty acid uptake being 47% higher than in non-treated control cells (Fig. 7B), an effect that is already described in the literature.^{15,17} A significant stimulating effect of insulin on fatty acid uptake could also be demonstrated in HepG2 cells (Fig. 7A), although less pronounced than in adipocytes.

Caffeine treatment of HepG2 cells caused a significant inhibition of fatty acid uptake ranging from 10% (30 μM and 300 μM) to 16% (1500 μM). In contrast, treatment of 3T3-L1 cells with caffeine enhanced fatty acid uptake (Fig. 7A). A 1.2-fold significant increase was observed by addition of caffeine at 30 μM. No effect was noticed after exposure of 3T3-L1 adipocytes to higher caffeine concentrations.

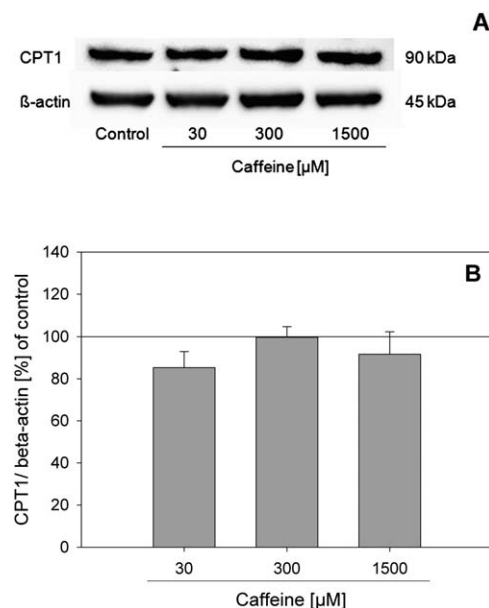


Fig. 6 CPT1 protein levels of HepG2 cells after exposure to caffeine (24 h) examined by Western blot analysis. A representative CPT1 blot with β -actin as internal control to ensure equal protein loading is displayed (A). CPT1/ β -actin band intensity ratios after caffeine treatment were related to the corresponding ratios of untreated controls (100%) (B). Results are shown as mean $\pm$ SEM ($n = 4$).

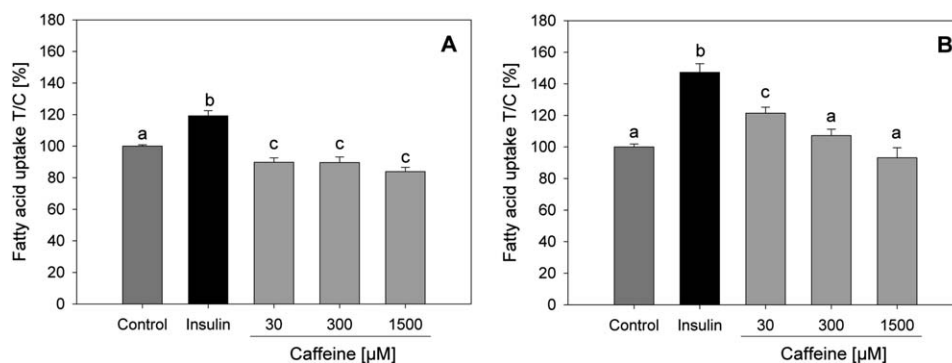


Fig. 7 Fatty acid uptake of HepG2 cells (A) and differentiated 3T3-L1 adipocytes (B) determined by addition of the BODIPY®-dodecanoic acid fluorescent fatty acid analog after 30 min pre-incubation with caffeine. Insulin (100 nM) was used as a positive control. Results represent mean $\pm$ SEM ($n = 4$) calculated from the AUC values obtained after 60 min of fluorescence measurement and normalized to untreated controls (100%). Significances between treatments were tested by one-way ANOVA with SNK post hoc test for pair-wise comparison ($p < 0.05$) and indicated with the letters a to c.

Lipid accumulation

The effect of caffeine on lipid accumulation in differentiated adipocytes was investigated by Oil Red O staining of incorporated oil droplets. However, no effect of caffeine on intracellular lipid accumulation was observed after 10 days of treatment (Fig. 8).

Discussion

This study examined the impact of caffeine on mitochondrial energy metabolism and cellular energy supply to investigate the potential effect of caffeine on cellular energy metabolism. ATP production occurs primarily in mitochondria by oxidative phosphorylation within the respiratory chain. During coupled respiration, the amount of oxygen consumed is proportional to the amount of ATP produced. We demonstrate that caffeine directly inhibited mitochondrial oxygen consumption in HepG2 cells, at a concentration of 30 μM that may be reached as peak plasma concentration after consumption of 3–4 cups of coffee.¹⁰ In contrast, baseline plasma caffeine concentration after regular coffee intake was shown to be approximately 0.4 μM .¹⁸ An inhibitory effect was also found by de la Cruz *et al.* after addition of caffeine to isolated rat liver mitochondria.¹⁹ However, similar

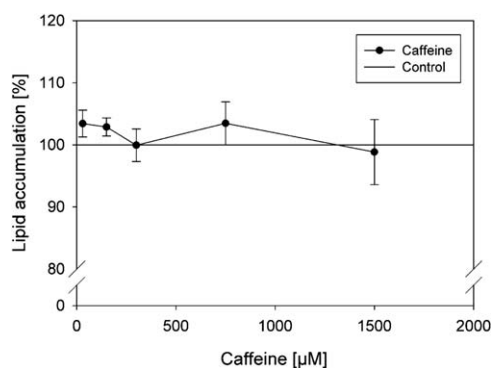


Fig. 8 Lipid accumulation in differentiated 3T3-L1 adipocytes after 10 days of exposure to caffeine. Oil droplets were stained with Oil red O. A one-way ANOVA was performed to test for dose-dependency. Data are expressed as mean $\pm$ SEM for 3 individual experiments.

effects to those observed in this study were only reached after administration of at least 10 mM caffeine. Oxygen consumption studies by Dulloo *et al.* in interscapular brown adipose tissue from rats could not ascertain any effect of caffeine in concentrations up to 250 μM ²⁰ and found respiratory stimulation only after treatment with concentrations higher than 2 mM.²¹ A stimulating effect of respiration by direct addition of caffeine at 15 mM was also demonstrated in human muscle fibers,²² indicating that effects of caffeine on mitochondrial respiration are partly opposed and strongly dose-dependent in various tissues.

So far, no data are available on the effects of caffeine on mitochondrial respiration after prolonged exposure. We revealed that caffeine also decreased basal respiration after 24 h incubation in a dose-dependent manner. As mitochondrial oxygen consumption is related to ATP production, these results indicate a reduced aerobic ATP production in human liver cells due to caffeine. This hypothesis is supported by the observed increase of mitochondrial membrane depolarisation. Caffeine induced a reduction of the mitochondrial membrane potential directly and more pronounced after 24 h treatment, suggesting a reduced ATP production. However, the half-life of caffeine in the plasma ranges between 2.5 and 4.5 h in healthy subjects due to rapid metabolism by the liver.²³ The observed long-term effects after exposure of HepG2 cells to caffeine, therefore, may also be mediated by endogenously generated metabolites.

Caffeine might also exhibit thermogenic effects by acting as a mild uncoupling agent that directly induces proton leaks and/or stimulates UCP-2 protein expression. We provide evidence that caffeine increases energy expenditure by inducing thermogenesis. Besides this, it was previously shown that high, non-physiological doses of caffeine (60 mg kg^{-1}) after subcutaneous injection upregulated UCP gene expression in brown adipose tissue and skeletal muscles of obese yellow KK mice.⁹

Regarding these findings, caffeine seems to alter mitochondrial energy production on the one hand by inhibiting oxygen consumption and increasing depolarisation, respectively, both resulting in a reduced ATP production, and, on the other hand, by increasing thermogenesis.

Quantification of cellular adenosine nucleotides after 24 h treatment of HepG2 cells with caffeine revealed a significantly higher ECP for caffeine at 30 μM and even an ECP value similar

to this of controls for caffeine at 1500 μM . Since caffeine at 1500 μM reduced mitochondrial respiration, showed strong effects on mitochondrial membrane depolarisation and induced uncoupling by UCP-2, alternative pathways to supply the cellular energy or ATP demand might have been activated. An alternative way to produce ATP *via* oxidative phosphorylation is lactate production under anaerobic conditions. Oxidation of 1 mol glucose under aerobic conditions yields in 36 molecules ATP, whereas anaerobic ATP formation *via* lactate generates only 2 molecules ATP per mol of glucose. Although anaerobic ATP formation is less efficient, it represents a faster way in short-term energy supply predominantly found in muscle cells. It is known from the literature that energy production in cancer cells is shifted to lactic acid fermentation, namely known as the Warburg effect,²⁴ taking place even under normal oxygen conditions. Therefore, we examined lactate release of HepG2 cells after 24 h exposure to caffeine. We found that lactate release was significantly reduced after incubation with caffeine at 1500 μM , whereby lower caffeine concentrations did not show any impact on lactate release, leading to the conclusion that a shift of energy production to the lactate pathway might not contribute to the high energy supply. A further substrate used by cancer cells to produce ATP is glutamine. By “glutaminolysis”, glutamine enters the citric acid cycle and (1) can be completely used for aerobic ATP production in the mitochondrial respiratory chain or (2) further converted to pyruvate, resulting in lactate production.²⁵ As utilisation of glutamine for ATP formation within mitochondrial oxidative phosphorylation would raise oxygen consumption and the alternative way would lead to augmented lactate production, we can exclude a shift to glutamine as substrate for the high energy supply prevalent after treatment of HepG2 cells with caffeine.

Moreover, caffeine is supposed to affect lipid metabolism *in vitro*^{12,13} and *in vivo*.^{13,26} A previous study by Shimoda *et al.* showed that caffeine administration resulted in a reduction of serum triglycerides in mice after olive oil-load in the short-term, but no effect of caffeine on CPT1 activity in mice liver mitochondria could be found in the long-term.²⁶ As CPT1 is the rate-limiting enzyme that is involved in long-chain fatty acid transport into mitochondria, we investigated the effect of caffeine on CPT1 protein levels to determine whether the increased ATP concentration presented in this study after caffeine treatment is a consequence of fatty acid oxidation. However, no effect of caffeine on CPT1 expression in HepG2 cells could be observed after 24 h incubation. Indeed, we found a direct impact of caffeine on cellular fatty acid uptake. Independent from the caffeine concentration, the fatty acid uptake of HepG2 cells was diminished, pointing to a reduced activity of fatty acid transport proteins. As energy producing pathways did not seem to be increased rather than reduced by caffeine, another possibility to maintain cellular energy potential is a depression of metabolic activity, suggesting that caffeine might cause energy restoration. Hypometabolism, as is found during hibernation, is characterized by reduction of metabolic rates and energy consuming pathways to endure harsh environmental conditions.²⁷ Moreover, an increase of uncoupling protein mRNA expression in various tissues, suggesting a contribution to thermogenesis during hibernation of ground squirrels, has been shown.²⁸ Similarly, we here ascertained the induction of UCP-2

by caffeine in HepG2 cells, a fact that is consistent with observations during hibernation.

A typical feature of hibernation is also the seasonal weight gain, primarily due to fat storage in adipocytes in preparation of hibernation.²⁹ Grant hypothesised that human obesity might be a preparation for hibernation that in the end does not take place.³⁰

Thus, we further investigated the effect of caffeine on fatty acid uptake in differentiated 3T3-L1 adipocytes. In contrast to HepG2 cells, fatty acid uptake in 3T3-L1 cells was increased by 21% after administration of caffeine at 30 μM , whereas treatment with higher caffeine concentrations did not show differences to controls. This finding would support the hypothesis of Grant,³⁰ but interestingly, higher fatty acid uptake did not lead to an elevated lipid accumulation in 3T3-L1 cells after 10 day long-term exposure to caffeine, which, in contrast, disagrees with the hypothesis that caffeine may induce additional fat accumulation.

Experimental

Materials and chemicals

Unless stated otherwise, all materials, chemicals and reagents were purchased from Sigma-Aldrich, Austria.

Cell culture

HepG2 cells. The human hepatocellular carcinoma cell line HepG2 was cultured in RPMI-1640 medium (supplemented with 10% FBS, 4 mM L-glutamine and 1% penicillin/streptomycin) and maintained at 37 °C and 5% CO₂ in a humidified incubator. For studying the long-term effect of caffeine, cells were seeded one day prior 24 h treatment with caffeine (30 μM , 300 μM and 1500 μM) in incubation medium (RPMI-1640 with 4 mM L-glutamine and 1% penicillin/streptomycin). Controls were treated with incubation medium only. For determination of the direct effect, caffeine was added directly during assay performance.

3T3-L1 adipocytes. Mouse 3T3-L1 pre-adipocytes were cultured in the basal medium (high-glucose DMEM supplemented with 10% FBS, 4 mM L-glutamine and 1% penicillin/streptomycin). For differentiation, pre-adipocytes were grown two days post-confluence in basal medium and differentiation was initiated by replacing the basal medium to the differentiation medium (basal medium supplemented with 10 $\mu\text{g mL}^{-1}$ insulin, 1 μM dexamethasone and 0.5 mM isobutylmethylxanthine) for two days. Then, the medium was changed to the maturation medium (basal medium supplemented with 10 $\mu\text{g mL}^{-1}$ insulin) and cells were maintained for further two days. After additional three to five days of subsequent culture in basal medium, differentiated cells showing the typical adipocyte phenotype (accumulation of lipid droplets) were used for the measurement of direct effects of caffeine. For long-term studies, differentiated 3T3-L1 adipocytes were incubated in basal medium in the absence or presence of caffeine (30 μM , 300 μM and 1500 μM) for 10 days exchanging the incubation medium every second day.

Cell viability

HepG2 cells. Cell viability was assessed by the WST-1 assay (Roche Diagnostics, Switzerland) and performed according to the manufacturer's protocol to exclude cytotoxic effects of treatments. As caffeine at 3000 μM was toxic to HepG2 cells, this concentration was excluded in further analyses (data not shown).

3T3-L1 adipocytes. Toxicity was determined by the MTT assay. After 10 days of exposure, the incubation medium was removed and MTT working solution (1 : 6 dilution of 5 mg mL^{-1} MTT stock solution in RPMI-1640 medium) was added. Cells were incubated for 4 h during which viable cells reduced the yellow MTT reagent to a purple formazan. Afterwards, the medium was removed and DMSO was added to dissolve the formazan product. Optical density was measured with a microplate reader (infinite M200, Tecan, Austria) at 550 nm using a reference wavelength of 690 nm to correct for unspecific absorbance. No toxic effects could be observed up to caffeine concentrations of 1500 μM (data not shown).

Mitochondrial oxygen consumption measurement in intact cells

Oxygen consumption measurements were carried out in intact HepG2 cells with slight modifications of the method described by Loiseau *et al.*³¹ For measurement of mitochondrial oxygen consumption following 24 h treatment with caffeine, HepG2 cells were washed with PBS and harvested by trypsinization. The cell suspension was centrifuged at $110 \times g$ for 5 min. The pellet was resuspended in at 37 °C air-equilibrated RPMI-1640 medium supplemented with 4 mM L-glutamine to a final concentration of exactly 10×10^6 cells mL^{-1} . Immediately, 1 mL of cell suspension was transferred to the measurement chamber of a Clark-type oxygen electrode (Hansatech Instruments, UK) and maintained at 37 °C with constant stirring. The oxygen consumption measurement was started by recording the basal endogenous respiration rate. Then, oligomycin (8 $\mu\text{g mL}^{-1}$) was added to inhibit ATP-synthase and the non-phosphorylating respiration rate was determined. To induce the maximal respiratory activity, the uncoupler FCCP was added at a concentration of 1 μM . As FCCP acts biphasically in intact cells, a titration curve was performed to assess the optimal FCCP concentration that induced maximal uncoupled respiration (data not shown). Finally, the respiratory complexes I and III were inhibited by the addition of rotenone (1.25 μM) and antimycin A (3 μM), respectively. For measurement of the direct effect of caffeine on mitochondrial respiration, HepG2 cells were starved for 30 min prior to cell harvest and cell preparation was carried out as described above. After recording the initial basal oxygen consumption, caffeine was directly added to the cell suspension and the caffeine-induced respiration rate was assessed for 5 min. Oligomycin, FCCP, rotenone and antimycin A were subsequently added, as described above.

The linear rate of oxygen consumption during each phase of measurement was calculated. Respiration rates were related to the basal respiration rates of controls or the initial respiration rates when examining the direct effect of caffeine, respectively. Moreover, the mitochondrial respiratory control ratio (RCR) of intact cells was estimated as the ratio of oxygen consumed during

uncoupling with FCCP to that during ATP-synthase inhibition after oligomycin addition.³¹

Mitochondrial membrane potential ($\Delta\Psi_{\text{m}}$)

Changes of the mitochondrial membrane potential were assessed using the JC-1 probe (BioVision, Inc., US) based on the method described by Cossarizza and Salvioi.³² For determination of $\Delta\Psi_{\text{m}}$, 24 h pre-treated cells were washed with PBS and harvested with a detachment solution (PBS supplemented with 2 mM EDTA and 5 mM glucose). After centrifugation at $300 \times g$ for 5 min, the cell pellet was resuspended in the staining medium (MEM medium supplemented with 2 mM L-glutamine and 1% penicillin/streptomycin). The JC-1 dye dissolved in DMSO was added to a final concentration of 4 μM and samples were incubated for 15 min at 37 °C. To calibrate the method, a 5 min pre-incubation with 50 μM CCCP, an uncoupler that strongly decreases the mitochondrial membrane potential, was performed. Samples were centrifuged at $300 \times g$ for 5 min and washed once with washing buffer (PBS supplemented with 5% FBS). After another centrifugation at $300 \times g$ for 5 min, the stained cell pellets were resuspended in the washing buffer to a maximum concentration of 500 cells μL^{-1} . Yellow fluorescence, as shown by JC-1 aggregates in the mitochondrial matrix of polarised cells, as well as green fluorescence, emitted by JC-1 monomers in the cytosol of depolarised cells, were assessed with the EasyCyte 8HT flow cytometer (Millipore GesmbH, Austria). The direct effect of caffeine on the mitochondrial membrane potential was determined by adding caffeine to the cell suspension and allowing a 5 min incubation prior to staining with the JC-1 dye.

Uncoupling protein-2 (UCP-2) protein expression

The cellular UCP-2 content of HepG2 cells was determined by ELISA. After 24 h treatment with caffeine, cells were washed quickly with ice-cold PBS and harvested by scraping in lysis buffer (50 mM Tris, 25 mM NaCl, 1 mM EDTA, 1 mM NaF, 1% Igepal, pH 7.4) supplemented with 1mM PMSF, 1mM sodium *ortho*-vanadate and protease inhibitor cocktail. After homogenization and centrifugation at $16\,000 \times g$ for 10 min at 4 °C, the supernatant was used for quantification of UCP-2 by means of a commercial ELISA kit (USCN Life Sciences, China). Results were normalized for total protein content of the supernatants determined by the bicinchoninic acid assay using bovine serum albumin (BSA) as a standard (Pierce® BCA Protein Assay Kit, Thermo Scientific, Austria).

Cellular content of AMP, ADP and ATP

The cellular content of the adenosine nucleotides AMP, ADP and ATP were measured in HepG2 cell extracts by HPLC-DAD. Therefore, 24 h pre-treated cells were washed with PBS and ice-cold perchloric acid (0.3 M) was added immediately. After scraping off the cells from the plate, cell lysates were centrifuged at $16\,000 \times g$ for 10 min at 4 °C. The pH of the acid extract was adjusted to pH 6 with sodium carbonate (1 M). Prior to HPLC injection, a solid phase extraction (SPE) was performed using the strata-X-AW polymeric weak anion column (Phenomenex, Germany) according to the manufacturer's protocol (application

note CN-039) with slight modifications. After activation of the column, first with methanol, then a mixture of formic acid/methanol/water (2/25/73) and finally water, cell extracts were loaded followed by washing steps with water and a methanol/water mixture (50/50). For elution, a mobile phase of ammonium hydroxide/methanol/water (2/25/73) was used and the eluted phase was passed through a 0.22 μm nylon filter (Carl Roth, Germany). Samples were injected into a Dionex Ultimate 3000 series UHPLC system consisting of a binary pump, a degasser, an autosampler and a column compartment connected to a diode array detector (Dionex, Austria). Data was collected using the Chromeleon 6.8 software (Dionex, Austria). The separation was achieved by gradient elution with mobile phase A consisting of 10 mM tetra-butyl-ammonium bisulphate, 10 mM potassium dihydrogen phosphate in water (pH 7.0) and 10% acetonitrile filtered through 0.2 μm regenerated cellulose (Carl Roth, Germany) while mobile phase B consisted of 100% acetonitrile (HPLC grade). The gradient was as follows: 0 min 100% A, 0% B; 1 min 100% A, 0% B; 8 min 92% A, 8% B; 13.5 min 70% A, 30% B; 18 min 100% A, 0% B; 29 min 100% A, 0% B. The column was a Phenomenex Luna® C18 (5 μm , 100 Å, 250 $\times$ 3 mm) LC column running at 25 °C. The mobile phase flow rate was 0.5 mL min⁻¹. The absorbance of the adenosine nucleotides was monitored at 259 nm. For quantification, an external six-point calibration curve was recorded for each standard (AMP 0.5–50 μM , ADP 0.5–50 μM , ATP 1–100 μM). Recovery was determined by standard addition to cell extracts prior to SPE and included in the final calculation (AMP 69.8% $\pm$ 7.1, ADP 91.8% $\pm$ 8.0, ATP 95.1% $\pm$ 9.6). From the final concentrations, the total adenosine nucleotides (TAN) value was calculated by TAN = ATP + ADP + AMP as well as the energy charge potential (ECP) by ECP = [ATP + 0.5(ADP)]/TAN as described by Pradet and Raymond.³³

Lactate release

Lactate release was determined by LC-MS after 24 h incubation of HepG2 cells with caffeine. The incubation medium was removed and centrifuged at 16 000 $\times g$ at 4 °C for 20 min. Quantification of lactate in incubation medium was performed by the external standard method. Commercially available sodium lactate was used as an external standard for a five-point calibration curve within the range 1–50 $\mu\text{g mL}^{-1}$ obtaining correlation coefficients of ≥ 0.999 . All analyses were conducted in duplicates as described by Sickmann *et al.*³⁴ on the Shimadzu Prominence HPLC system equipped with a quaternary pump and a single-quadrupole mass spectrometer with ESI as interface (Shimadzu, Austria). Data integration was performed with Shimadzu LC-MS software LabSolutions Version 5. The analyses were accomplished isocratically at 0.5 mL min⁻¹ on a Luna® 5 μm C18 100 Å LC column 250 $\times$ 3 mm (Phenomenex, Germany) as stationary phase at 25 °C. The mobile phase consisted of 20% methanol and 80% ultrapure water. A volume of 5 μL was injected and the total run time was 5 min. Prior injection the samples passed through a 0.22 μm nylon filter (Carl Roth, Germany). The mass spectrometer was operated in negative-ion mode. Lactate was determined with selected ion monitoring (SIM) for specific m/z of 89. For SIM mode, a nebulizing gas (N_2) flow of 1.5 L min⁻¹, a drying gas (N_2) flow of 5.0 L min⁻¹,

a desolvation line temperature of 250 °C, a heat block temperature of 200 °C and an interface voltage of 4.5 kV was selected. For reliable identification of lactate, in-source fragmentation was applied to standards and samples revealing a prominent fragment ion at m/z of 45 after a decarboxylation reaction. To achieve in-source fragmentation a desolvation line voltage of –20 V and a Qarray® DC voltage of –40 V was used.

Western blot analysis of carnitine palmitoyl transferase 1 (CPT1)

After incubation of HepG2 cells with caffeine (30 μM , 300 μM and 1500 μM) for 24 h, cell lysates were prepared as described for UCP-2 protein expression and maintained under constant agitation for 30 min at 4 °C. Then, cell lysates were centrifuged at 16 000 $\times g$ for 10 min at 4 °C and the protein content of the cleared supernatant was determined by the Bradford assay.³⁵ Western blot analysis of CPT1 was performed as described by Mazzarelli *et al.*³⁶ with slight modifications. Protein extracts were boiled in loading buffer for 5 min at 95 °C and a total of 40 μg protein was subjected to a 10% SDS-PAGE. After separation, proteins were transferred by semi-dry blotting for 1.5 h to PVDF membranes (PeqLab Biotechnolgy, Austria), previously activated in methanol for 2 min and ice-cold blotting buffer (48 mM Tris, 39 mM glycine, 0.08% SDS, 20% MeOH) for 5 min. The membranes were blocked with 5% skim milk for 1 h for detection of CPT1 followed by overnight incubation at 4 °C with the primary CPT1 antibody (rabbit polyclonal anti-CPT1A H95, Santa Cruz Biotechnology, Inc., Germany) 1 : 500 diluted in 5% skim milk. The membranes were washed with TBS-T buffer and incubated for 1 h at room temperature with the secondary antibody (anti-rabbit IgG-HRP, Santa Cruz Biotechnology, Inc., Germany) 1 : 4000 diluted in 5% skim milk. After another washing with TBS-T buffer, membranes were developed using the SuperSignal chemoluminescent detection kit (Thermo Scientific, Austria) and visualized with the Fusion device (Vilber Lourmat, Germany). β -Actin was used as a loading control. Sample preparation, protein separation and protein transfer were conducted as described for CPT1 detection. Membranes were blocked with 5% BSA for 1 h and incubated with the 1 : 2000 in 5% BSA diluted primary β -actin antibody (rabbit anti- β -actin 13E5 mAb, Cell Signaling Technology®, Germany) for 1.5 h at room temperature. After washing with TBS-T buffer the secondary antibody (anti-rabbit IgG-HRP, Cell Signaling Technology®, Germany) 1 : 1000 diluted in 5% BSA was added for 1 h at room temperature. The membranes were washed with TBS-T buffer, developed and detected as described above for CPT1. Band intensities were calculated by applying the ImageJ software.

Fatty acid uptake

HepG2 cells. The fatty acid uptake was determined by the QBT™ Fatty Acid Uptake Assay Kit (Molecular Devices Corporation, Germany) employing a BODIPY®-dodecanoic acid fluorescent fatty acid analog. HepG2 cells were deprived with serum-free medium for 1 h at 37 °C followed by addition of caffeine diluted in a HBSS/Hepes buffer. For controls only HBSS/Hepes was added and cells were allowed to incubate for

30 min at 37 °C. As a positive control, insulin at a final concentration of 100 nM was used. Then, the fluorescent fatty acid analog diluted in HBSS/Hepes buffer supplemented with 0.2% fatty acid free BSA was added and fluorescence signals were recorded for 60 min at 515 nm after excitation at 485 nm with the infinite M200 microplate reader (Tecan, Austria). The area under curve (AUC) was calculated for each sample to determine the cellular fatty acid uptake and results were expressed as percentage of controls.

3T3-L1 adipocytes. Differentiated adipocytes were seeded on the experimental day and left to adhere for 4 h prior to deprivation with serum-free medium for 1 h at 37 °C. The measurement of fatty acid uptake in adipocytes was performed according to the procedure as described for HepG2 cells.

Lipid accumulation

Lipid accumulation in differentiated 3T3-L1 adipocytes was determined by Oil Red O staining after 10 days of exposure to caffeine. The incubation medium was removed and a freshly prepared 10% formalin solution was added. After 5 min, the formalin solution was renewed and cells were fixed for another hour. After washing with 60% isopropanol, the cells were stained for 30 min with the Oil Red O working solution, consisting of six parts of the 3.5% Oil Red O in isopropanol (w/v) stock solution and four parts ultrapure water. The cells were washed four times with ultrapure water, left to dry and the stained oil droplets were dissolved in 100% isopropanol. Quantification was carried out by absorbance measurement at 520 nm with a microplate reader (infinite M200, Tecan, Austria). Lipid accumulation is presented as the percentage of control cells.

Statistical analysis

Data are presented as mean $\pm$ SEM. All experiments were done in multiple replicates as stated in the figures (n = number of biological replicates). Dose-dependent effects were determined by one-way analysis of variance (ANOVA) after performing a Nalimov outlier test. The Student–Newman–Keuls (SNK) test was applied as a post hoc test for pair-wise comparison. Significant differences between treatment groups ($p < 0.05$) are indicated in the figures with distinct letters.

Conclusions

Caffeine affects mitochondrial energy production in HepG2 cells in the short-term and in the long-term by enhancing thermogenesis, probably through mitochondrial uncoupling resulting from (1) a direct induction of proton leaks and (2) enhanced expression of uncoupling proteins, *e.g.* UCP-2. The cellular energy loss due to augmented thermogenesis was not compensated by either intensified mitochondrial oxidative phosphorylation or ATP formation, or a shift to anaerobic energy production. Therefore, we conclude that caffeine provokes energy restoration that might be the result of attenuated energy consuming pathways.

Compared to HepG2 cells, adipocytes may react differently to caffeine exposure. In the presence of fatty acids, caffeine did enhance fatty acid uptake, although no effect on lipid

accumulation was observed. This result provides evidence for tissue specific actions of caffeine that need further investigation to judge the overall anti-obesity effect of caffeine.

Although the results of this *in vitro* study suggest an alteration of mitochondrial energy production and energy supply by caffeine, *in vivo* studies have to be considered to demonstrate this effect.

Abbreviations

AUC	area under curve
CCCCP	carbonylcyanide <i>m</i> -chlorophenylhydrazone
CPT1	carnitine palmitoyl transferase 1
ECP	energy charge potential
FATP	fatty acid transport protein
FCCP	carbonylcyanide <i>p</i> -trifluoromethoxyphenylhydrazone
HBSS	Hank's buffered salt solution
OM	oligomycin
RCR	respiratory control ratio
SNK	Student–Newman–Keul
TAN	total adenosine nucleotides
UCP	uncoupling protein

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2.2 “N-methylpyridinium, a degradation product of trigonelline upon coffee roasting, stimulates respiratory activity and promotes glucose utilization in HepG2 cells”

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N-Methylpyridinium, a degradation product of trigonelline upon coffee roasting, stimulates respiratory activity and promotes glucose utilization in HepG2 cells

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N-Methylpyridinium (NMP) is a thermal degradation product of trigonelline formed upon coffee roasting and hypothesized to exert several health benefits in humans. Since for trigonelline evidence for hypoglycemic effects exists, we examined whether NMP also affects mechanisms of glucose utilization and cellular energy formation. For this purpose, the impact of trigonelline and NMP on respiratory activity, extracellular acidification, cellular adenosine nucleotides, energy supply from fatty acids and glucose as well as thermogenesis in HepG2 cells was analyzed. A 24 hour incubation with nanomolar concentrations of NMP enhanced oxygen consumption rates, resulting in increased ATP levels. Glucose was identified as the prevalent energy substrate as its uptake was augmented up to $18.1\% \pm 7.44\%$ by NMP at $0.09 \mu\text{M}$, whereas the uptake of fatty acids decreased upon NMP treatment. Cellular glucose uptake was also stimulated by trigonelline administration; however, a shift to the anaerobic energy production pathway was monitored. Both pyridine derivatives induced thermogenesis, although trigonelline presumably promoted proton leaks, while NMP increased the concentration of the uncoupling protein-2. We provide evidence that both compounds appear to stimulate cellular energy metabolism in HepG2 cells. Human intervention studies are warranted to ensure these effects *in vivo*.

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Introduction

Coffee is a globally important commodity and favoured for its pleasant aroma and its stimulating effects on the central nervous system. The annual production of green coffee is forecast to reach about 9 million tons in the marketing year 2012/2013.¹ However, roasting of green coffee beans is essential in order to generate coffee's pleasurable aroma and taste. Upon coffee roasting, the chemical composition of coffee beans undergoes profound changes depending on the roasting methods and roasting temperatures applied. Thereby, *e.g.*, chlorogenic acids, representing the predominant polyphenols in green coffee, are extensively degraded² and new compounds like melanoidins are formed.³

Besides caffeine, trigonelline is a highly abundant alkaloid in green coffee beans, but also largely decomposed upon thermal treatment into N-methylpyridinium (NMP), nicotinic

acid and methylnicotinate.^{4,5} Lang *et al.* quantified the concentrations of these pyridine derivatives in medium dark coffee brew as well as in plasma and urine samples of 13 healthy volunteers after coffee consumption.⁶ Coffee brew contained about $2310 \mu\text{M}$ trigonelline and $491 \mu\text{M}$ NMP. After consumption of 350 mL freshly prepared coffee brew, peak plasma concentrations of trigonelline varied in males and females: in males, peak plasma concentrations of about $5.5 \mu\text{M}$ were reached after approximately 2 h, whereas females exhibited peak plasma concentrations of $6.5 \mu\text{M}$ 3 h post-administration. Values in the same order of magnitude were analyzed for NMP, reaching plasma peak concentrations of about $0.8 \mu\text{M}$ one hour after coffee consumption. Interestingly, NMP is hardly metabolised and nearly 70% of the ingested dose is excreted about 8 h after coffee consumption.⁶ However, NMP can be detected in the urine up to 72 h post-load.⁷

Although coffee displays the primary dietary source for trigonelline, other foods such as alfalfa sprouts, chick peas, lentils, peas and pumpkin also contain trigonelline.^{8,9} In contrast, the dietary occurrence of NMP is presumably restricted to coffee as it represents a pyrolytic product generated upon roasting by decarboxylation of trigonelline.^{10,11}

Several health benefits of trigonelline and NMP have been proposed previously. Trigonelline is supposed to possess

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hypoglycemic effects.¹² Van Dijk *et al.* monitored a reduction of early plasma glucose response during an oral glucose tolerance test after administration of 500 mg trigonelline to overweight men.¹³ Results from animal feeding experiments support the hypothesis of trigonelline being able to elicit anti-diabetic effects by lowering blood glucose and triglyceride levels, and by improving insulin-sensitivity in diabetic rats.¹⁴ Moreover, reduced triglyceride concentrations in liver and adipose tissue were reported after trigonelline administration to diabetic KK-A(y) obese mice.¹⁵ Although there is some evidence for the potential of trigonelline to improve diabetic symptoms, its impact on cellular energy production pathways is poorly understood.

Research on NMP and NMP-rich dark roast coffee, so far, has been dedicated to its chemopreventive action,^{16,17} antioxidant capacity¹⁸ and gastric-acid secretion inhibiting potential.¹⁹ A human intervention trial comparing the modulating effects of a light roast and a dark roast coffee on the antioxidant defence in humans demonstrated a body weight loss of the obese subjects in this study after a 4-week consumption of an NMP-rich dark roast coffee.²⁰ However, there is no knowledge about the potential of *N*-methylpyridinium to affect mechanisms of cellular energy metabolism, which may promote a reduction in body weight after coffee consumption.

Therefore, we aimed to elucidate the impact of trigonelline and NMP on mitochondrial energy formation, focussing on mechanisms of ATP production. Respiratory chain activity was assessed in the human hepatocarcinoma cell line HepG2 for up to 24 h. We further investigated whether free fatty acids or glucose represents the prevalent energy source. Moreover, we studied the potential of these structurally related compounds to stimulate thermogenesis.

Results

Cellular respiration, extracellular acidification and impedance of HepG2 cells

Metabolic activity was monitored online in HepG2 cells by means of the Bionas Discovery 2500 analyzing system that allows a simultaneous determination of respiration, extracellular acidification and cellular impedance. Fig. 1 displays a typical graph obtained from the online recording Bionas system. After a 4 h adaptation period that allowed the cells to adapt to a repeated medium exchange, the treatment period was started. Although only a regular so-called “running medium” (RM) was added during the control runs, minimal variations in oxygen consumption, extracellular acidification and cellular impedance between biological replicates were observed, though not exceeding a coefficient of variance of 12%, 15% and 5%, respectively, over the time span of 24 h. After application of the test compounds concomitantly with the RM, a two hour regeneration period was performed prior to addition of the uncoupling agent carbonylcyanide *p*-tri-fluoromethoxyphenyl-hydrazone (FCCP) that was used as a positive control for maximum oxygen consumption during uncoupling of respiration. FCCP addition caused a respiratory boost, reaching about $331\% \pm 54.6\%$ ($p < 0.001$) of the initial

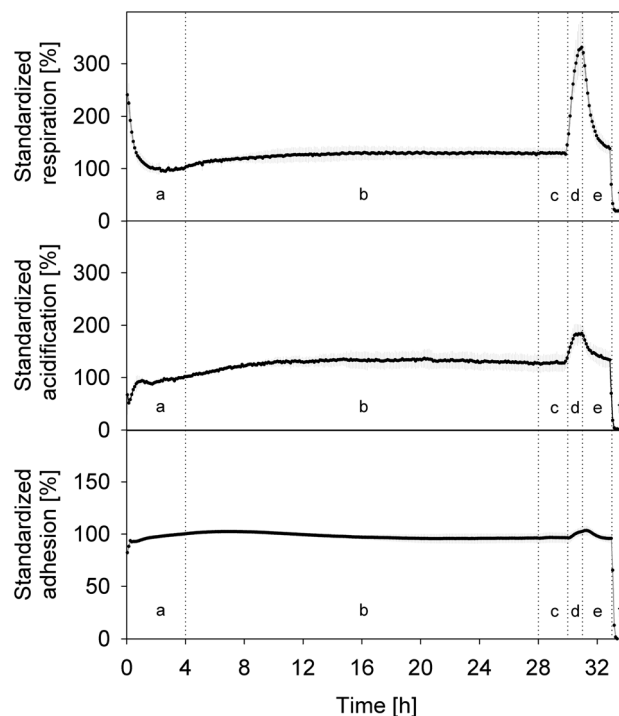


Fig. 1 Respiration, extracellular acidification and adhesion of HepG2 cells within the experimental setup including a 4 h adaptation period (a), 24 h exposure to regular running medium (b), 2 h regeneration (c), 1 h uncoupling by addition of 500 nM FCCP (d), 2 h regeneration (e) and 1 h period with 0.2% Triton X-100 (f) monitored by means of the Bionas Discovery 2500 analyzing system. Graphs result from data points taken every six minutes that are presented as mean $\pm$ SD. Three individual experiments were conducted, each with two running biomodules in parallel including data of three to five sensors for acidification and respiration as well as one sensor for adhesion of each biomodule.

oxygen consumption rate (100%) analysed after the run-in period. After another regeneration period of two hours, respiratory activity reached initial values of about $138\% \pm 12.2\%$, which were comparable to those before FCCP addition ($127\% \pm 9.21\%$, $p > 0.05$). Uncoupling of the mitochondrial respiratory chain by FCCP also increased extracellular acidification up to $184\% \pm 15.2\%$ ($p < 0.001$). Cellular adhesion was only slightly augmented by FCCP addition. The final period in which 0.2% Triton X-100 in RM was added to induce cell death led to a strong decline in respiratory activity, extracellular acidification and adhesion.

Mitochondrial respiratory activity of HepG2 cells after treatment with trigonelline or *N*-methylpyridinium

To evaluate the effects of trigonelline (Fig. 2A) and NMP (Fig. 2B) on oxygen consumption, extracellular acidification and cellular adhesion, HepG2 cells were treated with concentrations of 0.09 μ M and 90 μ M of either compound for 24 h. The resulting graphs were used for calculating area under curve (AUC) values (Fig. 3). Treatment of the cells with trigonelline did neither alter respiration rates nor acidification within 24 h (Fig. 3A and B). Taking into account only the time span from 20 h to 24 h, incubation with trigonelline increased oxygen

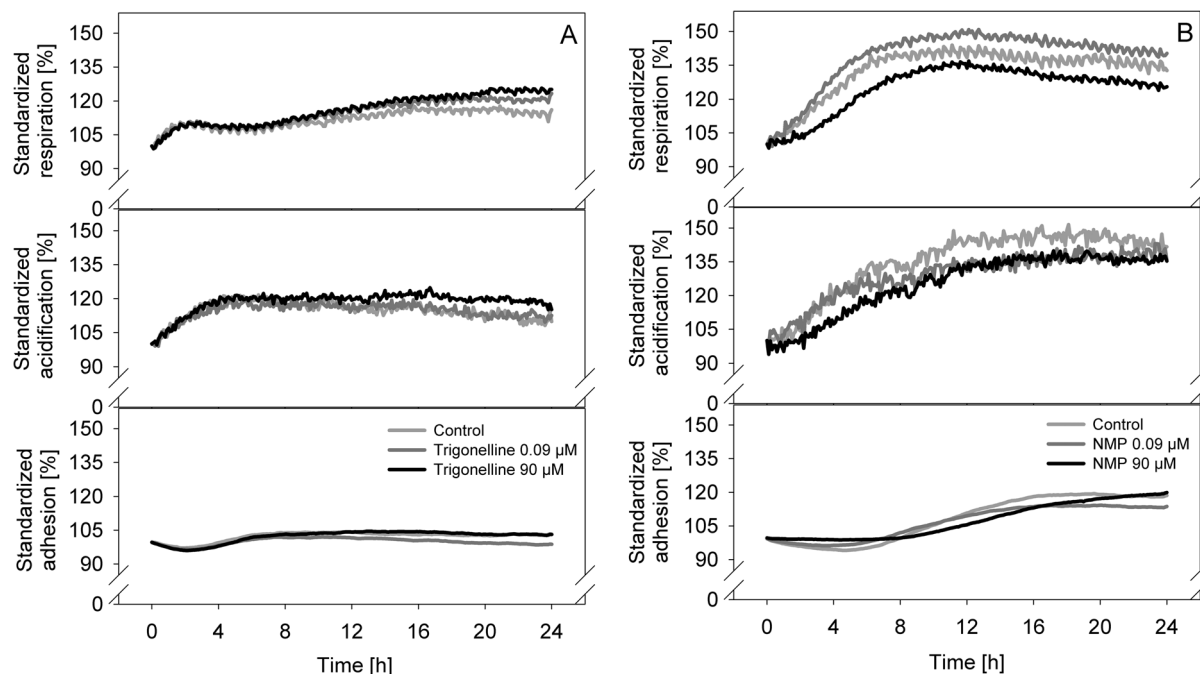


Fig. 2 Effects of trigonelline (A) and NMP (B) on respiration, extracellular acidification and adhesion of HepG2 cells monitored for 24 h with the Bionas Discovery 2500 analyzing system. Graphs are presented as the mean of two parallel running biomodules, including data of three to five sensors for acidification and respiration as well as one sensor for adhesion of each biomodule. Data points were taken every six minutes.

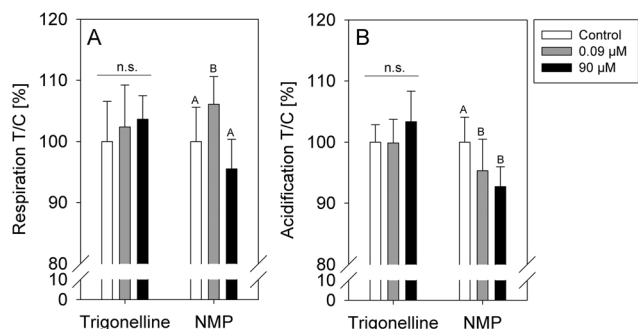


Fig. 3 Mean AUC relative to control (100%) calculated from respiration (A) and acidification (B) curves of HepG2 cells after 24 h treatment with trigonelline and NMP. Results are displayed as mean $\pm$ SD of two parallel running biomodules including data of three to five acidification and respiration sensors, respectively. A one-way ANOVA with SNK *post hoc* test was applied and significant differences are indicated with distinct letters ($p < 0.05$); n.s. not significant.

consumption rates from $100\% \pm 7.10\%$ of untreated controls to $103\% \pm 5.13\%$ ($p > 0.05$) at $0.09 \mu\text{M}$ trigonelline and $108\% \pm 5.22\%$ ($p < 0.05$) at $90 \mu\text{M}$ trigonelline, respectively, pointing to a manifested stimulation of mitochondrial respiration after the pro-longed incubation time. Extracellular acidification also augmented within the last 4 h of treatment from $100\% \pm 3.37\%$ of controls to $101\% \pm 6.01\%$ ($p > 0.05$) and $107\% \pm 6.36$ ($p < 0.05$) after exposure to trigonelline at $0.09 \mu\text{M}$ and at $90 \mu\text{M}$.

In contrast, cellular respiration was stimulated by $0.09 \mu\text{M}$ NMP after the 24 h incubation period, and resulted in a mean increase of $6.11\% \pm 4.50\%$ compared to non-treated control cells ($p < 0.05$), whereas administration of $90 \mu\text{M}$ NMP had no

effect (Fig. 3A). Mean acidification rates were dose-dependently reduced ($p < 0.05$) by $4.67\% \pm 5.16\%$ and $7.28\% \pm 3.25\%$ after treatment with $0.09 \mu\text{M}$ and $90 \mu\text{M}$ NMP, respectively (Fig. 3B).

During the last 4 h of treatment, the stimulatory effect of NMP on the respiratory activity of the cells was maintained at $106\% \pm 4.52\%$ ($p = 0.05$) and $95.5\% \pm 5.70\%$ ($p > 0.05$) in the presence of $0.09 \mu\text{M}$ and $90 \mu\text{M}$, respectively, when compared to untreated controls ($100\% \pm 6.97\%$). In contrast, changes in acidification rates were attenuated within the last 4 h, although not different from controls ($p > 0.05$). Cellular impedance was also neither affected by trigonelline nor by NMP treatment (Fig. 2A and B).

Cellular content of AMP, ADP and ATP

The adenosine nucleotides AMP, ADP and ATP were quantified in cellular extracts after 24 h incubation with either trigonelline or NMP (Table 1). Moreover, the energy charge potential (ECP) was calculated from the concentrations of ADP and ATP in relation to total adenosine nucleotides (TAN) in order to estimate the cellular energy status after compound treatment (Fig. 4).

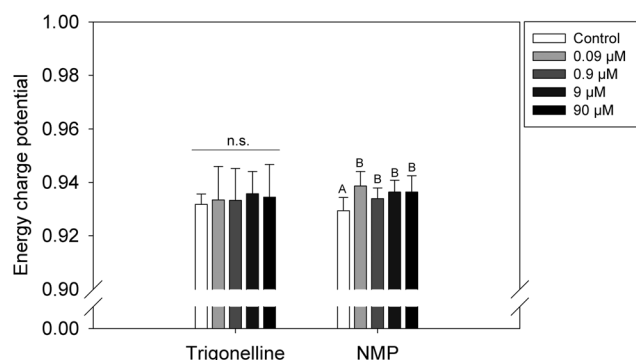
Incubation with trigonelline (0.09 , 0.9 and $90 \mu\text{M}$) reduced cellular AMP contents ($p < 0.05$). ADP concentrations were decreased by trigonelline at $90 \mu\text{M}$, while ATP concentrations were not affected by trigonelline treatment at any concentration tested. Consequently, the ECP remained unchanged after trigonelline exposure.

In contrast, AMP levels augmented after incubation with $0.9 \mu\text{M}$ NMP ($p < 0.05$) and ADP levels did so after incubation with 0.9 and $90 \mu\text{M}$ NMP ($p < 0.05$). Independent of the NMP concentration applied, an increase of cellular ATP ranging

Table 1 Adenosine nucleotides (AMP, ADP, ATP) normalized to untreated controls (100%)

		AMP	ADP	ATP
Trigonelline	Control	100 ± 0.51 ^a	100 ± 0.33 ^a	100 ± 0.15 ^a
	0.09 μM	82.5 ± 12.1 ^{b,c}	95.0 ± 12.8 ^{a,b}	101 ± 13.5 ^a
	0.9 μM	71.1 ± 9.28 ^c	92.2 ± 9.61 ^{a,b}	97.2 ± 16.3 ^a
	9 μM	90.0 ± 19.2 ^{a,b}	105 ± 12.0 ^a	111 ± 15.4 ^a
	90 μM	77.2 ± 3.51 ^{b,c}	82.6 ± 17.6 ^b	94.3 ± 11.4 ^a
NMP	Control	100 ± 1.26 ^a	100 ± 0.81 ^a	100 ± 1.63 ^a
	0.09 μM	97.0 ± 8.17 ^a	105 ± 2.68 ^{a,b}	118 ± 9.10 ^b
	0.9 μM	113 ± 4.69 ^b	115 ± 8.88 ^b	123 ± 8.18 ^b
	9 μM	104 ± 18.2 ^{a,b}	109 ± 15.9 ^{a,b}	120 ± 21.4 ^b
	90 μM	102 ± 7.52 ^{a,b}	116 ± 8.34 ^b	123 ± 17.2 ^b

Results are displayed as mean ± SD, $n \geq 3$, significant differences are indicated with letters a–c ($p < 0.05$).

**Fig. 4** Energy charge potential (ECP) calculated from adenosine nucleotides after 24 h treatment with trigonelline and NMP, respectively. Results are presented as mean ± SD ($n = 4$). For statistical analysis, a one-way ANOVA with SNK *post hoc* test was performed and significant differences ($p < 0.05$) are indicated with distinct letters; n.s. not significant.

between 118% and 123% in comparison to untreated controls (100%) ($p < 0.05$) was monitored. As a result, ECP values were elevated after NMP treatment (0.09–90 μM).

Fatty acid uptake of HepG2 cells

Short-term effects of trigonelline and NMP on fatty acid uptake were determined in HepG2 cells as a marker of cellular substrate utilization for mitochondrial energy production. The cells were pre-treated with either trigonelline or NMP for 30 min prior to determination of cellular fatty acid uptake. No changes in fatty acid uptake were observed after exposure to varying concentrations of trigonelline (Fig. 5A). In contrast, cellular fatty acid uptake was reduced after incubation with NMP (Fig. 5A). The strongest decline was evoked by NMP at a concentration of 0.9 μM, resulting in an 18.0% ± 8.11% decrease in cellular fatty acid uptake ($p < 0.05$).

Glucose uptake of HepG2 cells

The impact of trigonelline and NMP on the uptake of 2-NBDG, a fluorescent glucose analog, was assessed as an indicator of energy substrate utilization from glucose.

Although, HepG2 cells predominately express glucose transporter type-1 and type-2,²¹ both not insulin-responsive, the 30 min pre-treatment with insulin slightly augmented 2-NBDG uptake from 100% ± 3.92% of untreated controls to 112% ± 6.31% ($p < 0.001$, data not shown). Addition of trigonelline in a concentration of 90 μM increased glucose uptake by 13.3% ± 8.88% compared to non-treated controls ($p < 0.05$) (Fig. 5B). Enhanced glucose uptake was also monitored after incubation with NMP (Fig. 5B). The strongest effect was demonstrated after addition of the lowest NMP concentration (0.09 μM), resulting in a mean increase of 2-NBDG uptake by 18.1% ± 7.44% ($p < 0.001$), while the highest tested concentration of 90 μM led only to a rise of 8.50% ± 5.80% ($p < 0.05$) compared to untreated control cells.

Mitochondrial membrane potential ($\Delta\Psi m$)

Depolarization of the mitochondrial membrane is associated with an uncoupling of the respiratory chain from ATP production and results in energy dissipation.²² Fig. 6 illustrates the amount of depolarized cells directly after addition and after 24 h incubation with trigonelline or NMP.

Direct addition of trigonelline did not alter the mitochondrial membrane potential (Fig. 6A). In contrast, after the 24 h trigonelline treatment (0.09 μM, 0.9 μM and 90 μM), an increase in depolarized cells was measured, reaching a maximum of 19.9% ± 12.7% at a concentration of 90 μM (Fig. 6B).

NMP treatment neither affected the mitochondrial membrane potential directly nor after 24 h of incubation, revealing a comparable amount of depolarized cells as in the untreated control cell population (Fig. 6A and B).

Protein expression of uncoupling protein-2 (UCP-2)

UCP-2 concentrations of HepG2 cells remained unchanged after 24 h exposure to trigonelline (Fig. 7). In contrast, 24 h treatment with NMP at 0.09 μM and 0.9 μM increased the UCP-2 protein expression by 34.4% ± 25.7% ($p < 0.05$) and 35.3% ± 27.8% ($p < 0.05$), respectively (Fig. 7). Addition of higher NMP concentrations had no effect on UCP-2 protein expression.

Discussion

The study presented here examined the effect of the structurally related coffee components trigonelline and NMP on mechanisms of energy metabolism in HepG2 cells.

Since trigonelline is thermally degraded upon coffee roasting, trigonelline contents of coffee beans strongly depend on the roasting times and the roasting temperatures applied.²³ While lightly roasted (260 °C, 120 s) Arabica coffee is reported to contain about 7.5 g trigonelline per kg coffee dry matter, dark roasts (260 °C, 300 s) only contain about 1.6 g kg⁻¹ dry matter.⁴ In contrast, the amount of NMP increases with the intensity of the roasting process.²⁴ Since trigonelline has been shown to affect glucose homeostasis in human volunteers¹³ and in diabetic rats,¹⁴ this study addressed the question whether trigonelline, more abundant in light roast coffee, and NMP, highly prevalent in dark roast coffee, exhibit effects on cellular energy

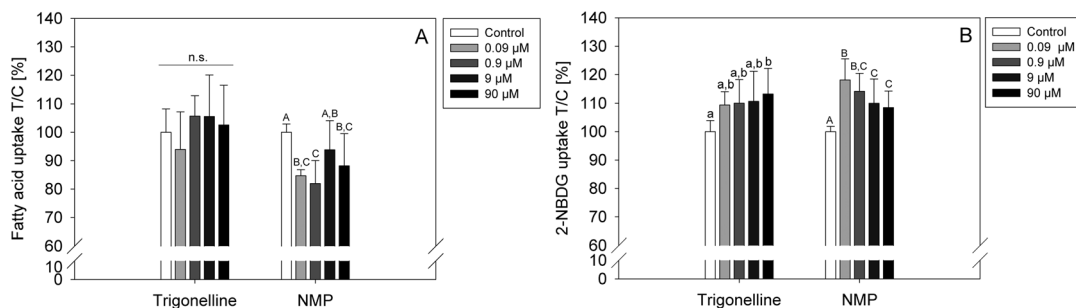


Fig. 5 Fatty acid uptake (A) as well as 2-NBDG uptake (B) of HepG2 cells after 30 min pre-incubation with trigonelline and NMP was assessed. Results were normalized to untreated controls (100%) and are represented as mean $\pm$ SD ($n = 3$). Dose-dependent effects were tested by a one-way ANOVA with SNK *post hoc* test for pair-wise comparison ($p < 0.05$). Significant differences are indicated with distinct letters; n.s. not significant.

metabolism. The results revealed that exposure of HepG2 cells to trigonelline or NMP increased oxygen consumption rates. Cellular oxygen consumption largely represents mitochondrial electron transport chain performance at complex IV and, therefore, reflects mitochondrial activity. Tight coupling of mitochondria is characterized by ATP production that is mechanistically coupled with mitochondrial respiratory activity: an electrochemical gradient that is established by the activity of the electron transport chain complexes is used by the enzyme ATP synthase for phosphorylation of ADP to ATP.

Although administration of trigonelline (90 μ M) to HepG2 cells modified respiration rates, the stimulating effect did only reach the level of significance during the final 4 h of the treatment period. During the same time span, extracellular acidification was augmented. This effect might have been a consequence of an enhanced anaerobic glycolysis. We, therefore, investigated whether glucose or fatty acids are utilized as energy substrates. 2-NBDG uptake was increased after administration of 90 μ M trigonelline, whereas fatty acid uptake remained unchanged at all concentrations tested.

In addition, the ECP values, representing a cell's energy status, based on the ratio of ATP and ADP to TAN, were not affected by any of the treatments with trigonelline. This might

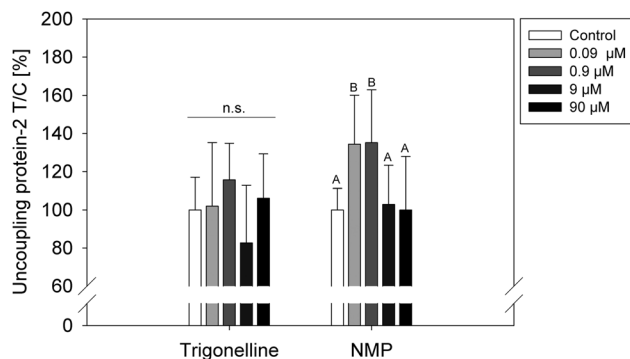


Fig. 7 UCP-2 protein expression was determined in HepG2 cells after 24 h incubation with different concentrations of trigonelline and NMP. UCP-2 concentrations were normalized to untreated controls (100%). Results are displayed as mean $\pm$ SD ($n = 4$). A one-way ANOVA with SNK *post hoc* test was applied to determine concentration-dependent effects ($p < 0.05$) that are indicated with distinct letters; n.s. not significant.

have been a consequence of the observed shift to anaerobic energy formation, resulting in fewer moles ATP per mol glucose than generated from aerobic energy production, or the

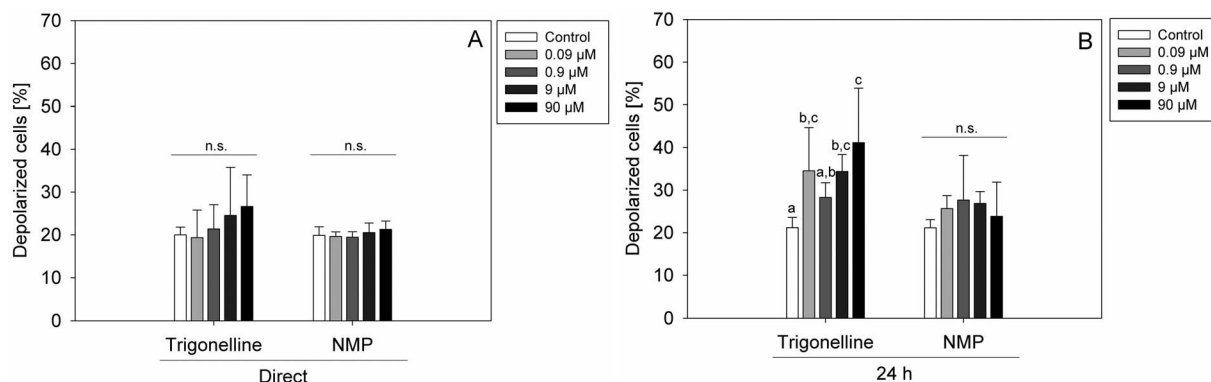


Fig. 6 Mitochondrial membrane depolarization of HepG2 cells after direct addition (A) and 24 h incubation (B) with trigonelline and NMP determined by flow cytometry after staining with the membrane potential sensitive dye JC-1. Data are shown as mean $\pm$ SD of at least three biological replicates. Dose-dependency of trigonelline and NMP was assessed by a one-way ANOVA with SNK *post hoc* test and considered to be significant at $p < 0.05$. Concentration-dependent effects ($p < 0.05$) are indicated with distinct letters; n.s. not significant.

induction of thermogenesis. Therefore, we hypothesized that trigonelline favors the utilization of glucose as an energetic substrate for ATP production *via* a shift to the anaerobic pathway of lactate production, also because extracellular acidification was increased after trigonelline treatment.

Hypoglycemic effects of trigonelline have already been proposed in the literature. In humans, administration of 500 mg trigonelline reduced early plasma glucose and insulin response during an oral glucose tolerance test in overweight men.¹³ This effect has been hypothesized not to be mediated by incretin hormones.²⁵ Protective effects against the development of streptozotocin-induced diabetes in male Wistar rats were observed after 48-week treatment with 40 mg trigonelline per kg body weight,²⁶ a dose corresponding to the daily intake of 2.8 g trigonelline for a 70 kg human. Improvement of glucose tolerance and insulin resistance was also found by Yoshinari *et al.* who demonstrated an increased activity of glucokinase in the liver of trigonelline-fed rats in comparison to controls.⁹ The enzyme glucokinase catalyzes the phosphorylation of glucose to glucose-6-phosphate, which is the initial step of glycolysis, and thereby promotes glucose oxidation for energy production. Regarding these findings, our *in vitro* study further supports the beneficial effects of a high-dose trigonelline administration on glucose utilization. However, it is hardly possible to obtain plasma concentrations of 90 μM trigonelline by regular coffee intake as peak plasma concentrations after consumption of 350 mL medium dark roast coffee brew were shown to reach only about 5.5 to 6.5 μM .⁶

So far, there is no knowledge about the potential of NMP to modify parameters of cellular energy metabolism. In this study, incubation of HepG2 cells with NMP stimulated oxygen consumption over a time period of 24 h. Assessment of adenosine nucleotides confirmed the stimulating effect of NMP on ATP formation, leading to an augmented energy charge potential. We, therefore, conclude that NMP stimulates aerobic energy/ATP formation *via* oxidative phosphorylation. The NMP-induced reduction of extracellular acidification, an outcome measure of lactate release, further supports this hypothesis.

In order to also identify whether fatty acids or glucose are utilized as energy substrates, we further examined the impact of NMP on the cellular uptake of the latter. *Via* β -oxidation and glycolysis, fatty acids and glucose may serve for the formation of NADH/H^+ and/or FADH_2 which can be used by the electron transport chain to establish the electrochemical gradient needed for ATP-synthase activity. Exposure of HepG2 cells to nanomolar NMP concentrations significantly decreased fatty acid uptake. In contrast, 2-NBDG uptake was increased by NMP, clearly pointing to glucose as the prevalent energy substrate used.

It is noteworthy that the respiratory chain activity and glucose uptake in human liver cells were more effectively stimulated when NMP was applied in lower concentrations. Changes in the metabolic activity were evoked by NMP at 0.09 μM , a concentration approximately 9-fold lower than peak plasma concentrations after acute ingestion of 350 mL freshly brewed medium dark coffee,⁶ although corresponding to

steady-state plasma concentrations of regular coffee drinkers that range between 47 and 163 nM (unpublished data).

Besides evaluating the ability of trigonelline and NMP to modify parameters of cellular energy production, we studied their impact on parameters of thermogenesis. Induction of thermogenesis is the result of a less well-uncoupled electron transport chain and is accompanied by a reduced proton gradient between the mitochondrial matrix and the mitochondrial inner membrane. Proton leaks across the inner mitochondrial membrane or expression of uncoupling proteins might contribute to these effects which result in mitochondrial thermogenesis.²⁷ In the HepG2 cells studied, trigonelline and NMP might have evoked mitochondrial thermogenesis. However, they appear to act on different mechanisms. After 24 h of cell treatment with trigonelline, the mitochondrial membrane potential was decreased, possibly by induction of proton leaks as the UCP-2 protein content was not affected. In contrast, administration of NMP in plasma representative concentrations increased the UCP-2 protein expression. However, we provide evidence that both compounds exhibit thermogenic effects and might increase energy expenditure.

In conclusion, this study demonstrated that the coffee constituents trigonelline and NMP exert distinct effects on cellular energy metabolism. While NMP was effective in concentrations reachable in humans by moderate coffee consumption, higher concentrations of trigonelline were mostly necessary. Both pyridine derivatives might improve plasma glucose clearance by promoting cellular glucose uptake and thereby might also exert effects on glucose metabolism and energy provision. Moreover, both compounds affected mechanisms of thermogenesis and might contribute to a coffee-induced increase in energy expenditure that is assumed to be partly responsible for the body-weight modulating effect of coffee.²⁰ Human intervention studies have to be conducted to ensure these effects.

Experimental

Materials and chemicals

All chemicals, reagents and materials were obtained from Sigma-Aldrich (Austria), unless stated otherwise. Trigonelline hydrochlorid (98% purified) was purchased from Carl Roth GmbH (Germany). *N*-Methylpyridinium iodide was synthesized from methyl iodide and pyridine, and purified by crystallization as described previously.^{5,16}

Cell culture

HepG2 (human hepatocellular carcinoma) cells were cultured at 37 °C and 5% CO_2 in a humidified incubator. RPMI-1640 medium supplemented with 10% FBS, 4 mM L-glutamine and 1% penicillin-streptomycin was used as culture medium. Cells were incubated with the test compounds trigonelline and *N*-methylpyridinium at concentrations ranging from 0.09–90 μM dissolved in the incubation medium (culture medium without FBS supplementation) unless stated otherwise.

Cell viability

To exclude cytotoxic effects of treatments, cell viability was determined by the WST-1 assay (Roche Diagnostics). Analyses were carried out according to the manufacturer's protocol. Neither trigonelline nor NMP exhibited toxic effects on HepG2 cells at concentrations up to 90 μM (data not shown).

Cellular respiration, extracellular acidification and cell impedance measurement

Online-monitoring of cellular respiration, extracellular acidification and cell impedance of HepG2 cells in response to trigonelline and NMP treatment was conducted by means of a Bionas Discovery 2500 analyzing system (Bionas GmbH, Germany).²⁸ The system consisted of six biomodules located in an incubation hood, each capable of inserting one sensor chip. The biomodules were connected to an autosampler *via* a pumping system for provision of running medium (RM).

Each sensor chip provides a pair of interdigitated electrode structures for impedance measurement, five ion-sensitive field-effect transistors for extracellular pH measurement as well as five Clark-type electrodes for oxygen consumption measurement. After 10 minutes of disinfection with 70% ethanol and rinsing with sterile water and culture medium, the cells were seeded in culture medium directly on the sensor chips and led to adhere under standard cell culture conditions at 37 °C and 5% CO₂ in a humidified incubator. Then, cell-coated chips were inserted into the biomodules. The pumping system allowed continuous supply of running medium with or without test compounds at a flow of 56 $\mu\text{L min}^{-1}$ and was running in a three minute rhythm, allowing three minutes of medium exchange followed by three minutes of sensory measurement, resulting in one data point every six minutes. Data collection was conducted using the Bionas Discovery Data Analyzer software. Raw data of each sensor were normalized to the initial value reached after the run-in period (100%). For comparison of treatment effects, the area under curve (AUC) was calculated for each sensor and normalized to the mean AUC of untreated controls (100%).

Trigonelline and NMP were administered to HepG2 cells at concentrations of 0.09 μM and 90 μM . Biomodules were analyzed in duplicate. Sensor chips were reused three to four times and cleaned with trypsin-EDTA solution, PBS, 70% ethanol and ultrapure water after each measurement.

HepG2 cells were seeded at a density of 150,000 cells per chip in 250 μL culture medium one day prior to the experiment. For analysis, sensor chips were inserted into the biomodules tempered at 37 °C. The experimental setup was as follows: a 4 h run-in period for adaptation of cells to the RM (RPMI-1640 without bicarbonate and without FBS, supplemented with 2 mM L-glutamine, 1% penicillin-streptomycin and 1 mM HEPES) and the measurement conditions; 24 h of continuous exposure to trigonelline or NMP in RM or RM only; 2 h of regeneration with RM only; 1 h of exposure to the uncoupling agent carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) at 500 nM diluted in RM to stimulate respiratory activity; 2 h of regeneration with RM only and 1 h final period with RM

containing 0.2% Triton X-100 (Carl Roth GmbH, Germany) to induce cell detachment from the sensor surface of the chips.

Cellular content of AMP, ADP and ATP

The adenosine nucleotides AMP, ADP and ATP were quantified in HepG2 cell extracts after 24 h treatment with trigonelline or NMP by HPLC-DAD according to the method described by Riedel *et al.*²⁹ Individual adenosine nucleotides were normalized to untreated controls (100%). Furthermore, adenosine nucleotide concentrations of the cellular extracts were summarized to total adenosine nucleotides (TAN) by $\text{TAN } [\mu\text{mol L}^{-1} \text{ extract}] = \text{AMP } [\mu\text{mol L}^{-1} \text{ extract}] + \text{ADP } [\mu\text{mol L}^{-1} \text{ extract}] + \text{ATP } [\mu\text{mol L}^{-1} \text{ extract}]$ and the energy charge potential (ECP) was calculated as the marker for the cellular energy status by $\text{ECP} = (\text{ATP } [\mu\text{mol L}^{-1} \text{ extract}] + 0.5 \text{ ADP } [\mu\text{mol L}^{-1} \text{ extract}]) / \text{TAN } [\mu\text{mol L}^{-1} \text{ extract}]$.³⁰

Fatty acid uptake

Fatty acid uptake was measured in HepG2 cells after 30 minutes of exposure to trigonelline and NMP by means of a QBT™ Fatty Acid Uptake Assay Kit (Molecular Devices Corporation, Germany) according to the protocol specified previously.²⁹

Glucose uptake

Glucose uptake was determined in HepG2 cells applying the 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) fluorescence glucose analog (life technologies, Austria). Cells were starved for one hour with DMEM (without glucose, L-glutamine and FBS). After a 30 min pre-incubation with trigonelline and NMP, the 2-NBDG fluorescence glucose analog was added to a final concentration of 200 μM and then incubated for 30 min. Insulin (100 nM) was used as a positive control. Cells were washed three times with ice-cold PBS to eliminate excess dye and fluorescence signals were recorded at an emission wavelength of 535 nm after excitation at 485 nm with an Infinite M 200 multiwell plate reader (Tecan, Austria). Glucose uptake is expressed as the percentage of untreated controls (100%).

Mitochondrial membrane potential ($\Delta\Psi_m$)

A flow cytometry based method using a JC-1 probe (BioVision, Inc., US) was applied to determine the mitochondrial membrane potential in HepG2 cells directly after addition and 24 h pre-incubation with trigonelline or NMP. Upon polarization of the mitochondrial membrane, the JC-1 dye forms aggregates in the mitochondrial matrix that emit yellow fluorescence, whereas upon depolarization, JC-1 monomers remain in the cytosol and show green fluorescence. The uncoupling agent carbonylcyanide *m*-chlorophenylhydrazone (50 μM) was used as a positive control to calibrate the method consistently inducing a depolarization of over 90%. Measurements were carried out according to the protocol described previously.²⁹

Uncoupling protein-2 (UCP-2) protein expression

The UCP-2 protein content of HepG2 cells after a 24 h incubation with trigonelline or NMP was analyzed by means of a commercial ELISA kit (USCN Life Sciences, China) and

normalized for the total protein content by the bicinchoninic acid assay (Pierce® BCA Protein Assay Kit, Thermo Scientific, Austria) as published previously.²⁹ To allow comparison of treatment effects, UCP-2 contents are shown as percentage of untreated control.

Statistical analysis

Results are displayed as mean $\pm$ SD. Multiple biological replicates (n) were conducted as stated in the figure legends. A Nalimov outlier test was performed prior to verification of statistical significances. Dose-dependent effects of test compounds were ascertained by one-way analysis of variance (ANOVA) with Student–Newman–Keuls (SNK) *post hoc* test. Differences between various concentrations on treatment are indicated with distinct letters ($p < 0.05$).

Conclusions

This *in vitro* study provides evidence for the coffee pyridine derivatives trigonelline and *N*-methylpyridinium affecting mechanisms of ATP synthesis and thermogenesis in HepG2 cells. We demonstrate that both compounds promote glucose metabolism and the utilization of glucose for ATP synthesis. However, mechanisms of anaerobic ATP synthesis *via* the lactate pathway were stimulated by trigonelline, while aerobic pathways *via* mitochondrial oxidative phosphorylation were intensified by NMP.

Furthermore, also energy expenditure was stimulated by trigonelline and NMP. Both compounds increased thermogenesis, even though parameters of mitochondrial heat production were differently affected.

Therefore, we conclude that trigonelline and its roasting-derived degradation product NMP exhibit stimulating effects on cellular energy metabolism by inducing the utilization of glucose as an energy substrate for ATP production, possibly to compensate the thermogenesis-related energy loss. Nevertheless, it has to be considered that underlying mechanisms seem to vary whether liver cells are exposed to trigonelline or NMP, though further studies are needed as it remains unclear which cellular signalling cascades and/or receptors are activated by the individual compounds.

Conflict of interest

The authors G. Bytof and I. Lantz are employees of Tchibo GmbH, Germany.

Abbreviations

2-NBDG	2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose
AUC	Area under curve
dm	Dry matter
ECP	Energy charge potential
FCCP	Carbonylcyanide <i>p</i> -trifluoromethoxyphenylhydrazone
NMP	<i>N</i> -Methylpyridinium
RM	Running medium

SNK	Student–Newman–Keul
TAN	Total adenosine nucleotides
UCP	Uncoupling protein

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2.3 “Structure-dependent effects of pyridine derivatives on intestinal fatty acid uptake: Regulation of nicotinic acid receptor and fatty acid transporter expression”

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Structure-dependent effects of pyridine derivatives on mechanisms of intestinal fatty acid uptake: Regulation of nicotinic acid receptor and fatty acid transporter expression

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Keywords:

pyridines, fatty acid uptake, Caco-2, nicotinic acid, nicotinic acid receptor, N-methyl-4-phenylpyridinium

Abbreviations:

2-NBDG, 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose; AUC, area under curve; FATP, fatty acid transport protein; HPRT-1, hypoxanthine guanine phosphoribosyl transferase; IFABP, intestinal fatty acid binding protein; HBSS, Hank's balanced salt solution; MPP, N-methyl-4-phenylpyridinium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NA, nicotinic acid; PPAR, peroxisome proliferator-activated receptor; TEER, trans-epithelial electrical resistance; UAR, unit area resistance

Abstract

Pyridines are widely distributed in foods. Nicotinic acid (NA), a carboxylated pyridine derivative, inhibits lipolysis in adipocytes by activation of the orphan nicotinic acid receptor (HM74A), and is applied to treat hyperlipidaemia. However, knowledge on the impact of pyridine derivatives on intestinal lipid metabolism is scarce. This study was performed to identify the structural determinants of pyridines for their effects on fatty acid uptake in enterocyte-like Caco-2 cells, and to elucidate the mechanisms of action. The impact of 17 pyridine derivatives on fatty acid uptake was tested. Multiple regression analysis revealed the presence of a methyl-group to be the structural determinant at 0.1 mM, whereas at 1 mM, the presence of a carboxylic group and the N-methylation presented further structural characteristics to affect the fatty acid uptake. NA, showing a stimulating effect on FA uptake, and N-methyl-4-phenylpyridinium (MPP), inhibiting FA uptake, were selected for mechanistic studies. Gene expression of the fatty acid transporters CD36, FATP2 and FATP4, and the lipid metabolism regulating transcription factors PPAR α and PPAR γ was upregulated upon NA treatment. Caco-2 cells were demonstrated to express the low affinity nicotinic acid receptor HM74 of which the gene expression was upregulated upon NA treatment. We hypothesize that the NA-induced fatty acid uptake might result from nicotinic acid receptor activation and related intracellular signaling cascades. In contrast, MPP increased trans-epithelial electrical resistance. We therefore conclude that NA and MPP, both sharing the pyridine motif core, exhibit their contrary effects on intestinal FA uptake by activation of different mechanisms.

Introduction

Pyridines are widely distributed in plants and foods. Coffee beverages, in particular, are rich in pyridine derivatives such as trigonelline, N-methylpyridinium, nicotinamide and nicotinic acid (NA) [1, 2]. N-methylpyridinium has recently been shown to possess bioactive properties [3-6]. Nicotinic acid, also known as niacin or vitamin B3, is an essential, pellagra-preventing nutrient whose physiological effects have been extensively described [7]. In gram amounts, nicotinic acid is used to treat hyperlipidaemia because it lowers plasma cholesterol [8] and reduces plasma free fatty acids by inhibiting lipolysis in adipocytes [9]. However, side effects of high dose intake of nicotinic acid, such as intense flushing due to increased prostaglandin D2 release, accompany its beneficial effects as plasma lipid lowering drug [10].

The mode of action of nicotinic acid was not elucidated until the deorphanization of the nicotinic acid receptor, a G-protein coupled receptor that appears in the two subtypes HM74A (GRP109A) and HM74 (GPR109B). HM74A is the high affinity receptor, and HM74, homologous with HM74A, but with 24 additional C-terminus amino acids, is the low affinity receptor [11-13]. These receptors have also been classified as hydroxy-carboxylic acid receptors since ligand receptor studies have shown that the carboxylic acid group determines the affinity of potential ligands [14]. β -hydroxybutyrate has been identified as the endogenous ligand for these receptors [15]. In contrast, nicotinamide, possessing a carboxamide group instead of a carboxylic group, was inactive in G-protein activation and showed an $EC_{50} > 1000 \mu M$, whereas nicotinic acid showed an EC_{50} of $1.42 \mu M$ in rat adipocytes [16].

Although evidence for the lipolysis inhibiting effect of nicotinic acid exists, the role of pyridine derivatives in fatty acid absorption at the intestinal level is poorly understood. Therefore, we aimed to elucidate the structural features of pyridine derivatives for their effects on intestinal fatty acid uptake and intended to verify the potential role of the nicotinic acid receptors as mediators for pyridine-induced changes *in vitro*. We selected 17 test compounds, either by synthesis or from commercial sources, sharing the pyridine core motif, and tested their potential to alter fatty acid uptake in differentiated Caco-2 cells. Upon differentiation, the colon carcinoma cell line Caco-2 undergoes profound morphological changes resulting in enterocyte-like cells. As Caco-2 cells are characterized by polarization and the development of a brush border membrane as well

as intercellular tight junctions, they present a suitable cellular model for intestinal uptake studies [17].

The absorption of long-chain fatty acids is facilitated by passive diffusion or active transport mediated by fatty acid transporters such as fatty acid translocase CD36 or fatty acid transport proteins (FATPs) [18]. Therefore, we assessed gene expression of CD36, FATP2, FATP4, intestinal fatty acid binding protein (IFABP), PPAR α and PPAR γ , as genes representative of those regulating fatty acid metabolism. We further analyzed nicotinic acid receptor gene expression. Trans-epithelial electrical resistance (TEER) was assessed to estimate cellular integrity.

Material and methods

Chemicals

Pyridine (1), nicotinic acid (2), 4-carboxypyridine (iso-nicotinic acid, 3), picolinic acid (4), trigonelline hydrochloride (9), nicotinamide (11), N-methylnicotinamide iodide (12), nicotinamide-N-oxide (16) and N-methyl-4-phenylpyridinium (17) were purchased from Sigma-Aldrich (Steinheim, Germany). N-methylpyridinium iodide (5), N-methyl-2-picolinium iodide (6), N-methyl-3-picolinium iodide (7), N-methyl-4-picolinium iodide (8), N-methyl-2-pyridone-5-carboxylic acid (10), N-methyl-2-pyridone-5-carboxamide (13), N-methyl-2-pyridone-3-carboxamide (14) and N-methyl-4-pyridone-5-carboxamide (15) were synthesized and purified by recrystallization or preparative HPLC, respectively, as reported [2, 19]. All other materials, chemicals and reagent were obtained from Sigma-Aldrich (Austria) unless stated otherwise.

Cell culture

The human colon cancer cell line Caco-2 cells was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1% penicillin/streptomycin at 37°C and 5% CO₂ in a humidified incubator. To avoid undesired initiation of differentiation, cells were passaged at a maximum of 90% confluency. For experiments, cells were seeded into 96-well plates, 6-well plates or

transwell plates reaching confluency after 2 days. Cell differentiation was obtained by subsequent culture for further 18 days including medium changes every second to third day. Then, enterocyte-like differentiated Caco-2 cells were used for further studies.

Fatty acid uptake

The fatty acid uptake was assessed in differentiated Caco-2 cells by the QBT™ Fatty Acid Uptake Assay Kit (Molecular Devices Corporation, Germany) that applies a BODIPY®-dodecanoic acid fluorescent fatty acid analog. Differentiated Caco-2 cells were starved with serum-free medium for one hour under standard cell culture conditions prior addition of pyridine derivatives (1 – 17) at 0.1 and 1 mM diluted in Hank's balanced salt solution (HBSS)/HEPES buffer. Controls were treated with HBSS/Hepes only. After a 30 min pre-incubation at 37°C, the fluorescent fatty acid analog diluted in HBSS/Hepes (supplemented with 0.2% essential fatty acid free bovine serum albumin) was added. Fluorescence signals were recorded at an emission wavelength of 515 nm after excitation at 485 nm with a microplate reader (infinite M200, Tecan, Austria) for 60 minutes. A time-signal intensity curve was plotted and thereof the area under curve (AUC) was calculated to determine treatment-dependent cellular fatty acid uptake. Results are described as percentage of controls.

Cell viability

Cytotoxic effects of test compounds were excluded by performance of the MTT assay that is based on the ability of viable cells to reduce the yellow tetrazole MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent to a purple formazan. Differentiated Caco-2 cells were starved with serum-free medium one hour prior addition of nicotinic acid (NA) and N-methyl-4-phenylpyridinium (MPP) diluted in HBSS/Hepes in a concentration range of 0.001 – 1 mM. Test substances were removed after 90 min exposure to cells. The MTT stock solution (5 mg/ mL) was diluted (1:6) in serum-free DMEM and led to incubate for about 13 min. After removal of the MTT solution, the resulting formazan was diluted in DMSO and absorbance was measured at 550 nm with a multiwell plate reader (Tecan infinite M200, Tecan, Austria). Results were further corrected for unspecific interferences recorded at a reference wavelength of 690 nm and viability was determined relative to untreated control cells (100%).

Glucose uptake

Glucose uptake was assessed using the 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) fluorescence glucose analog (life technologies, Austria). Differentiated Caco-2 cells were starved with glucose-free DMEM (without L-glutamine and FBS) for one hour at 37°C and 5% CO₂ in a humidified incubator. Then, cells were pre-incubated with nicotinic acid and N-methyl-4-phenylpyridinium, respectively, diluted in HBSS/Hepes buffer in concentrations ranging from 0.001 – 1 mM for 30 min prior addition of 2-NBDG (diluted in HBSS/Hepes) to a final concentration of 200 µM. After another 30 min incubation in which cellular absorption of the fluorescent glucose analog occurs, cells were washed three times with ice-cold PBS to remove excess dye and fluorescence emission was measured at 550 nm after excitation at 485 nm with a multiwell plate reader (Tecan infinite M200, Tecan, Austria). Results are expressed relative to untreated controls (100%).

RT-qPCR

Gene expression patterns of differentiated Caco-2 cells after treatment with nicotinic acid and N-methyl-4-phenylpyridinium were analyzed by quantitative real-time reverse transcription PCR. Differentiated Caco-2 cells were exposed to 1 mM NA and 1 mM MPP for 30 min, 60 min and 90 min. After a brief washing step with ice-cold PBS, cells were lysed and total RNA was extracted applying the peqGOLD Total RNA Kit (peqlab, Germany). RNA content and purity were analyzed with the NanoQuant Plate specified for the infinite M200 plate reader (Tecan, Austria) and a total of 2 µg RNA was used for cDNA transcription applying the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Austria). Real-time qPCR was conducted with 100 ng cDNA that were amplified with the Fast SYBR® Green Master Mix (Applied Biosystems, Austria). Analyses were performed in triplicates by means of a StepOnePlus Realtime PCR System (Applied Biosystems, Austria). Specific primers were designed using NCBI/Primer-BLAST or extracted from PrimerBank and purchased from Sigma Aldrich (Austria). Primer sequences for the target genes fatty acid transport protein-2 (FATP2), fatty acid transport protein-4 (FATP4), intestinal fatty acid binding protein (IFABP), fatty acid translocase (CD36), peroxisome proliferator-activated receptor (PPAR α and PPAR γ) and nicotinic acid receptor (HM74 and HM74A) are given in Table 1 and applied at a concentration of 100 nM each. Results were analyzed using the LinRegPCR software. Hypoxanthine guanine phosphoribosyl transferase (HPRT-1) was used as

reference gene and gene expression of target genes was calculated as a ratio to HPRT-1 expression [20]. Results are given as expression relative to HPRT-1 for comparison of gene expression patterns in undifferentiated and differentiated Caco-2 cells or fold changes when comparing alteration in gene expression after treatment with NA or MPP to untreated controls.

Table 1: qPCR primer pairs

Gene	Primer	Sequence (5'-3')	Product length (bp)
FATP2	FW	TGGAACCCACAGGTGCTACTC	116
	RV	ACCGAAGCAGTTCACCGATA	
FATP4	FW	GGCTGCCCTGGTGTACTATG	93
	RV	CCCACGATGTTTCCTGCTGA	
IFABP	FW	AACTGAACTCAGGGGGACCT	78
	RV	TCGTTTCCATTGTCTGTCCGT	
CD36	FW	TGTAACCCAGGACGCTGAGG	69
	RV	GAAGGTTCTGAAGATGGCACC	
PPAR α	FW	TTCGCAATCCATCGGCGAG	146
	RV	CCACAGGATAAGTCACCGAGG	
PPAR γ	FW	ACCAAAGTGCAATCAAAGTGGA	100
	RV	ATGAGGGAGTTGGAAGGCTCT	
HM74	FW	TGACATAAAGGCAGGCACGG	103
	RV	TGCAGCTAGTGAGTCCGATG	
HM74A	FW	GACAACTATGTGAGGCGTTGG	133
	RV	CCCGGAAATACCTGTCTACCG	
HPRT-1	FW	CCTGGCGTCGTGATTAGTGA	137
	RV	CGAGCAAGACGTTTCAGTCCT	

Calcium signaling

Intracellular calcium mobilization was determined in differentiated Caco-2 cells by means of the Fluo-4 Direct™ Calcium Assay Kit (Invitrogen, Austria). The fluo-4 dye was dissolved in the loading buffer to obtain a twice concentrated dye solution that was further diluted with the same volume of HBSS/Hepes buffer to the final loading dye. The cell culture medium was removed and the loading dye was added. After a 30 min incubation step at 37°C in a humidified incubator, cells were kept another 90 min at room temperature to allow dye uptake before starting the experiment. Nicotinic acid and N-methyl-4-phenylpyridinium, respectively, were diluted in HBSS/Hepes buffer and various concentrations of these compounds (0.001 – 1 mM) were added by direct injection applying the Tecan infinite M200 microplate reader (Tecan, Austria). Fluorescence signals after individual injections were recorded every 2 seconds at an emission wavelength of 520 nm after excitation at 490 nm. The AUC of fluorescence signals over

the first 20 seconds was calculated for individual measurements and normalized to the starting fluorescence signal of each cell. Results are expressed as AUC of untreated controls (100%).

Trans-epithelial electrical resistance (TEER)

The integrity of the epithelial barrier after treatment of differentiated Caco-2 cells with nicotinic acid and N-methyl-4-phenylpyridinium, respectively, was determined by means of the epithelial voltohmmeter EVOM (World Precision Instruments, Germany) connected to the EndOhm-24 chamber (World Precision Instruments, Germany) with 24 mm diameter suitable for Costar® Snapwell™ inserts (Corning Life Sciences, Germany). Caco-2 cells were differentiated in Snapwells™ each filled with 450 μ L culture medium in the upper compartment and 2.5 mL culture medium in the lower compartment. On the experimental day, cells were starved with serum-free medium for one hour prior exposure to the test compounds nicotinic acid and N-methyl-4-phenylpyridinium diluted in HBSS/Hepes buffer, respectively, that were added at a final concentration of 1 mM to the upper compartment only. Control cells were incubated only with medium containing 10% HBSS/Hepes buffer. TEER was measured in duplicate prior test compound addition as well as after 30 min, 60 min and 90 min of treatment. Therefore, Snapwells™ were transferred to the EndOhm chamber prefilled with 2.5 mL of serum-free medium and the corresponding resistance was recorded with the EVOM meter. Basal background resistance was assessed in cell-free Snapwells™. Results are expressed as unit area resistance (UAR, $\Omega \cdot \text{cm}^2$) by multiplying cell-based electrical resistance with the Snapwell™ membrane area.

Statistical analysis

Results are displayed as mean $\pm$ SD of at least three individual experiments (n) including multiple technical replicates unless stated otherwise. The Nalimov outlier test was performed prior estimation of significant differences between treatments. To evaluate the impact of individual pyridine derivatives on fatty acid uptake, a one-way ANOVA with Bonferroni post hoc test versus control was applied. Significant differences to untreated controls were indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. To extract the most important structural determinants of the tested compounds a multiple linear regression was calculated including the percentage of fatty acid uptake as dependent variable and

the functional groups as independent variable. The additional methyl group, the carboxamide group, the carboxyl group, the oxo-group and N-methylation were specified as functional characteristics and included in the regression model to characterize the structure of the pyridine derivatives applied. The presence of each functional group was given the value 1 whereas those molecules lacking a specific group were given the value 0 in a matrix based data set. One-way analysis of variance (ANOVA) with Holm-Sidak post-hoc test was applied to determine dose-dependent effects of test substances and two-way ANOVA with Holm-Sidak post-hoc test was accomplished to test for time and treatment dependency. Differences between samples were assumed at the 5% level of significance and are indicated in the figures with distinct letters. All statistical analyses were carried out using SigmaPlot software (Systat Software, Inc., USA).

Results

Effect of pyridine derivatives on fatty acid uptake

The ability of 17 pyridine derivatives (Fig. 1) to modify enterocyte-like Caco-2 long-chain fatty acid uptake was assessed and results are displayed in Figure 2. Pyridine, presenting the basic structure of the tested compounds, did not affected fatty acid uptake, neither at 0.1 mM nor at 1 mM. In contrast, treatment of the cells with the carboxylated position isomers nicotinic acid (2), iso-nicotinic acid (3) and picolinic acid (4) increased fatty acid uptake at a concentration of 1 mM by $19.0\% \pm 16.2\%$, $11.8\% \pm 9.38\%$ and $13.9\% \pm 3.82\%$, respectively. N-methylpyridinium (5) had no impact on fatty acid uptake. Introduction of an additional methyl group in ortho-position stimulated fatty acid uptake at 0.1 mM and 1 mM (compounds 6), but did not so when present in meta- or para-position (compounds 7, 8). Treatment of differentiated Caco-2 cells with trigonelline (9) or nicotinamide (compound 11) did not cause a significant change in fatty acid uptake. Furthermore, none of the pyridine derivatives that possess an additional oxo-group (compounds 10, 13, 14, 15) affected fatty acid uptake. Inhibition of fatty acid uptake was only observed after incubation with 0.1 mM N-methylnicotinamide (12) and 1 mM N-methyl-4-phenylpyridinium (17), resulting in a reduction by $-10.3\% \pm 11.9\%$ and $-13.3\% \pm 9.59\%$, respectively.

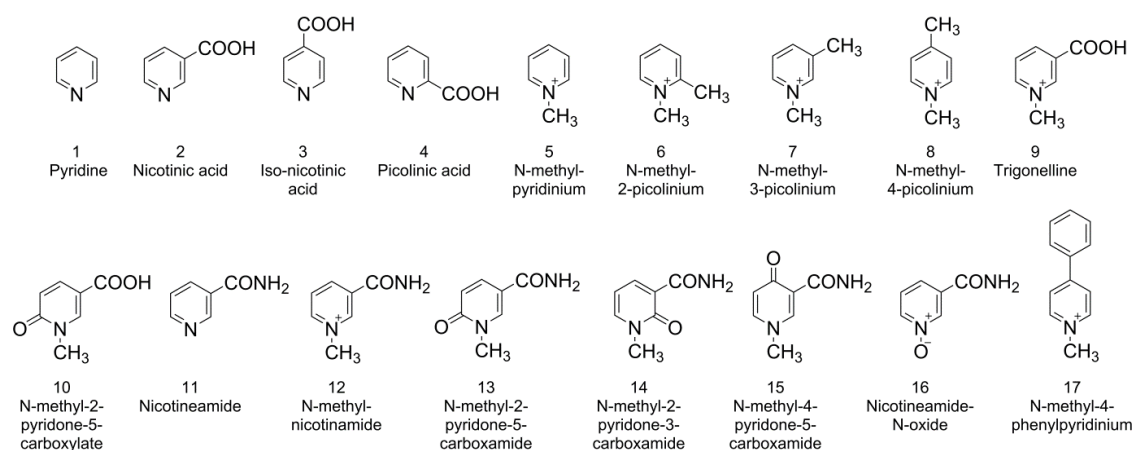


Figure 1: Chemical structures of the pyridine derivatives tested: 1) pyridine, 2) nicotinic acid, 3) iso-nicotinic acid, 4) picolinic acid, 5) N-methylpyridinium, 6) N-methyl-2-picolinium, 7) N-methyl-3-picolinium, 8) N-methyl-4-picolinium, 9) trigonelline, 10) N-methyl-2-pyridone-5-carboxylate, 11) nicotinamide, 12) N-methylnicotinamide, 13) N-methyl-2-pyridone-5-carboxamide, 14) N-methyl-2-pyridone-3-carboxamide, 15) N-methyl-4-pyridone-5-carboxamide, 16) nicotinamide-N-oxide, 17) N-methyl-4-phenylpyridinium.

Results

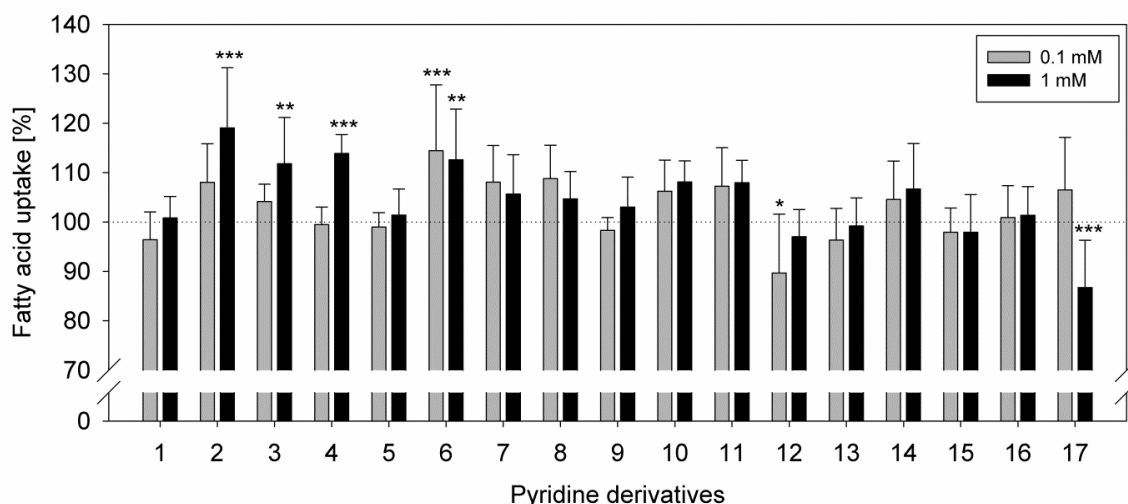


Figure 2: Fatty acid uptake by differentiated Caco-2 cells after 30 min pre-treatment with 0.1 mM and 1 mM of different pyridine derivatives compared to non-treated differentiated cells (=100 %): pyridine (1), nicotinic acid (2), iso-nicotinic acid (3), picolinic acid (4), N-methylpyridinium (5), N-methyl-2-picolinium (6), N-methyl-3-picolinium (7), N-methyl-4-picolinium (8), trigonelline (9), N-methyl-2-pyridone-5-carboxylic acid (10), nicotinamide (11), N-methylnicotinamide iodide (12), N-methyl-2-pyridone-5-carboxamide (13), N-methyl-2-pyridone-3-carboxamide (14), N-methyl-4-pyridone-5-carboxamide (15), nicotineamide-N-oxid (16) and N-methyl-4-phenylpyridinium (17). For data analysis, the AUC after a 60 min fluorescence signal record was calculated and results were normalized to untreated controls (100%). Results are displayed as mean $\pm$ SD of at least 3 individual experiments. Significant differences to untreated controls (dotted line) were determined by one-way ANOVA with Bonferroni post hoc test and are indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

To identify the most important structural determinants of the tested pyridines with regard to their ability to modify fatty acid uptake, a multiple linear regression was performed (Table 2). Fatty acid uptake was defined as the dependent variable whereas the functional groups were defined as independent variables. At a concentration of 0.1 mM, a weak relationship between fatty acid uptake and the presence of functional groups was observed since only 28% of the variability are explainable with the model (p -value of the regression = 0.123, $R = 0.710$, $R^2 = 0.505$, adjusted $R^2 = 0.280$). The additional methyl group was calculated to be the only determining structural characteristic ($p < 0.05$). In contrast, at a concentration of 1 mM, a better prediction of the fatty acid uptake by the regression equation is provided (p -value of the regression = 0.007, $R = 0.853$, $R^2 = 0.728$, adjusted $R^2 = 0.640$). Besides the additional methyl group ($p < 0.01$), the carboxylic group ($p < 0.05$) and the N-methylation ($p < 0.05$) presented further structural characteristics affecting the fatty acid uptake.

Table 2: Multiple linear regression analysis^a

Functional group	0.1 mM		1 mM	
	Coefficient	p-value	Coefficient	p-value
Constant	103 ± 3.85	<0.001	102 ± 3.54	<0.001
Additional methyl group	11.3 ± 4.43	0.027	14.3 ± 6.06	0.005
Carboxamid group	-3.51 ± 3.61	0.427	3.21 ± 3.91	0.429
Carboxyl group	0.48 ± 4.14	0.909	11.6 ± 3.80	0.011
N-methylation	-4.51 ± 6.26	0.237	-8.94 ± 3.32	0.021
Oxo group	4.65 ± 2.26	0.298	4.33 ± 3.91	0.291

^a at 0.1 mM: p-value of the regression = 0.123, R = 0.710, R² = 0.505, adjusted R² = 0.280; at 1 mM: p-value of the regression = 0.007, R = 0.853, R² = 0.728, adjusted R² = 0.640

Cell viability after treatment with nicotinic acid and N-methyl-4-phenylpyridinium

Cytotoxicity was evaluated in differentiated Caco-2 cells after treatment with the selected compounds nicotinic acid and N-methyl-4-phenylpyridinium. No cytotoxic effects were observed after exposure of differentiated Caco-2 cells to NA and MPP for 90 minutes at a concentration range of 0.001 - 1 mM (Fig. 3).

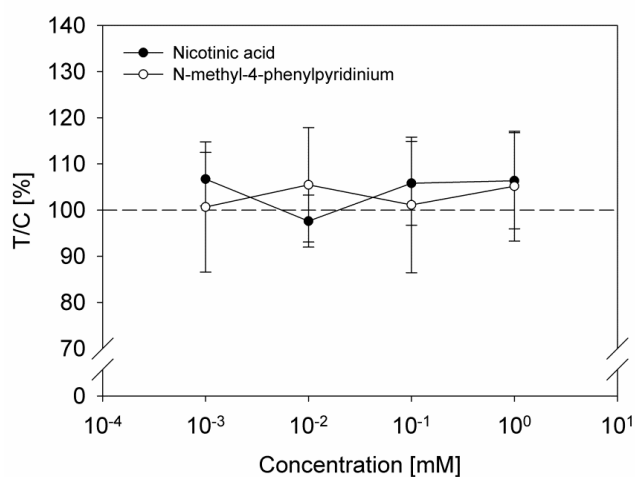


Figure 3: Cytotoxic effects of the selected compounds nicotinic acid and N-methyl-4-phenylpyridinium on differentiated Caco-2 cells after 90 min incubation. Results were normalized to untreated controls (100%) and are shown as mean ± SD of three individual experiments. One-way ANOVA was performed to exclude dose-dependent cytotoxic effects of test compounds ($p > 0.05$).

Impact of nicotinic acid and N-methyl-4-phenylpyridinium on cellular glucose uptake

The uptake of 2-NBDG, a fluorescent glucose analogue, was determined to estimate whether NA and MPP might affect other metabolic pathways, e.g. cause a switch to glucose as energy substrate or inhibit glucose uptake to compensate increased energy supply by fatty acids. However, no changes in glucose uptake of differentiated Caco-2 cells were observed by NA (Fig. 4A) or MPP treatment (Fig. 4B).

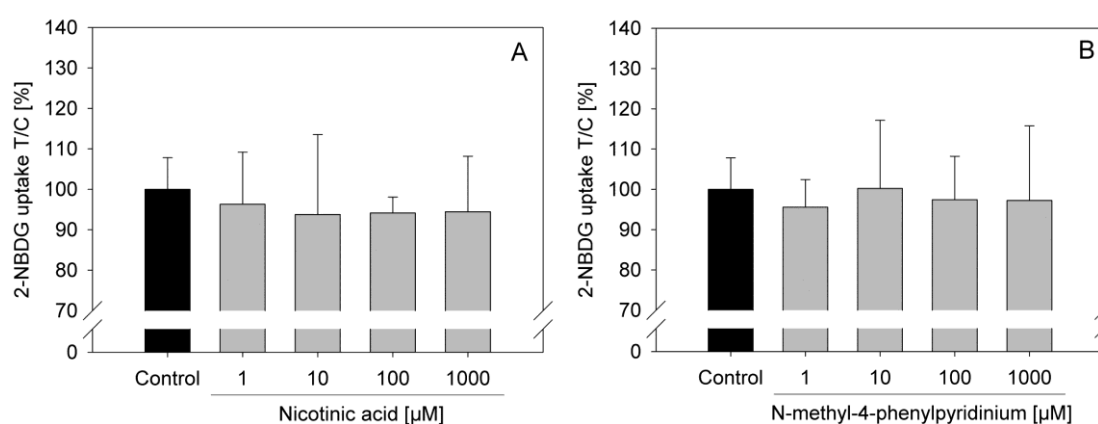


Figure 4: Cellular glucose uptake assessed by means of 2-NBDG uptake in differentiated Caco-2 cells after 30 min exposure to nicotinic acid (A) and N-methyl-4-phenylpyridinium (B). Results were normalized to untreated controls (100%) and are displayed as mean $\pm$ SD ($n \geq 3$). One-way ANOVA was applied to test for significant differences between treatments ($p > 0.05$).

Modulation of gene expression patterns of fatty acid transporters and genes involved in fatty acid metabolism

Intestinal fatty acid absorption is mediated by transport proteins or occurs by passive diffusion [18]. Several fatty acid transport proteins (FATPs) as well as fatty acid binding proteins (FABPs) have been identified in enterocyte-like Caco-2 cells [20]. First, changes in gene expression profiles of fatty acid transporters and genes involved in fatty acid metabolism were assessed in undifferentiated and enterocyte-like differentiated Caco-2 cells. Expression of FATP2, FATP4, CD36, PPAR α and PPAR γ could be verified in undifferentiated Caco-2 cells (Table 3). However, except for FATP2, significantly higher expression of these genes was found in differentiated cells (Table 3). IFABP was below the limit of detection in undifferentiated cells since ΔcT values were above 35.

Results

Table 3: Gene expression relative to HPRT-1 in undifferentiated (ud) and differentiated (d) Caco-2 cells (mean $\pm$ SD, n = 3)

Gene	udCaco-2	dCaco-2	dCaco-2/ udCaco-2	p-value
FATP2	0.013 $\pm$ 0.004	0.029 $\pm$ 0.006	2.22 $\pm$ 0.45	<0.001
FATP4	0.471 $\pm$ 0.104	0.431 $\pm$ 0.075	0.92 $\pm$ 0.16	0.365
IFABP	n.d. ^a	0.457 $\pm$ 0.055		
CD36	0.001 $\pm$ 0.001	0.028 $\pm$ 0.006	27.4 $\pm$ 6.27	<0.001
PPAR α	0.086 $\pm$ 0.009	0.378 $\pm$ 0.099	4.40 $\pm$ 1.15	<0.001
PPAR γ	0.330 $\pm$ 0.074	0.520 $\pm$ 0.070	1.58 $\pm$ 0.21	<0.001
HM74	0.002 $\pm$ 0.001	0.054 $\pm$ 0.007	22.7 $\pm$ 2.97	<0.001

^a not detectable

As fatty acid uptake was modified by nicotinic acid and N-methyl-4-phenylpyridinium, expression patterns of fatty acid transporters and genes involved in fatty acid metabolism were analyzed. Treatment-dependent relative gene expressions of CD36, FATP2, FATP4, IFABP, PPAR α and PPAR γ are illustrated in Figure 5. Expression of the fatty acid transporter CD36 was promoted by incubation with NA after 30 min and 60 min, resulting in a respective 1.45 $\pm$ 0.54 fold and 1.52 $\pm$ 0.15 fold higher gene expression in comparison to untreated controls (Fig. 5). FATP2 and FATP4 have also been upregulated upon nicotinic acid treatment (Fig. 5). Moreover, a slight increase in PPAR α and PPAR γ gene expression was found in differentiated Caco-2 cells after exposure to 1 mM nicotinic acid (Fig. 5). Additionally, time-dependent effects of 1 mM nicotinic acid on gene expression levels were evaluated. Expression of CD36, FATP4, PPAR α and PPAR γ showed significant differences between the time points 30 min and 90 min ($p < 0.001$) as well as 60 min and 90 min ($p < 0.01$), though not differing between the incubation times 30 min and 60 min ($p > 0.05$). In contrast, FATP2 was only affected by treatment ($p < 0.05$) and not by time ($p > 0.05$) while IFABP remained unaffected by nicotinic acid ($p > 0.05$).

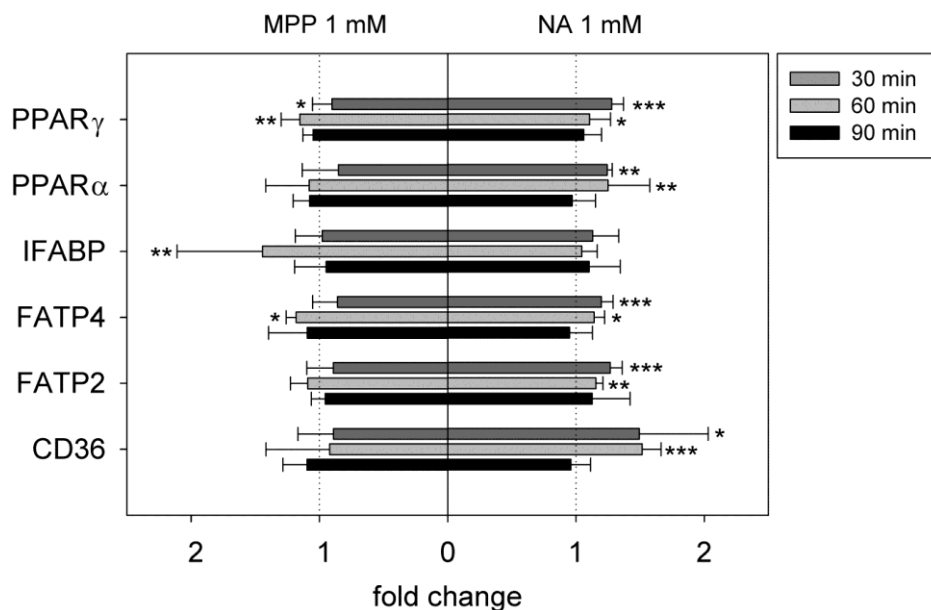


Figure 5: Relative expression of genes involved in fatty acid uptake and regulation of cellular fatty acid metabolism determined in differentiated Caco-2 cells after treatment with 1 mM nicotinic acid (NA) and 1 mM N-methyl-4-phenylpyridinium (MPP). CD36, FATP2, FATP4, IFABP, PPAR α and PPAR γ gene expression was determined after 30 min, 60 min and 90 min incubation with the test compounds. Data were normalized to the reference gene HPRT-1 and are given as fold changes of untreated controls (dotted line). Results represent mean $\pm$ SD ($n = 3$). A two-way ANOVA with Holm-Sidak post hoc test was performed to test for time- and treatment-dependency and considered as significant at $p < 0.05$. Significant differences to controls are indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The incubation of Caco-2 cells with N-methyl-4-phenylpyridinium also caused changes in the expression profile of the selected genes (Fig. 5). PPAR γ was slightly downregulated after 30 min followed by an upregulation after 60 min. IFABP and FATP4 gene expressions were only increased after 60 min of N-methyl-4-phenylpyridinium treatment. Moreover, time-dependent variations in gene expression levels were calculated for PPAR γ showing differences between all time points ($p < 0.05$), IFABP differing between 30 min and 60 min ($p < 0.01$) as well as 60 min and 90 min ($p < 0.01$), and FATP4 being different between 30 min and 60 min ($p < 0.01$) as well as 30 min and 90 min ($p < 0.01$). However, CD 36, FATP2 and PPAR α were neither affect by time or by treatment ($p > 0.05$).

Effect of nicotinic acid and N-methyl-4-phenylpyridinium on the nicotinic acid receptor expression

In humans the nicotinic acid receptor, a G-protein coupled receptor, appears in two subtypes: the HM74A or GRP109A and the HM74 or GPR109B subtype, of which HM74A lacks 24 amino acids at its C-terminus [13, 21]. Undifferentiated Caco-2 cells express only marginal amounts of the nicotinic acid receptor subtype HM74 relative to the reference gene HPRT-1 (hypoxanthine guanine phosphoribosyl transferase), that is increased upon differentiation to enterocyte-like cells (Table 3). In contrast, the HM74A subtype was neither detectable in undifferentiated nor in differentiated Caco-2 cells. Incubation of differentiated Caco-2 cells with 1 mM nicotinic acid led to a 2.38 ± 0.89 fold enhanced HM74 gene expression compared to untreated controls (1.00 ± 0.09) after 30 min (Fig. 6). This effect was maintained after 60 min, resulting in a 2.06 ± 1.09 fold increased HM74 expression in comparison to controls (1.00 ± 0.09). After 90 min, gene expression levels reached values similar to those of control cells. Time-dependent effects of nicotinic acid treatment was revealed for HM74 gene expression between 30 min and 90 min ($p < 0.001$) and between 60 min and 90 min ($p < 0.001$), although not varying between the time points 30 min and 60 min ($p > 0.05$). N-methyl-4-phenylpyridinium I not affect HM74 expression patterns (Fig. 6).

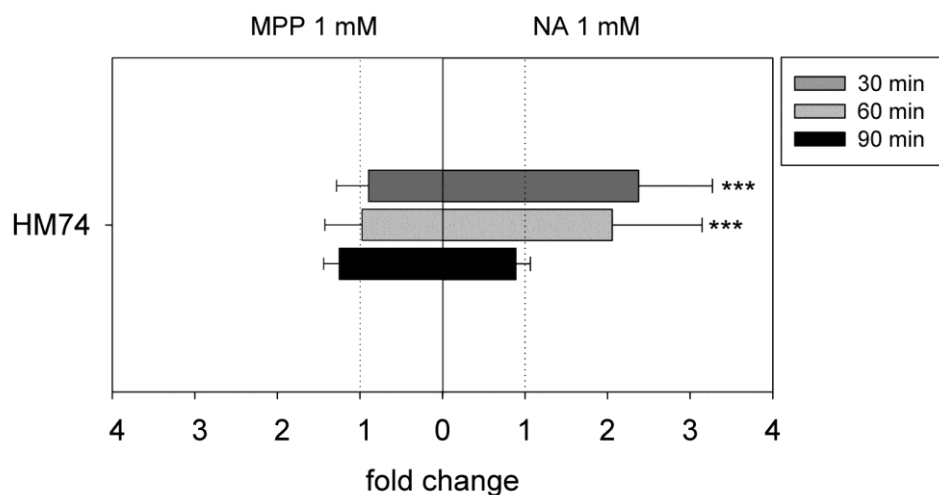


Figure 6: Gene expression of the nicotinic acid receptor HM74 determined in differentiated Caco-2 cells after treatment with 1 mM nicotinic acid (NA) and 1 mM N-methyl-4-phenylpyridinium (MPP) for 30 min, 60 min and 90 min. Data were normalized to the reference gene HPRT-1 and are given as fold changes of untreated controls (dotted line). Results represent mean $\pm$ SD of at least three biological replicates. A two-way ANOVA with Holm-Sidak post hoc test was performed to test for time- and treatment-dependency and considered as significant at $p < 0.05$. Significant differences to controls are indicated with *** $p < 0.001$.

Induction of intracellular calcium mobilization

Nicotinic acid receptors are G_i -type G-protein coupled receptors that, upon stimulation, inhibit intracellular cAMP accumulation, but also increase intracellular Ca^{2+} mobilization by activating the G-protein α -subunit $G_{\alpha 15}$ [12]. Direct addition of nicotinic acid to enterocyte-like Caco-2 cells enhanced intracellular calcium mobilization (Fig. 7). The highest tested concentration of 1 mM was applied to evaluate the expression of the nicotinic acid receptor HM74-stimulated calcium mobilization, resulting in a mean increase of $186\% \pm 98.6\%$ in comparison to untreated controls ($100\% \pm 11.9\%$). In contrast, no effect on intracellular calcium mobilization was found after N-methyl-4-phenylpyridinium treatment.

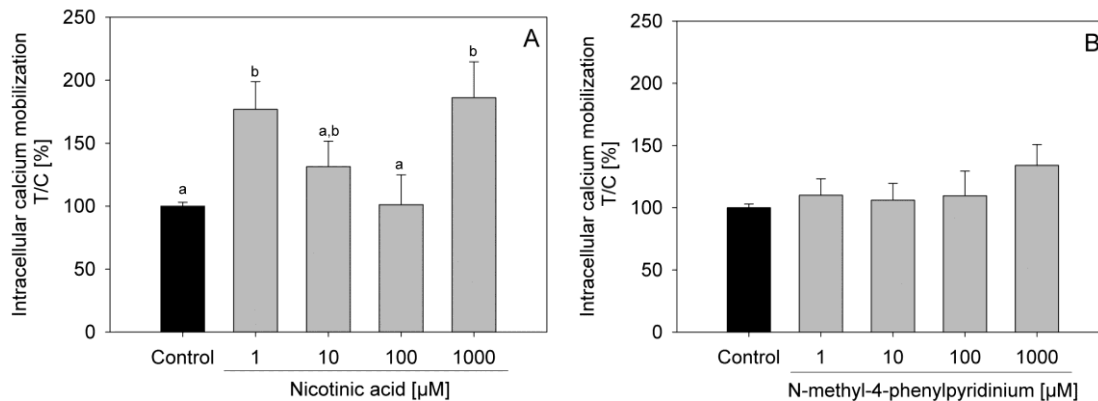


Figure 7: Intracellular calcium mobilization after direct addition of with nicotinic acid (A) and N-methyl-4-phenylpyridinium (B) to differentiated Caco-2 cells. Data are shown as mean $\pm$ SEM ($n \geq 3$). For statistical analysis one-way ANOVA with Holm-Sidak post hoc test was performed and significant differences ($p < 0.05$) between treatments are indicated with distinct letters.

Changes in trans-epithelial electrical resistance

Differentiated Caco-2 cells present a suitable cell model to study barrier function on intestinal epithelial cells as they form monolayers that are characterized by polarization, development of a brush border membrane and formation of intercellular tight junctions [17]. Analysis of the trans-epithelial electrical resistance provides evidence for the paracellular integrity of enterocyte-like Caco-2 cells.

Caco-2 cells demonstrated a mean TEER of $454 \pm 40.1 \Omega \times \text{cm}^2$ after 21 days of differentiation, a value that is comparable to those described in the literature [22].

Addition of incubation medium containing 10% HBSS/Hepes buffer to the control cells induced a slight decrease by $-27.4 \pm 2.81 \Omega \times \text{cm}^2$ after 30 minutes, but remained stable throughout the following measurement time points (Fig. 8). Incubation of differentiated Caco-2 cells with 1 mM nicotinic acid also reduced TEER by $-19.0 \pm 3.33 \Omega \times \text{cm}^2$ within 30 minutes when compared to the initial value prior to the addition of test compounds. TEER further decreased after 60 minutes ($-30.0 \pm 4.70 \Omega \times \text{cm}^2$), but was maintained in the same range after 90 minutes ($-32.9 \pm 12.9 \Omega \times \text{cm}^2$). However, monolayer integrity after nicotinic acid addition was not different from controls at any time point ($p > 0.05$). In contrast, a strong time-dependent increase in trans-epithelial electrical resistance was monitored after addition of 1 mM N-methyl-4-phenylpyridinium, showing the most pronounced effect with an average augmentation of $77.0 \pm 10.9 \Omega \times \text{cm}^2$ after 90 minutes. Changes in TEER provoked by N-methyl-4-phenylpyridinium addition significantly differed ($p < 0.001$) from those of controls at 30 min, 60 min and 90 min, respectively.

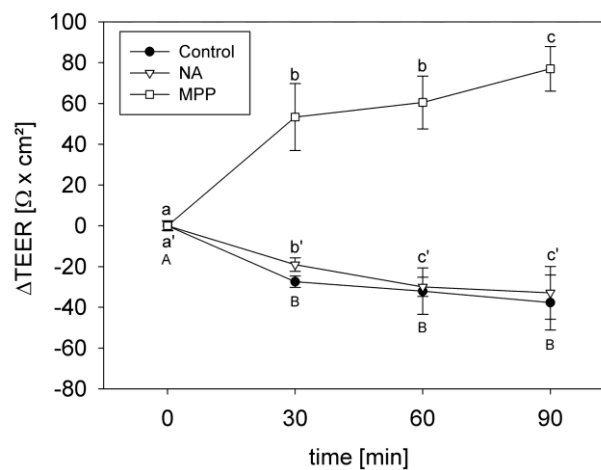


Figure 8: Trans-epithelial electrical resistance (TEER) of Caco-2 cells after treatment with incubation medium only (control), 1 mM nicotinic acid (NA) and 1 mM N-methyl-4-phenylpyridinium (MPP), respectively. Measurements were carried out before as well as after 30 min, 60 min and 90 min of incubation with NA or MPP. Results are shown as mean difference $\pm$ SD to initial TEER ($n = 3$). A two-way ANOVA with Holm-Sidak post hoc test was performed to estimate time- and treatment-dependent effects and considered as significant at $p < 0.05$. Significant differences ($p < 0.05$) between time points of each treatment are indicated with distinct letters.

Discussion

Nicotinic acid has been in use as a lipid-lowering drug for the last decades as it is effective in lowering the blood levels of low-density lipoprotein (LDL)-cholesterol, triglycerides, and lipoprotein (a), and in increasing levels high-density lipoprotein (HDL)-cholesterol [7,8]. However, its effects on intestinal fatty acid uptake have been incompletely investigated. In order to elucidate the structural determinants of pyridines responsible for their impact on fatty acid uptake by enterocyte-like Caco-2 cells, nicotinic acid (NA) and NA analogs were tested. Moreover, we intended to clarify the mechanisms of action by which two of the studied pyridines, nicotinic acid and N-methyl-4-phenylpyridinium (MPP), might modify intestinal fatty acid uptake.

Results from the multiple linear regression analysis of data obtained from the fatty acid uptake studies in differentiated enterocyte-like Caco-2 cells revealed an additional methyl-group as an important structural determinant for a compounds affecting fatty acid uptake when applied at a concentration of 0.1 mM. As a result, N-methyl-2-picolinium presents a promising pyridine derivative that may increase intestinal fatty acid uptake. At a concentration of 1 mM, the carboxylic group was also identified as significant structural determinant. Treatment of Caco-2 cells with the monocarboxylated pyridine derivative nicotinic acid and its position isomers iso-nicotinic acid and picolinic acid stimulated fatty acid uptake. In contrast, N-methylation was identified as an inhibitory characteristic. However, identification of the three structural determinants, the presence of (i) a methyl-group, (ii) a carboxylic group, and (iii) N-methylation, as important structural characteristics affecting fatty acid uptake in differentiated Caco-2 cells provide first evidence for their specific mode of action.

Nicotinic acid, which shows the most pronounced stimulating effect on fatty acid uptake, and N-methyl-4-phenylpyridinium, which inhibits fatty acid uptake, were selected for mechanistic studies.

Nicotinic acid is used pharmacologically to treat hyperlipidaemia. Oral administration of 200 mg nicotinic acid was shown to reduce plasma free fatty acids within minutes, followed by a rebound effect one hour after administration [7]. Therefore, up to 3 grams of nicotinic acid per day are administered to humans to achieve a prolonged reduction of plasma lipids and total cholesterol [23-25]. However, substantial side effects, such as skin flushing, are associated with high-dose administration of nicotinic acid [10]. Dietary

intake of nicotinic acid is far lower than that needed to obtain pharmacological effects. Lang *et al.* [1] estimated nicotinic acid contents in several foods and found about 3.45 µg/g in breakfast cereals, 5.79 µg/g in beef liver and up to 369 µg/g in roasted coffee. However, analytical quantification of nicotinic acid is challenging due to its rapid metabolism. Nicotinic acid has so far not been detected in generic human plasma samples [1], nor in plasma samples after administration of 350 mL freshly prepared coffee brew containing about 3.1 mg nicotinic acid [2]. Plasma nicotinic acid concentrations slightly above the limit of quantification (0.4 µM) were only reported in samples from a clinical pharmacokinetic study after bolus administration of 1000 mg nicotinic acid [26].

In this study, no toxic effects of nicotinic acid on differentiated Caco-2 cells were found in a concentration range of 0.001 - 1 mM for 90 min. Interfering effects of intestinal fatty acid and glucose uptake induced by nicotinic acid could be excluded as glucose uptake remained unchanged after administration nicotinic acid. Therefore, we focused on the modulation of the nicotinic acid receptors involved in lipid metabolism and modifications of gene expression patterns of fatty acid transporters and genes involved in the regulation of fatty acid metabolism.

With the identification of the nicotinic acid receptors, the anti-lipolytic effect of nicotinic acid in the adipose tissue could be explained [11-13]. Upon activation, these G-protein coupled receptors inhibit the enzyme adenylyl cyclase [27] and, hence, reduce the formation of cAMP [28]. Results from tissue distribution studies point to a predominant expression of nicotinic acid receptors in adipose tissue, lung, lymphocytes and spleen [11, 13]. More recently, expression of both receptor subtypes has been proven in several colon cancer cell lines, but had not been investigated in Caco-2 cells [29], although HM74A has recently been verified in a subclone of Caco-2, C2BBE1 cells [30]. Here, we proved evidence that undifferentiated Caco-2 cells also express the low affinity nicotinic acid receptor HM74 and that its expression is substantially increased upon differentiation to enterocyte-like cells. In contrast, the high affinity version HM74A was not detectable. We further demonstrated that incubation of differentiated Caco-2 cells with 1 mM nicotinic acid upregulated HM74 expression, indicating a stimulating effect of nicotinic acid on HM74 that might account for the increased fatty acid uptake. Knowles *et al.* [31] showed that activation of the nicotinic acid receptors in macrophages promotes prostaglandin D₂ production. Both subtypes, the low affinity version HM74 as well as the high affinity version HM74A, are involved in the initiation of the prostaglandin pathway [31]. Consequently, the nicotinic acid-induced prostaglandin D₂ formation and

subsequent PPAR γ activation might explain the enhanced transcription of fatty acid translocase/CD36 monitored in monocytoid cells after 3 hour treatment with 1 mM nicotinic acid [32].

In intestinal cells, several fatty acid transport proteins have been identified [20, 33, 34]. We first showed that expression of the analyzed genes was more abundant in differentiated Caco-2 cells compared to the undifferentiated cells, except for FATP4, thereby presenting characteristic metabolic changes of the enterocyte-like differentiated Caco-2 cells compared to undifferentiated cells. Administration of nicotinic acid upregulated CD36, FATP2 and FATP4 gene expression in enterocyte-like Caco-2 cells. This result clearly indicates a stimulating effect on fatty acid transporters. We also monitored the short-term upregulation of PPAR α and PPAR γ gene expression. PPAR α is a major regulator of lipid metabolism. Activation of the transcription factor PPAR α has been shown to increase CD36 expression in the liver and the intestine of mice [35]. Furthermore, PPAR α is associated with enhanced fatty acid uptake, utilization and catabolism [36].

Our *in vitro* data clearly support the hypothesis that, in intestinal cells, nicotinic acid is able to activate the nicotinic acid receptor HM74. Its activation may lead to prostaglandin D₂ formation, subsequent PPAR γ activation, and stimulation of CD36 expression, resulting in an enhanced intestinal fatty acid uptake. Although an increased intestinal fatty acid uptake may lead to higher levels of circulating free fatty acids and lipoproteins, the plasma lipid lowering effects of nicotinic acids are well-established. Thus, we hypothesize that an increased intestinal fatty acid uptake in the presence of nicotinic acid might be compensated by (i) repression of lipolysis in adipocytes or (ii) inhibition of hepatic diacylglycerol acyltransferase and triglyceride synthesis resulting in reduced secretion of lipoproteins from the liver [37].

We further demonstrated an inhibition of fatty acid uptake by Caco-2 cells by N-methyl-4-phenylpyridinium treatment. MPP has been shown to possess toxic effects [38]. On a cellular level, treatment of neural cells with N-methyl-4-phenylpyridinium caused dopaminergic neurodegeneration and bioenergetic dysfunction due to a repression of oxygen consumption as a consequence of mitochondrial respiratory complex 1 activity inhibition [39, 40]. However, in this study, MPP concentrations up to 1 mM for 90 min showed no toxic effects on Caco-2 cells. No changes in glucose uptake and nicotinic acid receptor HM74 expression were monitored after MPP treatment. Fatty acid transporter and PPAR γ gene expression were only slightly affected. These results led to the assumption that, although nicotinic acid and N-methyl-4-phenylpyridinium share the

pyridine core and both affect fatty acid uptake in differentiated enterocyte-like Caco-2 cells, the underlying mechanisms of action differ from each other. In particular, the inhibitory effect on fatty acid uptake demonstrated for N-methyl-4-phenylpyridinium is very unlikely based on inhibition of the nicotinic acid receptor.

In contrast, trans-epithelial electrical resistance (TEER) was increased after addition of N-methyl-4-phenylpyridinium to differentiated Caco-2 cells. An augmented TEER was also monitored after incubation of Caco-2 cells with short-chain fatty acids [41], quercetin [42] or apple extracts [43], thus reducing paracellular permeability and enhancing the cellular barrier function. Changes in TEER have been associated with an altered membrane protein composition, contributing to a stronger conjunction of cells [42, 43]. We therefore hypothesize that N-methyl-4-phenylpyridinium treatment reduced fatty acid uptake by modifying the paracellular permeability and the membrane integrity, diminishing passive diffusion. Moreover, TEER increased constantly after N-methyl-4-phenylpyridinium treatment. This result might be interpreted as counteraction of the cells to N-methyl-4-phenylpyridinium exposure to prevent adverse effects on mitochondrial function.

In conclusion, this study elucidated the effects of pyridine derivatives on fatty acid uptake in enterocyte-like differentiated Caco-2 cells. Multiple linear regression analysis revealed a methyl-group and a carboxylic group to be structural determinants for pyridines affecting the fatty acid uptake. Two of the studied compounds, nicotinic acid and N-methyl-4-phenylpyridinium, although sharing the pyridine core motif, affected fatty acid uptake in differentiated Caco-2 cells differently. Whereas the stimulating effect of high dose nicotinic acid on fatty acid uptake was very likely evoked by nicotinic acid receptor activation, resulting in an enhanced expression of genes involved in lipid metabolism as well as fatty acid transporters, N-methyl-4-phenylpyridinium reduced fatty acid uptake by increasing TEER and hence, impairing passive diffusion.

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2.4 “A 4-week consumption of medium roast and dark roast coffee affects parameters of energy status in healthy subjects”

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“A 4-week consumption of medium roast and dark roast coffee affects parameters of energy status in healthy subjects“

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Abstract:

Scope: This study intended to clarify whether two coffee brews, a market (MB) and a study blend (SB), containing equal amounts of caffeine, but differing in their contents of N-methylpyridinium, trigonelline and chlorogenic acids, differentially affect blood lipid profiles and glucose concentrations as well as blood platelet phosphodiesterase and lymphocyte energy charge potential in healthy volunteers.

Methods and results: In this double-blinded, randomized, controlled cross-over intervention study, 84 healthy normal-weight female and male volunteers consumed 750 mL of medium roast MB and dark roast SB coffee per day for 4 weeks. Following MB and SB coffee intervention, plasma free fatty acid concentrations equally increased ($p < 0.001$) by $140 \pm 187 \mu\text{M}$ and $178 \pm 160 \mu\text{M}$, respectively. Plasma glucose remained largely unchanged. Lymphocyte adenosine nucleotide analysis revealed a comparable rise in the energy charge potential, as calculated from ADP/ATP concentrations, adjusted to total adenosine nucleotides. Blood platelet phosphodiesterase activity was found decreased to about the same extent ($p < 0.001$). Levels of HDL-cholesterol, adiponectin, leptin, insulin and osteopontin were found to some extent differentially influenced by MB, respectively SB coffee.

Conclusion: The results of this intervention study indicate that MB and SB coffees, although differing in contents of N-methylpyridinium, trigonelline and chlorogenic acids, largely exert similar biological effects as monitored by the biomarkers tested.

Keywords: Coffee, Free Fatty Acids, HDL-cholesterol, ATP, Lymphocytes, Phosphodiesterase

Abbreviations: **ADA**, adenosine deaminase; **ADP**, adenosine diphosphate; **AMP**, adenosine monophosphate; **ATP**, adenosine triphosphate; **cAMP**, cyclic adenosine monophosphate; **CGA**, chlorogenic acid; **cT**, cycle threshold; **DMUA**, dimethyluric acid; **ECP**, energy charge potential; **FFA**, free fatty acid; **GAPDH**, glyceraldehyde 3-phosphate dehydrogenase; **HOMA-IR**, homeostatic model assessment of insulin resistance; **LEPR**, leptin receptor; **MB**, market blend; **MX**, methylxanthine; **NMP**, N-methylpyridinium; **ns**, not significant; **PDE**, phosphodiesterase; **RPL13a**, Ribosomal protein L13a; **RT**, room temperature; **SB**, study blend; **TAN**, total adenosine nucleotides; **UCP-2**, uncoupling protein-2; **WO**, wash-out

1 Introduction

Coffee beverages are mostly appreciated for their tasty flavor and their stimulating effects on the central nervous system. Furthermore, certain beneficial health effects are discussed that may contribute to the consumers' choice of coffee. Although it is still a matter of debate whether the overall effects to human health of regular coffee consumption are positive or negative, epidemiological as well as clinical data indicate that moderate coffee consumption is inversely associated with the risk of certain human cancers (Giri et al., 2011; Radoi et al., 2013; Sang, Chang, Li, & Jiang, 2013), Parkinson's disease (Popat et al., 2011) and diabetes mellitus type II (Bhupathiraju et al., 2013). Epidemiological data also suggest a potential effect of coffee consumption for promoting body weight maintenance (Greenberg, Axen, Schnoll, & Boozer, 2005; Lopez-Garcia et al., 2006). Since body weight maintenance reflects energy intake and energy expenditure, increased physical activity, enhanced thermogenesis or utilization of endogenous substrate result in a reduction of body weight at constant energy intake. The coffee constituent most widely studied for its stimulating effects on energy expenditure is caffeine. Controlled clinical studies demonstrated an increased resting energy expenditure in healthy, normal-weight or overweight subjects after consumption of regular coffee or intake of caffeine by means of indirect calorimetry (Acheson, Zahorska-Markiewicz, Pittet, Anantharaman, & Jequier, 1980; Dulloo, Geissler, Horton, Collins, & Miller, 1989; Hollands, Arch, & Cawthorne, 1981). Moreover, Astrup *et al.* (Astrup et al., 1990) reported a positive correlation between the caffeine dose and the integrated thermogenesis response, not seen for caffeine metabolites. Some authors suggest that the main mechanism of action of caffeine is to antagonize adenosine receptors, especially the A_{2A} subpopulation. Inhibition of intracellular cyclonucleotide phosphodiesterases (PDEs) and in consequence enhanced 3',5'-cAMP levels may also be of relevance, for instance by enhanced response to catecholamines (Randall D. J., Burggren W., & K., 1997). It is still under debate whether and to what extent PDE inhibition may contribute to the actions of caffeine. Evidence is growing, though, that most pharmacological effects of this xanthine result from adenosine receptor antagonism (Tunnicliffe, Eller, Reimer, Hittel, & Shearer, 2011).

Despite strong evidence for caffeine being the prime coffee constituent responsible for increased total energy expenditure, it is still not clear whether this effect is longer lasting. Previous studies focused primarily on short-term effects with intervention periods up to 24 hrs. Moreover, since coffee contains a multitude of compounds, other coffee

constituents may as well affect energy metabolism. Chlorogenic acids (CGAs), the most abundant group of polyphenols in coffee, are also supposed to have an impact on body weight gain. In a controlled human intervention study, a 12-week consumption of a CGA-enriched coffee resulted in a significant body weight reduction in overweight subjects (Thom, 2007). More recently, a cross-over study in pre-obese subjects showed that a 6-week supplementation of a green coffee extract (rich in CGA) was effective in reducing body weight and body fat (Vinson, Burnham, & Nagendran, 2012). CGA is hypothesized to be primarily responsive for modifying glucose homeostasis. During an oral glucose tolerance test, concomitant administration of 1 g CGA reduced glucose response after 15 minutes, compared to a water control (van Dijk et al., 2009). The authors hypothesized that this effect might have resulted from delayed intestinal absorption or enhanced transport into peripheral tissues. Furthermore, peritoneal injection of 80 mg CGA per kg body weight daily for 8 weeks to hamsters on a high fat diet decreased fasting serum levels of free fatty acids, glucose and insulin, compared to non CGA-treated controls (Li, Chang, Ma, & Yu, 2009). In cultured L6 skeletal muscle cells as model for peripheral tissue, incubation with 2 mM CGA increased glucose uptake up to 24 hrs via activation of AMP-activated protein kinase and translocation of glucose transporter type 4 (Ong, Hsu, & Tan, 2012). Concentrations of 0.1 - 100 μ M CGA were also reported to stimulate 2-[N-(7-nitrobenz-2-oxa-1,3-dioxol-4-yl)amino]-2-deoxyglucose uptake in 3T3-L1 adipocytes (Alonso-Castro, Miranda-Torres, Gonzalez-Chavez, & Salazar-Olivo, 2008). Thus, the above studies in muscle cells and adipocytes show CGA to increase glucose uptake in peripheral tissues resulting in an elevated cellular energy supply. However, an increased cellular glucose uptake does not provide a mechanism for CGA to promote body weight loss.

In humans, CGA is effectively metabolized resulting in peak plasma concentrations of CGAs and their conjugates within the nanomolar range after coffee consumption (Erk et al., 2012; Lang et al., 2013; Stalmach et al., 2009). However, it is still unclear to which extent CGA may contribute to the overall effect of coffee on body weight maintenance. Apart from caffeine and chlorogenic acid, the alkaloid trigonelline and its degradation product formed upon coffee roasting, N-methylpyridinium (NMP) (Stadler, Varga, Hau, Vera, & Welte, 2002), might also have an impact on energy metabolism in humans and contribute to the overall effect of coffee on body weight maintenance. For trigonelline, it was shown, that a 500 mg bolus intervention lowered peak plasma glucose concentrations compared to water control in healthy subjects 15 minutes after administration of 75 g glucose in an oral glucose tolerance test (van Dijk et al., 2009). Moreover, reduction in body weight in overweight subjects after a 4-week consumption of

dark roasted coffee high in NMP has been reported (Kotyczka et al., 2011). The molecular mechanisms by which trigonelline or NMP may help to maintain a healthy body weight is unknown yet.

Taken together, evidence for beneficial effects of coffee consumption is suggestive, although it is still unknown whether moderate coffee consumption as part of a regular diet affects resting energy expenditure. Likewise, mechanisms of thermogenesis or utilization of endogenous energy substrates like glucose or fatty acids are unknown, as are the coffee constituents that may contribute to these effects. To address the question whether regular coffee consumption affects mechanisms of energy metabolism, we conducted a randomized, cross-over intervention study. In this study, healthy volunteers were administered daily 750 mL of two different coffee beverages for 4 weeks. Both coffees contained comparable amounts of caffeine, but differed in their contents of CGA, trigonelline and NMP due to their different roast characters. Accordingly, a CGA- and trigonelline-rich medium roast market blend coffee and a NMP-rich study blend coffee were studied for effects on parameters of lipid, glucose and energy metabolism.

2 Material and methods

2.1 Study design and base-line characteristics of volunteers

This randomized double-blinded cross-over human intervention study was approved by the local ethic committee of Rhineland-Palatinate, no. 837.414.10 (7423). The study design, the base-line characteristics of the 84 volunteers participating in this study and the composition of the two coffees administered in this study are explained in detail in the publication of Bakuradze and coworkers (Bakuradze et al., 2014). In brief, volunteers who matched the following inclusion criteria were recruited to participate in the study: age 20 - 44 years, BMI 19 - 26 kg/m², healthy, coffee drinkers, non-smokers, no intake of pharmaceutical drugs and/or food supplements, no pregnancy. In total, 84 volunteers (mean age 25.6 ± 5.8 years, BMI 22.9 ± 1.9 kg/m²) with regular coffee consumption participated in the 20 week cross-over study. Volunteers were randomly assigned into two groups (group A: n = 43, 24 male, 19 female; group B: n = 41, 22 male, 19 female) with two wash-out and two coffee intervention periods, respectively, each lasting 4 weeks. During wash-out periods, volunteers consumed 750 mL water per day. During coffee intervention periods, 750 mL freshly brewed regular coffee (6 coffee pads à 7.5 g/

day), without milk and ad libitum sugar addition, were taken in three portions each of 250 mL per day. The volunteers were instructed to keep their dietary habits during the study, except for additional intake of coffee, caffeine containing products, dietary supplements and foods rich in polyphenols, such as dark chocolate and red berries, which should have been avoided.

The study design was as follows: week 1 - 4 first wash-out period, week 5 - 8 first coffee intervention period (group A: market blend coffee; group B: study blend coffee), week 9 - 12 second wash-out period, week 13 - 16 second coffee intervention period (group A: study blend coffee; group B: market blend coffee) and week 17 - 20 third wash-out period.

Body weight as well as body composition of the study subjects were assessed at the beginning and the end of each intervention period and nutrient intake was recalled from 7-day dietary protocols recorded in the last week of each intervention period (Bakuradze et al., 2014).

2.2 Coffee characterization

Details on the administered coffees and their preparation are published in this issue by Bakuradze and colleagues (Bakuradze et al., 2014). Briefly, a medium roast market blend (MB) coffee and a dark roast study blend (SB) coffee were administered in one of the two coffee intervention periods. MB coffee was blended from equal portions of five commercially available regular ground coffees (Rubach et al., 2014). SB coffee was produced by blending predominantly dark-roast coffee with significant portions of light-roast coffee (by Tchibo GmbH, Überseering 18, 22297 Hamburg, Germany). While SB represented a pure Arabica blend, one of the five commercial coffees used for the MB coffee turned out to contain an unknown quantity of Robusta coffee, as indicated by the occurrence of 16-O-methylcafesol (Speer & Kölling-Speer, 2006). Caffeine, N-methylpyridinium, trigonelline and chlorogenic acid were quantified in the MB and the SB coffee, respectively, according to the methods described previously (Lang, Wahl, Stark, & Hofmann, 2012; Weiss et al., 2010). The concentrations of the selected coffee constituents are given in Table 1.

Table 1. Concentration of selected constituents in the market blend (MB) and study blend (SB) coffee

Compound	Marked blend coffee	Study blend coffee
Caffeine [mg/g]	12.4 ± 0.1	12.8 ± 0.2
N-methylpyridinium [mg/g]	0.39 ± 0.0	1.20 ± 0.0
Trigonelline [mg/g]	6.27 ± 0.1	3.42 ± 0.2
Chlorogenic acid ^{a)} [mg/g]	19.2 ± 0.3	10.0 ± 0.3

Values are given as mean ± SD per gram dry matter

^{a)} sum of 3-, 4- and 5-caffeoylquinic acid

2.3 Blood sample collection

Blood samples were drawn from the study subjects after an overnight fast from 8 p.m. until 8 a.m. at the beginning and the end of each intervention period, as described elsewhere (Bakuradze et al., 2014). Blood was collected in EDTA-coated tubes (Sarstedt, Germany) and centrifuged at 1,800xg at 4 °C for 15 min. The resulting plasma was used for the quantification of leptin, adiponectin, osteopontin, free fatty acids as well as caffeine and its metabolites. For quantitative glucose analyses, plasma samples were obtained by centrifugation 1,800xg for 15 min at 4° C of blood samples withdrawn in fluoride- and EDTA-coated tubes (Sarstedt, Germany). Insulin, triglycerides, total cholesterol, HDL-cholesterol and LDL-cholesterol were determined in serum samples. Blood samples were drawn into EDTA-coated serum tubes (Sarstedt, Germany) and centrifuged at 2,000xg at 20 °C for 10 min. All plasma and serum samples were stored in aliquots at -80 °C until analysis of biochemical parameters. For cAMP and PDE assays, platelets were isolated from platelet-rich plasma, divided in two different tubes, and centrifuged (800xg, 15 min, RT). Supernatants were aspirated and stored for adenosine deaminase (ADA) assays. Pellets were gently re-suspended in appropriate buffer.

2.4 Quantification of methylxanthines and 1,7-dimethyluric acid

Methylxanthines and 1,7-dimethyluric acid were quantified according to the method published previously by Lang et al. (Lang et al., 2013).

2.5 Leptin, adiponectin, glucose, insulin, osteopontin and free fatty acids assays

Plasma leptin and serum insulin levels were determined using commercially available sandwich ELISAs (DRG Diagnostics GmbH, Germany). The ELISA kits for the quantification of plasma adiponectin and osteopontin were purchased from Ray Biotech, Inc., USA. Plasma glucose was measured photometrically and free fatty acids (FFA) were quantified fluorometrically, both by means of commercially available kits (Cayman Europe, Estonia). All analyses were carried out according to the manufacturer's protocols. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated according to the equation $\text{HOMA-IR} = \text{fasting insulin } [\mu\text{U/mL}] \times \text{fasting glucose } [\text{mg/dL}] / 405$.

2.6 Triglycerides, total cholesterol, HDL-cholesterol and LDL-cholesterol

Total cholesterol, triglycerides, HDL and LDL cholesterol levels were quantified by commercially available enzymatic assay kits (Abbott Diagnostics, Germany).

2.7 Leptin receptor expression

Leptin receptor expression was determined in isolate peripheral blood mononuclear cells. Cell isolation from whole blood and RNA extraction was conducted as described previously (Volz et al., 2012). The Quantitect Reverse Transcription Kit (QIAGEN, Germany) was used to obtain cDNA out of 1 µg RNA and quantitative real-time PCR was performed according to the manufacturer's manuals (Quantitect SYBR Green PCR and Quantitect Primer Assays, QIAGEN; Germany). The applied primer assays were Hs_LEPR_1_SG QT00006524 (leptin receptor), Hs_GAPDH_1_SG QT00079247 (GAPDH), Hs_RPL13A_1_SG QT00089915 (RPL13a). All samples were determined at least in duplicates and controls without templates were included. PCR reaction protocol: 15 min at 95°C, 40 cycles of 15 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C. Relative transcription was calculated using the $2^{-\Delta\Delta C_t}$ method with Ct-values normalized on the endogenous control genes GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and RPL13a (ribosomal protein L13a).

2.8 Quantification of AMP, ADP and ATP

The cellular concentration of AMP, ADP and ATP was quantified in isolated lymphocytes. Anti-coagulated EDTA-blood was diluted with the same amount of PBS and transferred into ficoll-paque filled Leucosep®-tubes (Greiner Bio-One, Germany). After centrifugation (1,000xg, 10 min, RT), the lymphocyte fraction was aspirated and transferred into washing medium (RPMI-1640 medium supplemented with 10 % FBS, 2 mM L-glutamine and 1 % penicillin/ streptomycin). Samples were re-centrifuged (250xg, RT, 10 min), the supernatant discarded and the washing process repeated twice. Immediately after isolation, ice-cold 0.3 M perchloric acid was added to the lymphocytes and cells were re-suspended. The cell lysate was centrifuged (16,000xg, 10 min, 4 °C) and the supernatant adjusted to pH 6 with sodium carbonate (1 M). Quantification of AMP, ADP and ATP in the lymphocyte extracts was carried out by HPLC-DAD according to a method described previously (Riedel et al., 2012). The total adenosine nucleotides (TAN) were calculated from the cellular AMP, ADP and ATP concentrations by $TAN [\mu\text{mol/L extract}] = AMP [\mu\text{mol/L extract}] + ADP [\mu\text{mol/L extract}] + ATP [\mu\text{mol/L extract}]$. Data of AMP, ADP and ATP are given as percentage of TAN. To avoid confounding by high inter-individual variations in lymphocyte counts, AMP, ADP and ATP concentrations analyzed in the lymphocyte extract of each study subject were adjusted to the total concentration of adenosine nucleotides. The energy charge potential (ECP) was estimated by $ECP = (ATP [\mu\text{mol/L extract}] + 0.5 ADP [\mu\text{mol/L extract}]) / TAN [\mu\text{mol/L extract}]$ (Pradet & Raymond, 1983).

2.9 Uncoupling protein-2 assay

Uncoupling protein-2 (UCP-2) concentrations were determined in lymphocytes isolated as described in the previous section (Quantification of AMP, ADP and ATP). Lymphocytes were re-suspended in ice-cold PBS and frozen immediately. For analysis, lymphocyte extracts were treated with ultrasonification, and sonicates were centrifuged (16,000xg, 10 min, 4 °C). Supernatants were used for quantification of UCP-2 by means of a commercial sandwich ELISA Kit (USCN Life Sciences, China). UCP-2 contents were normalized for protein contents of the supernatants applying the bicinchoninic acid assay using bovine serum albumin as standard (Pierce® BCA Protein Assay Kit, Thermo Scientific, Austria).

2.10 Adenosine deaminase activity and adenosine concentrations

Plasma adenosine deaminase (ADA) activity was measured using a commercially available assay kit (Diazyme Laboratories, USA). Quantification of adenosine in blood samples was performed by HPLC-ESI-MS/MS with slight modifications of the method described by Doležalová *et al.* (Dolezalova, Krijt, Chladek, Nemcova, & Hoza, 2005). Blood samples were deproteinized immediately after collection with an equal volume of 1 M perchloric acid, to prevent potential enzymatic degradation or production of adenosine. Samples were cooled in ice-water and centrifuged (4,000xg, 5 min, 4 °C). The clear supernatant was transferred to a test tube and the internal standard containing 1.6 µM [1',2',3',4',5'-¹³C₅]-adenosine (Omicron, USA) was added. The solution was neutralized by addition of a buffer solution (2.5 M K₃PO₄, 1.3 M KOH). After a short centrifugation step (4,000xg, 5 sec) the potassium perchlorate precipitate was removed and the supernatant was diluted with distilled water and loaded on a preconditioned solid phase extraction column (StrataX 33u Polymeric Reversed Phase, 30 mg/ mL, Phenomenex, Germany). After a washing step with distilled water, adenosine was eluted with methanol. The eluate was concentrated to a volume of about 100 µL using a vacuum centrifuge and diluted with 0.02 % acetic acid (1:4). Aliquots were analyzed using HPLC-ESI-MS/MS. The chromatographic separation of adenosine was accomplished with an Aqua C18 column (250 × 4.6 mm, 5 µ, Phenomenex, Germany) using an isocratic solvent system of 0.1% formic acid in water (v / v) and methanol (85:15; v / v) at a flow rate of 0.5 mL/ min. Analyte and internal standard were detected by means of a triple-quadrupole tandem mass spectrometer API 3200™ (Applied Biosystems, Germany) with positive electrospray ionization. Adenosine was quantified by determining the ratio of the adenosine peak area and the isotope-labeled standard peak area.

2.11 cAMP concentration and PDE activity in platelets

Intracellular cAMP was determined with the Perkin Elmer's LANCE® Kit (Massachusetts, USA.) following the manufacturer's protocol. For PDE-activity assays, platelets were re-suspended in Tris buffer (50 mM Tris/HCl, pH 7.4, 10 mM MgCl₂, 0.1 mM EDTA, 5 mM benzamidine, 0.5 mM β-mercaptoethanol and a protease inhibitor mix) and lysed in a Wheaton glass tissue grinder („tight“ Pistill, 40 strokes). After centrifugation (100,000xg, 4 °C, 40 min) the supernatant was removed and directly subjected to the PDE assay. PDE activity was determined according to a modified method by Poch *et al.* (Poch,

1971). Briefly, the PDE-containing preparations were incubated at 37 °C with the cAMP[³H] in a buffer solution (30 mM Tris/HCl, pH 7.4, 9 mM MgCl₂, 3 mM 5'-AMP and 3 μM cAMP). The incubation period was adjusted by performing a pre-test applying different incubation times since the cAMP turnover should not exceed about 20% ensuring steady state enzyme kinetics. The enzyme reaction was stopped with ZnSO₄ and 5'-AMP[³H] was precipitated by adding Ba(OH)₂. Tubes were centrifuged (20,000xg, 4°C, 5 min) and the supernatant containing the non-hydrolysed cAMP[³H] subjected to liquid scintillation counting. PDE3 and PDE4 isoenzyme activity were typed by using specific inhibitors (10 μM milrinone for PDE3 and 50 μM rolipram for PDE4).

2.12 Statistical analysis

Statistical analyses were carried out using the software R 2.13.1 (GNU General Public License) and SigmaPlot software (Systat Software, Inc., USA). All statistical hypotheses were tested against two-sided alternatives at the 5% level of significance. The statistical model that corresponds to the chosen cross-over design includes effects of group, treatment and time, as well as interaction of treatment and time and carry-over effect. Details of the statistical model and the appropriate statistical analysis methods are described by Jones and Kenward (Jones & Kenward, 1988). Prior application of t-Tests, the assumption of normality was verified by the Shapiro-Wilk test. In case of rejection of the normality assumption, data were transformed logarithmically. If normality could not be achieved by the transformation, data were analyzed by Wilcoxon tests. In the presence of significant interactions or carry over effects, the data of the second intervention period were excluded from calculation of treatment effects. Effectiveness of both coffees was compared by two sample t-Test (or Wilcoxon's rank sum test). To compare data before and after consumption of either market blend (MB) or study blend (SB) coffee one-sample t-Tests (or Wilcoxon's signed rank test) on the basis of intra-individual differences between measurements after and before coffee consumption were performed. The statistical model allows estimation of product effect differences for the comparison of the two coffee blends, although the effects of single coffee blends cannot be distinguished from time effects in the cross-over design. Nevertheless, time effects which can be calculated between wash-out periods and intervention periods, respectively, may serve as indicators for seasonal influences. The number of volunteers was n = 43 in group A and n = 41 in group B, unless stated otherwise. All biochemical parameters were analyzed on the basis of the mean of 2 - 3 technical replicates. Data are either presented as means ± standard deviation (SD) for normally distributed data, or

otherwise as medians (interquartile range). Spearman correlation coefficients were calculated based on the changes of the first wash-out period to the first intervention period. Significant correlations were determined by means of t-Tests.

3 Results

3.1 Plasma concentrations of methylxanthines and 1,7-dimethyluric acid

Results of quantified methylxanthines and 1,7-dimethyluric acid in the plasma of healthy volunteers are represented in Table 2. The statistical analyses include both intervention periods of the measured methylxanthines, except for caffeine for which a significant carry-over effect was calculated ($p < 0.05$). Caffeine, theophylline, paraxanthine, 2-methylxanthine, 7-methylxanthine and 1,7-dimethyluric acid were below the limit of detection in the plasma of all volunteer after the wash-out periods (Lang et al., 2013). However, theobromine was detectable in plasma samples after the wash-out periods. Following each of the 4 week intervention with MB and SB coffee, plasma concentrations of all methylxanthines increased as compared to the preceding wash-out period ($p < 0.001$).

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Table 2. Methylxanthines quantified in the plasma of healthy volunteers after different interventions

	Group	WO	Market blend	MB vs WO <i>p</i> -value	WO	Study blend	SB vs WO <i>p</i> -value	MB vs SB <i>p</i> -value	Inter-action <i>p</i> -value	Carry-over <i>p</i> -value
Caffeine [μM]	A	0	19.5 (8.93-26.4)	<0.001	-	-	<0.001	ns	ns	0.010
	B	-	-		0	11.8 (7.02-23.2)				
Theo- bromine [μM]	A	2.46 ± 2.86	6.11 ± 5.85	<0.001	2.20 ± 2.20	4.28 ± 2.39	<0.001	ns	ns	ns
	B	2.20 ± 2.32	3.22 ± 2.00		2.81 ± 3.28	5.54 ± 4.50				
Theo- phylline [μM]	A	0	2.57 (1.40-3.26)	<0.001	0	2.27 (1.40-3.25)	<0.001	ns	ns	ns
	B	0	1.81 (1.32-2.95)		0	2.31 (1.39-3.64)				
Para- xanthine [μM]	A	0	11.7 (8.00-15.4)	<0.001	0	10.8 (7.42-16.3)	<0.001	0.032	ns	ns
	B	0	9.16 (6.21-12.5)		0	12.2 (7.79-15.9)				
3-MX [μM]	A	0	0 (0-0.36)	<0.001	0	0.24 (0-0.42)	<0.001	ns	ns	ns
	B	0	0 (0-0.43)		0 (0-0.04)	0 (0-0.62)				
7-MX [μM]	A	0 (0-0.20)	0.34 (0.17-0.64)	<0.001	0 (0-0.34)	0.45 (0.30-0.64)	<0.001	ns	ns	ns
	B	0 (0-0.40)	0.42 (0.30-0.59)		0 (0-0.41)	0.44 (0.23-0.75)				
1,7- DMUA [μM]	A	0	0 (0-0.19)	<0.001	0	0.33 (0-0.59)	<0.001	ns	ns	ns
	B	0	0.28 (0-0.38)		0	0 (0-0.20)				

Values are displayed as mean ± SD or median (interquartile range) of group A (n = 43) and group B (n = 41). WO - wash-out; MB - market blend coffee; SB - study blend coffee

3.2 Blood lipid profile, plasma adiponectin and leptin concentrations

Plasma free fatty acid concentrations of the study subjects are shown in Figure 1A. Due to significant interaction effects ($p < 0.05$), the second cross-over period was excluded from statistical analyses. During the first intervention period, plasma free fatty acid levels were increased after administration of MB coffee and SB coffee in comparison to the preceding wash-out period by $140 \pm 187 \mu\text{M}$ ($p < 0.001$) and $178 \pm 160 \mu\text{M}$ ($p < 0.001$), respectively. No difference in the effectiveness was observed since both coffee interventions increased free fatty acid levels to a similar extent. However, as a significant time effect ($p < 0.05$) within the treatment periods was calculated, the increase in plasma free fatty acids cannot exclusively be deduced to the administration of either MB or SB coffee.

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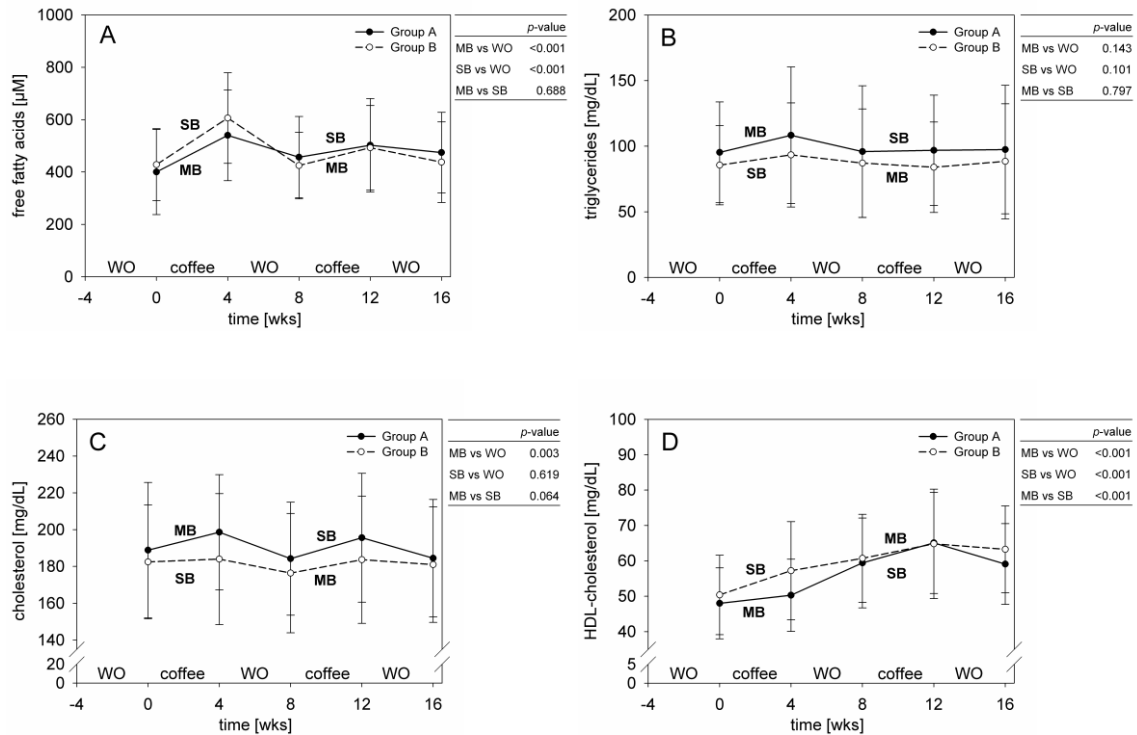


Figure 1. (A) Plasma free fatty acid, (B) serum triglyceride, (C) serum cholesterol and (D) serum HDL-cholesterol concentrations of healthy volunteers during the intervention study (WO - wash-out; MB - market blend coffee; SB - study blend coffee). Data are displayed as mean $\pm$ SD (group A: $n = 43$; group B: $n = 41$). For statistical analyses of plasma free fatty acids the second cross-over period was excluded due to significant interaction ($p < 0.05$). Significances between consumption of either MB or SB coffee and wash-out periods were tested by one-sample t-Test either of the original data or after logarithmical transformation of data sets. Treatment comparison was conducted by two-sample t-Test of normally distributed data sets either of the original data or logarithmically transformed data. Differences between treatments were considered to be significant at $p < 0.05$.

The statistical analysis of triglycerides, HDL-cholesterol and LDL-cholesterol included data from both intervention periods of the cross-over study, whereas data from the second intervention period were excluded for total cholesterol due to a significant carry-over effect ($p < 0.05$). Triglyceride levels of the volunteers remained unchanged during both interventions (Fig. 1B) and total cholesterol increased only after consumption of the MB coffee (Fig. 1C). In contrast, after the 4-week consumption of both coffees serum HDL-cholesterol levels were found enhanced when compared to the preceding wash-out periods (Fig. 1D). Administration of SB coffee increased HDL-cholesterol serum concentrations by 6.24 ± 7.78 mg/dL ($p < 0.001$), whereas administration of MB coffee resulted in an increase of 3.17 ± 6.93 mg/dL ($p < 0.001$). This rise in serum HDL-

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cholesterol was more pronounced for the SB coffee ($p < 0.001$). However, none of the administered coffees affected serum LDL-cholesterol concentrations ($p > 0.05$) (data not shown).

Table 3 gives the concentrations of the adipose tissue-derived hormones adiponectin and leptin. There were no differences in adiponectin plasma concentrations after consumption of either MB or SB coffee compared to the preceding wash-out periods. However, treatment comparison revealed a significant difference for both coffees affecting plasma adiponectin ($p < 0.01$). Volunteers showed higher plasma adiponectin concentrations after consumption of the MB coffee than after consumption of the SB coffee. Statistical analysis for the impact of coffee consumption on plasma leptin concentrations excluded the second intervention period, since significant interaction effects were calculated ($p < 0.05$). Consumption of the MB coffee resulted in higher ($p < 0.01$) median leptin plasma concentration compared to the preceding wash-out period (Table 3). However, significant time effects ($p < 0.05$) were observed. Therefore the increase in leptin cannot exclusively be assigned to the consumption of MB coffee. In contrast, administration of the SB coffee did not affect leptin levels (Table 3). Changes induced by consumption of the MB coffee significantly differed from those demonstrated for the SB coffee.

Table 3. Plasma adiponectin and leptin concentrations as well as relative leptin receptor expression in healthy volunteers

	Group	WO	Market blend	MB vs WO <i>p</i> -value	WO	Study blend	SB vs WO <i>p</i> -value	MB vs SB <i>p</i> -value	Inter-action <i>p</i> -value	Carry-over <i>p</i> -value
Adiponectin [μg/mL]	A	10.5 ± 6.06	10.5 ± 5.06	ns	9.19 ± 6.17	9.20 ± 4.63	ns	0.003	ns	ns
	B	8.59 ± 3.96	8.87 ± 3.92		9.33 ± 4.41	8.35 ± 4.41				
Leptin [ng/mL]	A	2.26 (0.84-4.69)	3.56 (1.88-7.12)	0.002	-	-	ns	0.032	0.001	ns
	B	-	-		3.56 (1.07-5.18)	2.89 (0.90-6.46)				
LEPR delta cT	A	8.72 (8.20-9.39)	8.93 (8.24-9.23)	ns	8.92 (8.41-9.18)	8.82 (8.36-9.35)	0.043	ns	ns	ns
	B	9.05 (8.39-9.42)	8.71 (8.25-9.14)		8.42 (8.04-9.06)	9.05 (8.46-9.72)				
LEPR relative transcription	A	1 (0.57-1.24)	0.85 (0.72-1.19)		0.93 (0.72-1.29)	1.02 (0.70-1.40)				
	B	0.85 (0.57-1.24)	0.86 (0.70-1.46)		1 (0.58-1.18)	0.83 (0.58-1.18)				

Values are displayed as mean ± SD or median (interquartile range) of group A (n = 43) and group B (n = 41). LEPR was normalized to GAPDH and RPL13a and statistical analysis was based on the delta cT values. WO - wash-out; MB - market blend coffee; SB - study blend coffee

Effects of coffee consumption on leptin receptor transcription in lymphocytes were analyzed by means of delta Ct values and revealed decreased expression after administration of the SB coffee solely (Table 3). Consumption of the MB coffee had no impact on leptin receptor expression and no difference in the effectiveness of the coffees was observed (Table 3). Moreover, time effects ($p < 0.05$) might have had an impact on leptin receptor expression alterations.

3.3 Plasma glucose, serum insulin, HOMA-IR and plasma osteopontin

Plasma glucose concentrations showed significant interaction effects ($p < 0.05$) within the cross-over study, resulting in the exclusion of the second intervention period for treatment comparisons. Neither administration of MB nor SB coffee had an impact on glucose concentrations in the first intervention period (Fig. 2A). The second intervention period was also excluded from the statistical analysis for the parameters insulin, HOMA-IR and osteopontin due to carry-over effects ($p < 0.05$). The 4-week administration of the MB coffee but not the SB coffee increased insulin concentrations in comparison to the preceding wash-out period, resulting in a discriminative effect of both coffees (Fig. 2B). The calculated HOMA-IR augmented by 0.20 ± 0.77 ($p < 0.05$) after MB coffee intervention but remained unchanged after consumption of the SB coffee (Fig. 2C). Hence, consumption of the MB coffee effectively increased the HOMA-IR values in comparison consumption of the SB coffee ($p < 0.05$). The MB and SB coffee also evoked discriminative effects on plasma osteopontin levels ($p < 0.001$) (Fig. 2D). While intervention with the MB coffee reduced osteopontin concentrations by 7.81 ± 21.8 ng/mL ($p < 0.05$), administration of the SB coffee elevated osteopontin concentrations by 16.9 ± 30.7 ng/mL ($p < 0.001$).

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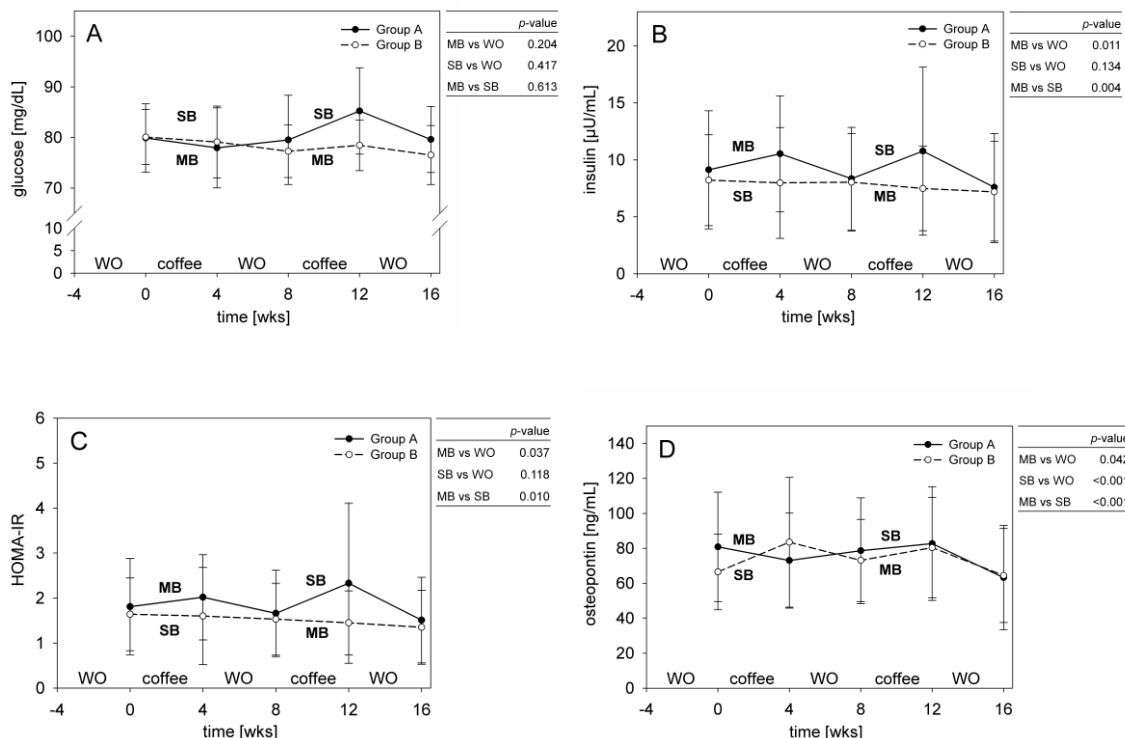


Figure 2. (A) Plasma glucose, (B) serum insulin, HOMA-IR (C) and plasma osteopontin (D) concentrations of healthy volunteers during the intervention study (WO - wash-out; MB - market blend coffee; SB - study blend coffee). Data are displayed as mean $\pm$ SD (group A: $n = 43$; group B: $n = 41$). For statistical analyses the second cross-over period was excluded for insulin, HOMA-IR and osteopontin due to significant carry-over effects ($p < 0.05$) and glucose due to significant interaction ($p < 0.05$). Significances between consumption of either MB or SB coffee and wash-out periods were tested by one-sample t-Test either of the original data or after logarithmical transformation of data sets. Treatment comparison was conducted by two-sample t-Test of normally distributed data sets either of the original data or logarithmically transformed data. Differences between treatments were considered to be significant at $p < 0.05$.

3.4 Plasma adenosine and ADA activity, and platelet cAMP concentrations and PDE activity

Subjects' adenosine concentrations determined in a subpopulation ($n = 20$) were not altered after consumption of the MB coffee, but increased about $+4.63 \pm 6.16$ nM after consumption of the SB coffee when compared to the preceding wash-out periods ($p < 0.01$) (Table 4). No differences between coffee effects could be determined by group comparison. In contrast, changes in ADA activities were higher after SB than after MB coffee consumption ($p < 0.05$) (Table 4). Regarding only the first intervention period, cAMP concentrations slightly increased. However, significant time effects ($p < 0.05$) were

calculated and an unexpected decrease after the second wash-out period was observed (Table 4). A significant carry-over effect was calculated for PDE activity. Therefore, the second intervention period was excluded from the statistical analysis. After the first intervention period, a similar marked decline by -32.2 ± 12.6 pmol cAMP(H3)/min*mg protein and -34.8 ± 10.3 pmol cAMP(H3)/min*mg protein in PDE activity ($p < 0.001$) after consumption MB and SB coffee, respectively, was demonstrated (Table 4).

However, significant time effects within wash-out periods ($p < 0.05$) and within treatment periods ($p < 0.05$) have to be taken into consideration for adenosine, ADA activity and PDE activity, that may have contributed to the changes observed after administration of MB and SB coffee.

Table 4. Plasma adenosine and adenosine deaminase activity as well as cAMP concentrations and phosphodiesterase activity in platelets of healthy volunteers

	Group	WO	Market blend	MB vs WO <i>p</i> -value	WO	Study blend	SB vs WO <i>p</i> -value	MB vs SB <i>p</i> -value	Inter-action <i>p</i> -value	Carry-over <i>p</i> -value
Adenosine ^a [nM]	A	15.0 ± 8.52	17.8 ± 3.48	ns	15.3 ± 3.19	16.8 ± 3.78	0.004	ns	ns	ns
	B	18.5 ± 2.52	18.7 ± 2.44		12.4 ± 3.78	21.2 ± 2.24				
ADA activity [U/L]	A	8.14 ± 2.90	8.23 ± 6.06	ns	6.35 ± 1.85	7.10 ± 3.59	ns	0.022	ns	ns
	B	5.77 ± 2.50	5.97 ± 1.69		7.83 ± 1.89	8.61 ± 2.77				
cAMP [nmol/mg protein]	A	3.71 ± 0.96	4.90 ± 1.37	<0.001	9.11 ± 2.65	2.81 ± 0.83	<0.001	0.002	ns	ns
	B	9.09 ± 2.83	1.71 ± 0.88		3.01 ± 0.87	4.03 ± 1.71				
PDE activity [pmol cAMP (3H)/min*mg protein]	A	47.0 ± 10.7	14.8 ± 7.13	<0.001	-	-	<0.001	ns	ns	<0.001
	B	-	-		44.0 ± 10.4	9.14 ± 4.48				

^a Determination in a subpopulation of group A (n = 11) and group B (n = 9)

Values are mean ± SD of group A (n = 43) and group B (n = 41). WO - wash-out; MB - market blend coffee; SB - study blend coffee

3.5 Adenosine nucleotides and energy charge potential in lymphocytes

Table 5 shows the proportion of adenosine nucleotides AMP, ADP and ATP quantified in isolated lymphocytes. As significant interaction or carry-over effects ($p < 0.05$) were calculated, the second intervention period was excluded from statistical analysis for the adenosine nucleotides. The cellular AMP/TAN ratio was not affected by administration of

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the MB coffee, but increased after consumption of the SB coffee in comparison to the preceding wash-out period. Thereby, 2.00 ± 4.65 of TAN were shifted to AMP. This change induced by the SB coffee was significantly different ($p < 0.05$) from that of the MB coffee. A decrease in ADP/TAN ratios compared to the preceding wash-out periods was observed after the 4-week consumption of both coffees, although a treatment comparison revealed that the decline was even more pronounced after consumption of the SB coffee ($p < 0.001$). Administration of MB coffee led to a $-3.64 \pm 5.56\%$ reduction of the ADP/TAN ratio, whereas administration of SB coffee resulted in a $-9.75 \pm 5.88\%$ decreased ADP/TAN ratio. In contrast, the ATP/TAN ratio was elevated in isolated lymphocytes by $+3.66 \pm 5.81\%$ and $+7.76 \pm 4.98\%$ after consumption of the MB and SB coffee, respectively, showing a clear shift in the cellular adenosine nucleotide distribution towards ATP. A comparison of the changes induced by both coffees revealed the consumption of the SB coffee to be more effective in increasing the ATP/TAN ratio ($p < 0.001$). The calculated ECP values as indicators of the cellular energy status were increased after consumption of both coffees, although no differences ($p > 0.05$) in the effectiveness could be demonstrated (Table 5). However, significant time effects within the treatment periods ($p < 0.05$) for ADP/TAN, ATP/TAN and ECP have to be considered.

Table 5. Cellular AMP, ADP and ATP content normalized to total adenosine nucleotides (TAN), ECP values and UCP-2 content quantified in isolated human lymphocytes from healthy volunteers after different interventions

	Group	WO	Market blend	MB vs WO <i>p</i> -value	WO	Study blend	SB vs WO <i>p</i> -value	MB vs SB <i>p</i> -value	Inter- action <i>p</i> -value	Carry- over <i>p</i> -value
AMP/ TAN[%]	A	7.19 ± 2.54	7.17 ± 2.14	ns	-	-	0.003	0.036	0.020	0.002
	B	-	-		7.84 ± 3.05	9.84 ± 3.34				
ADP/ TAN [%]	A	29.7 ± 5.50	26.1 ± 3.64	<0.001	-	-	<0.001	<0.001	<0.001	ns
	B	-	-		34.2 ± 5.06	24.5 ± 4.33				
ATP/ TAN [%]	A	63.1 ± 4.92	66.8 ± 4.46	<0.001	-	-	<0.001	<0.001	<0.001	ns
	B	-	-		57.9 ± 3.31	65.7 ± 4.95				
ECP	A	0.78 (0.77-0.79)	0.80 (0.78-0.82)	0.002	-	-	<0.001	ns	<0.001	0.018
	B	-	-		0.75 (0.74-0.76)	0.78 (0.77-0.80)				
UCP-2 ^a [pg/mg protein]	A	136 ± 81.1	104 ± 47.1	ns	138 ± 51.1	281 ± 115	0.003	ns	ns	ns
	B	181 ± 124	236 ± 79.5		125 ± 43.3	100 ± 53.3				

Values are mean $\pm$ SD or median (interquartile range) of group A ($n = 43$) and group B ($n = 40$) and ^a group A ($n = 42$) and group B ($n = 38$). WO - wash-out; MB - market blend coffee; SB - study blend coffee

The protein content of the uncoupling protein-2 in isolated lymphocytes (Table 5) increased after the SB coffee intervention when compared to the preceding wash-out period, and resulted in an average increase by 63.3 ± 122 pg/mg protein ($p < 0.01$). In contrast, consumption of the MB coffee had no impact on the UCP-2 content. As time effects within treatment periods ($p < 0.05$) were calculated, significant differences between coffee intervention and wash-out period cannot exclusively be deduced to the consumption of the SB coffee.

3.6 Correlation analyses

Spearman correlation coefficients were calculated between parameters related to energy metabolism and body fat. A significant correlation ($p < 0.05$) was calculated for plasma concentrations of FFAs and the ATP/TAN ratio ($\rho = 0.253$) (Fig. 3). No correlation was found for plasma glucose and ATP/TAN ($\rho = 0.07$, $p > 0.05$). In male volunteers negative correlations between body fat and ATP/TAN ($\rho = -0.34$, $p < 0.05$) as well as body fat and ECP ($\rho = -0.31$, $p < 0.05$) were ascertained.

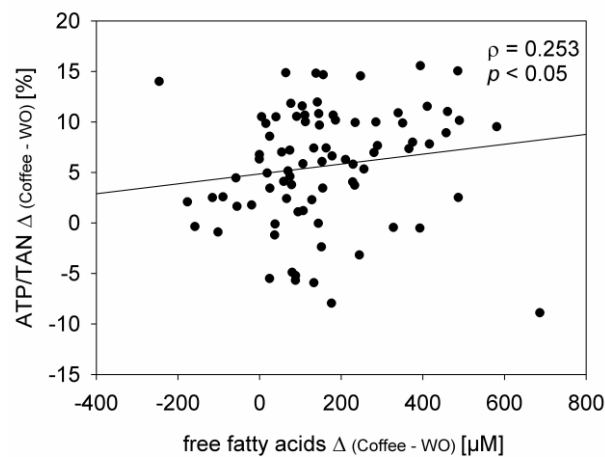


Figure 3. Correlation of FFA and ATP/TAN. Spearman correlation coefficients were calculated from the differences between the values after the first coffee intervention period and the first wash-out period ($n = 84$). Correlations were considered to be significant at $p < 0.05$.

4 Discussion

This human intervention study investigated the effect of a 4-week coffee consumption on parameters of lipid, glucose and energy metabolism in healthy subjects. The effects of two regular coffee beverages differing in their concentrations of chlorogenic acid (CGA), trigonelline and N-methylpyridinium (NMP) were studied. So far, no controlled cross-over human intervention study on 4-week effects of coffee consumption with discriminative analysis of two administered coffees has been conducted. The compositional differences of the coffee beverages were obtained by either blending five commercially available regular ground coffees (market blend) or blending mainly dark-roast coffee with significant proportions of light roast coffee (study blend). Both coffees contained comparable amounts of caffeine. Hence, concentrations of caffeine metabolites (methylxanthines) equally increased in the plasma of the volunteers after both coffee interventions. At the end of the wash-out periods, however, theobromine and its metabolites, 3-methylxanthine and 7-methylxanthine, were detected in the plasma of the study subjects, in spite of the caffeine-abstinent diet in the preceding wash-out period. This result might reflect the consumption of theobromine-containing foods. Study subjects were asked to abstain from caffeine-containing beverages like cola, tea (black, green and white), malt coffee and dark chocolate during the study, whereas milk chocolate was allowed to be consumed. Thus, milk chocolate intake might explain baseline theobromine concentrations demonstrated after the wash-out periods. Coffee consumption during the wash-out periods was excluded since no increase in urine concentrations of NMP, as specific indicator of coffee consumption, was seen (Bakuradze et al., 2014; Lang, Wahl, Stark, & Hofmann, 2011). Following the coffee intervention periods, not only methylxanthines, but also NMP concentrations increased, clearly indicating compliance of the study subjects (Bakuradze et al., 2014).

In the present study, coffee intake induced alterations in body composition and nutrient intake as described in detail by Bakuradze et al. (Bakuradze et al., 2014). In brief, body weight was not changed after 4-weeks of MB consumption, but decreased slightly by SB intervention. No difference between the effectiveness of both coffees was observed. In contrast, body fat was reduced after consumption of both coffees compared to the preceding wash-out periods, however more pronounced after SB coffee intervention. Total energy, protein and fat intake were reduced by SB coffee intervention when compared to the wash-out periods but remained unchanged by MB coffee consumption. Treatment comparison showed no difference between the effectiveness of both coffees.

According to our hypothesis, that a 4-week consumption of regular coffee promotes body weight maintenance in humans by mobilization of endogenous energy substrates, plasma glucose and free fatty acid concentrations in the study subjects were analyzed.

At the end of each intervention period, plasma free fatty acid concentrations of overnight-fasted volunteers were elevated compared to the preceding wash-out period, independent of the type of coffee consumed. This result is in accordance with data on acute effects of coffee consumption on plasma free fatty acids published by Bellet *et al.* reporting plasma free fatty acids to be increased by 94% in healthy volunteers 4 hours after consumption of 500 mL caffeinated coffee containing 250 mg caffeine whereas free fatty acid concentrations after consumption of decaffeinated coffee were not affected (Bellet, Kershbaum, & Finck, 1968). These observations in combination with our results support the assumption that caffeine might be one of the coffee constituents that stimulates the utilization of free fatty acids as endogenous energy substrates. We suggest that caffeine may increase glycerol degradation and lipolysis, as reported for 3T3-L1 adipocytes in culture (Murosaki *et al.*, 2007).

We also observed changes in the cholesterol profile of healthy volunteers after consumption of the SB and the MB coffee. Serum total cholesterol and HDL-cholesterol concentrations increased after the 4-week consumption of both coffees. In contrast, LDL-cholesterol remained unaffected. We here demonstrate for the first time a positive, enhancing long-term effect of coffee consumption on HDL-cholesterol. The SB coffee, containing higher amounts of NMP but lower concentrations of CGA and trigonelline, was more effective in increasing serum HDL-cholesterol than the MB coffee, suggesting the beneficial effect on serum lipid profile to depend on the coffee constituent profile.

Cheung *et al.* (Cheung, Gupta, & Ito, 2005) described a short-term increase, one hour after coffee consumption (without milk or sugar), in total cholesterol and HDL-cholesterol in humans. In contrast, Superko *et al.* (Superko, Bortz, Williams, Albers, & Wood, 1991) found no effect on total cholesterol and HDL-cholesterol after an 8-week coffee consumption period. In that study, plasma LDL-cholesterol concentrations were raised, but only after consumption of decaffeinated coffee. Moreover, a cross-sectional study demonstrated a positive association between coffee consumption and total cholesterol as well as LDL-cholesterol, and an inverse association with triglycerides by recall of dietary habits over one month (Yamashita *et al.*, 2012). Taken together, most studies report an increase in total cholesterol after coffee consumption and suggest the diterpenes kahweol and cafestol to be the cholesterol-elevating factors in coffee (Urgert

& Katan, 1997). Filtering coffee removes these diterpenes which may be of relevance for the varying effects observed in the studies mentioned above (Urgert et al., 1996).

Regarding the impact of coffee consumption on the regulation of lipid metabolism, the adipocyte-derived hormones adiponectin and leptin play a central role in glucose and lipid metabolism (Rosen & Spiegelman, 2006). In the present study, administration of MB coffee resulted in higher plasma adiponectin levels compared to administration of SB coffee. A positive association between coffee consumption and adiponectin concentrations has also been reported in cross-sectional studies (Imatoh et al., 2011; Yamashita et al., 2012). In an animal model, chlorogenic acid has been shown to increase adiponectin levels when administered as a supplement with a high-fat diet (Cho et al., 2010). Whether the higher contents of chlorogenic acid of the MB coffee studied in this work contributed to its more pronounced effect on plasma adiponectin levels cannot be answered unequivocally. Although an association might be possible, plasma concentrations of chlorogenic acid metabolites did not differ among the volunteers following MB vs. SB treatments (Winkler et al., 2014).

Moreover, in the present study consumption of the MB coffee elevated leptin concentrations, while consumption of the SB coffee did not. Leptin release from the adipose-tissue is known to stimulate catabolic pathways by activating the leptin receptor in the hypothalamus, especially in the arcuate nucleus, inducing the repression of orexigenic and concomitantly stimulating anorexigenic pathways (Rosen & Spiegelman, 2006). In peripheral tissues, adiponectin and leptin alter the metabolic state by activating the AMP-activated protein kinase thereby stimulating mitochondrial activity and fatty acid oxidation (Carling, 2004). Moreover, expression of the transcription factor peroxisome proliferator-activated receptor- α that promotes fatty acid oxidation, was only elevated in the lymphocytes of the subjects after consumption of the MB coffee in our study providing additional evidence for the MB coffee to modify lipid metabolism (Winkler et al., 2014).

Parameters of glucose metabolism were hardly affected by a 4-week coffee consumption. Insulin concentrations were only increased after consumption of the MB coffee, while no changes were observed after SB coffee intake. This suggests that SB coffee may have provided compounds counteracting the reducing effect on insulin-sensitivity reported for caffeinated coffee (van Dam, Pasman, & Verhoef, 2004). However, fasting plasma glucose levels remained unchanged after consumption of both coffees. We further estimated plasma concentrations of the pro-inflammatory cytokine osteopontin that is also associated with insulin resistance. Osteopontin concentrations

were found increased upon obesity in humans (Gomez-Ambrosi et al., 2007) and neutralization of its action led to improved insulin sensitivity in obese mice (Kiefer et al., 2010). While no investigations have focused on the impact of coffee consumption on osteopontin concentrations in humans, we provide a first hint to coffee-derived modifications in its plasma levels. While the 4 week consumption of the MB coffee reduced osteopontin concentrations, they were elevated after intervention with the dark roast SB coffee suggesting coffee constituents highly present in one or the other coffee that exert opposite effects on osteopontin concentrations.

Regarding these findings in healthy fasted humans, coffee consumption promotes the utilization of free fatty acids and not of glucose as energy substrate. Correlation analyses support this hypothesis, as a significant positive correlation was found between plasma FFAs and ATP/TAN ratio, as marker of the cellular energy status. In contrast, there was no association between glucose and ATP/TAN ratio. Moreover, the negative correlations between body fat and ATP/TAN as well as body fat and ECP ($p = -0.31$, $p < 0.05$) in male volunteers indicate the usage of endogenous body fat for maintenance of energy supply.

In comparison to the wash-out periods, in a fasting state, regular coffee consumption increased cellular energy supply. ECP values rose in lymphocytes after consumption of both coffees, and a shift from ADP/TAN to ATP/TAN ratio was observed. In previously published short-term studies, caffeinated coffee was shown to stimulate energy metabolism and energy expenditure compared to decaffeinated coffee (Acheson et al., 1980; Hollands et al., 1981). Therefore, caffeine is supposed to be one of the active coffee constituents that was also shown to increase the ECP and ATP levels in HepG2 cells in culture (Riedel et al., 2012). In the present study, we were able to demonstrate a shift in the adenosine nucleotide profile in human lymphocytes, which may be used as surrogate biomarker for the cellular energy status after long-term consumption of caffeinated coffee. As both coffees contained comparable amounts of caffeine, and increased the cellular energy charge potential to a similar extent, the increase in energy supply might be attributed primarily to caffeine. The stimulatory effect of caffeinated coffee on ATP production might result from induction of the cAMP signaling pathway. Methylxanthines, such as caffeine, theobromine and theophylline at concentrations higher than 0.1 mM inhibit 3',5'-cyclic nucleotide phosphodiesterase which results in elevated cAMP levels (Butcher & Sutherland, 1962). Enhanced cAMP levels may mediate a continuous energy demand, with the potential consequence of stimulated energy producing pathways. We observed a strong inhibitory effect of caffeinated coffee

on PDE activity in platelets. Additionally, adenosine plasma concentrations were increased after SB coffee consumption, potentially stimulating cAMP production in platelets. Since platelet, cAMP levels increased only in the first intervention period, not in the second, significant time effects precluded the further consideration of the second cross-over period, where elevated cAMP levels were not observed.

In addition, we demonstrated an induction of UCP-2 protein expression in human lymphocytes after SB coffee consumption. Caffeine is commonly accepted as the thermogenic agent in coffee (Astrup et al., 1990). A stimulating effect of caffeine on UCP-2 protein contents (Riedel et al., 2012) and UCP-2 gene expression (Kogure et al., 2002) was found in cell culture (1.5 mM caffeine) and animal models (60 mg caffeine/ kg body weight s.c.) after treatment with concentrations above the physiological range. In healthy subjects, peak plasma concentrations of 40 - 50 μ M are reached after consumption of 3 - 4 cups of coffee (Verhoef, Pasman, Van Vliet, Urgert, & Katan, 2002). Therefore, we assume that other coffee constituents may promote uncoupling protein expression after 4-week consumption of 750 mL coffee in our study. Recently, it was demonstrated that NMP treatment increased UCP-2 protein expression in HepG2 cells in culture (Riedel et al., 2014). Since the SB coffee contained higher amounts of NMP than the MB coffee, our findings support this *in vitro* result and suggest a thermogenic action of NMP-rich coffee in humans.

However, data interpretation of this study is limited due to some confounding factors. The cross-over study was designed to compare the effectiveness of two coffee beverages, differing in their constituent profile, on biomarkers of energy status. Coffee interventions were lacking a water control group. Thus, effects caused by individual coffee brews could only be estimated by comparing data with those of the preceding wash-out period. Here, time effects within wash-out periods and within treatment periods as well as interaction and carry-over effects had to be considered that might have affected the changes observed in some biomarkers. Moreover, consumption of hot coffee during intervention periods, instead of cold water as done during wash-out periods, might have affected outcome measures, since drinking of warm/hot drinks influences physiological responses (Quinlan, Lane, & Aspinall, 1997). In addition, adenosine was quantified only in the plasma of a subpopulation.

In conclusion, 4-week coffee consumption was shown to alter indicators of energy metabolism in healthy normal-weight subjects. Coffee consumption increased free fatty acid utilization and reduced body fat content (Bakuradze et al., 2014). An increased lymphocyte energy charge potential, possibly resulting, at least in part, from reduced

PDE activity might have been resulted in an augmented free fatty acid oxidation. Since both coffee beverages largely evoked similar effects, caffeine is hypothesized to play a pivotal role for those coffee-induced changes in energy metabolism that promote body weight maintenance. However, some biochemical parameters, e.g. HDL-cholesterol, leptin, adiponectin, insulin and osteopontin, were differentially affected by MB and SB coffee consumption. Therefore, we suggest to likewise consider further coffee constituents when evaluating health-beneficial effects of coffee.

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All authors have approved the final article.

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2.5 “Influence of a four week coffee intervention on nutrient intake, body composition, biomarkers associated with satiety and DNA integrity in humans”

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Influence of a four week coffee intervention on nutrient intake, body composition, biomarkers associated with satiety and DNA integrity in humans

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Abbreviations

BIA, Bioelectrical impedance analysis; **BMI**, body-mass index; **CCK**, cholecystokinin; **5-CQA**, 5-caffeoyl quinic acid; **FFM**, fat free mass; **FM**, fat mass; **GIP**, glucose-dependent insulinotropic polypeptide; **GLP-1**, glucagon-like peptide-1, **5-HT**, 5-hydroxytryptamine (serotonin); **MB**, market blend coffee; **NMP**, N-methylpyridinium; **PBMC**, peripheral blood mononuclear cells; **ROS**, reactive oxygen species; **SB**, study blend coffee; **tGSH**, total glutathione; **TBW**, total body water; **WBC**, white blood cells.

Abstract

Recent epidemiological studies suggest that coffee, one of the most widely consumed beverages worldwide, may reduce the risk of degenerative diseases such as diabetes type 2, cardiovascular disease and certain types of cancer. These effects have partly been ascribed to the antioxidant and body weight-reducing capacity of coffee. In a four week double-blind randomized cross-over intervention study with 84 healthy subjects effects associated with coffee-consumption on body weight/composition and food intake and on satiety markers (serotonin, ghrelin) and on DNA integrity were monitored. Brews of two different coffees (study blend, SB, and market blend, MB), both about equal in their contents of caffeine but differing in contents of chlorogenic acids and N-methylpyridinium, were consumed (3 x 250 mL per day). The consumption of both coffees was associated with a decrease in body fat over the whole study period (both $p < 0.001$), more pronounced after SB consumption. During intervention with MB, plasma serotonin increased ($p < 0.001$) whereas plasma ghrelin decreased ($p < 0.001$) in comparison to the preceding wash-out period. Consumption of both coffee brews was associated with DNA protective effects ($p < 0.001$). These findings suggest beneficial health effects associated with consumption of both coffees.

Keywords

Coffee, body fat, food intake, serotonin, ghrelin, DNA protection, human intervention study

1. Introduction

Coffee is a worldwide consumed beverage prepared from the dried and roasted seeds of *Coffea* varieties. Evidence from epidemiology suggests coffee consumption to be associated with the prevention or delay of certain non-communicable diseases, such as diabetes type 2, cardiovascular disease and some types of cancer (Bidel et al., 2010; Zhang et al., 2009). These beneficial health effects have been attributed in part to the adenosine receptor inhibitory effects of caffeine (Muller & Jacobson, 2011), and in part to antioxidative effects of green bean phenolics and chlorogenic acids (CQA) (Bakuradze et al., 2010) or of compounds generated during roasting (Boettler et al., 2011; Somoza et al., 2003). However, only few within the multitude of coffee constituents have been elucidated yet with respect to structure-dependent biological activity (Arab, 2010; Hoelzl et al., 2010).

The antioxidative activity of coffee is ascribed mostly to caffeoylquinic acids (CQA), as well as to certain compounds generated by roasting, including N-methylpyridinium (NMP) and other products of the Maillard reaction (Antony et al., 2000; Borrelli et al., 2002; Del Castillo et al., 2002; Farah et al., 2006; Lang et al., 2008; R. H. Stadler, Varga, Hau, et al., 2002; R. H. Stadler, Varga, Milo, et al., 2002). It has been proposed that certain coffee constituents, due to their ability to scavenge reactive oxygen species may protect the organism against damage mediated by oxidative stress (Bakuradze, et al., 2010).

Moderate decreases in body weight have also been reported to be associated with regular coffee consumption, in conjunction with decreased diabetes risk in normal and overweight population groups (Greenberg et al., 2006; Kempf et al., 2010; Thom, 2007; Tunnicliffe & Shearer, 2008). Consumption of a study coffee rich in both, original constituents and roast products has been found to be associated with moderate reduction of body weight and body fat in healthy subjects (Bakuradze et al., 2011). The reduction of energy and nutrient intake observed in this study suggests coffee constituents to influence the regulation of hunger and satiety in addition to increasing energy expenditure (Bracco et al., 1995; Greenberg, et al., 2006).

Caffeine was reported to induce thermogenesis and lipolysis and to increase metabolic rate in humans (Bracco, et al., 1995; Greenberg, et al., 2006; Tagliabue et al., 1994). However, similar effects on body weight were also observed in studies with decaffeinated coffee, suggesting non-caffeine compounds such as CQA to be of relevance as well (Thom, 2007; Vinson et al., 2012). Constituents of green coffee beans

such as CQAs have been found to inhibit glucose absorption and the activity of hepatic glucose-6-phosphatase, thus limiting the release of glucose, resulting in insulinemia (Hemmerle et al., 1997; Johnston et al., 2003; Thom, 2007). CQAs may also alter the secretion of gut peptides, such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic polypeptide (GIP), both of which are supposed to play important roles in short-term regulation of energy intake (Johnston, et al., 2003; Tunnicliffe & Shearer, 2008). Food intake is subject to a complex regulation involving the gastrointestinal tract, the hypothalamus and signaling networks mediated by various neurotransmitters and hormones (Halford & Harrold, 2012; Lopaschuk et al., 2010; Wren et al., 2000; Wurtman & Wurtman, 1995). In regulation of hunger and satiety serotonin (5-HT) and ghrelin are considered to play an important role. 5-HT is an indolamine also secreted by neuronal cells in the intestine in response to intraluminal stimuli (Burton-Freeman et al., 1999). An increase of 5-HT is considered to entail a reduction of food intake. The hormone ghrelin, secreted by gastric cells, stimulates appetite and food intake in rodents and humans.

In order to elucidate the long-term effects of coffee consumption on energy metabolism of healthy coffee drinkers a 20 week intervention study in cross-over design (four weeks of coffee intervention) was carried out with 84 healthy male ($n = 46$) and female ($n = 38$) volunteers. Aims of the present study were 1.) to investigate coffee-associated effects on body weight/composition, food intake and satiety and 2.) to monitor DNA protective effects associated with coffee consumption. These effects were studied using a study blend coffee (SB) characterized by elevated levels of dark roast coffee constituents and of green bean constituents, such as chlorogenic acids. Effects were compared against those exerted by a market blend coffee (MB), reflecting the average composition of normal household coffee commercially available in Germany.

2. Materials and Methods

2.1 Subjects and study design

This study was approved by the local ethic committee of Rhineland-Palatinate, no. 837.414.10 (7423). All volunteers gave their informed written consent. They were subjected to baseline medical examination (including blood collection for clinical parameters) and anthropometric measurements. Male and female volunteers were recruited ($n=90$) who matched the following inclusion criteria: ages 20-44 y, BMI 19-26

kg/m², healthy, coffee drinkers, non-smokers, no intake of pharmaceutical drugs and/or food supplements, no pregnancy. Six individuals dropped out because of illness or for private reasons. The remaining 84 volunteers (age 25.6 ± 5.8 y, BMI 22.9 ± 1.9 kg/m²) completed the 20 weeks intervention study (time interval: January 18/19th - June 6/7th 2011), designed as follows: study weeks 1-4 (1st wash-out phase), weeks 5-8 (1st coffee phase), weeks 9-12 (2nd wash-out phase), weeks 13-16 (2nd coffee phase) and weeks 17-20 (3rd wash-out phase). The study was a prospective, randomized study with crossover design (Figure 1), whereby the participants were randomly divided into two groups following computer generated number allocation (group A, n= 43, 24♂, 19♀; group B, n=41, 22♂, 19♀). During the first four weeks of coffee intervention, group A consumed MB and group B SB coffee. In the second intervention phase group A received SB and group B MB coffee.

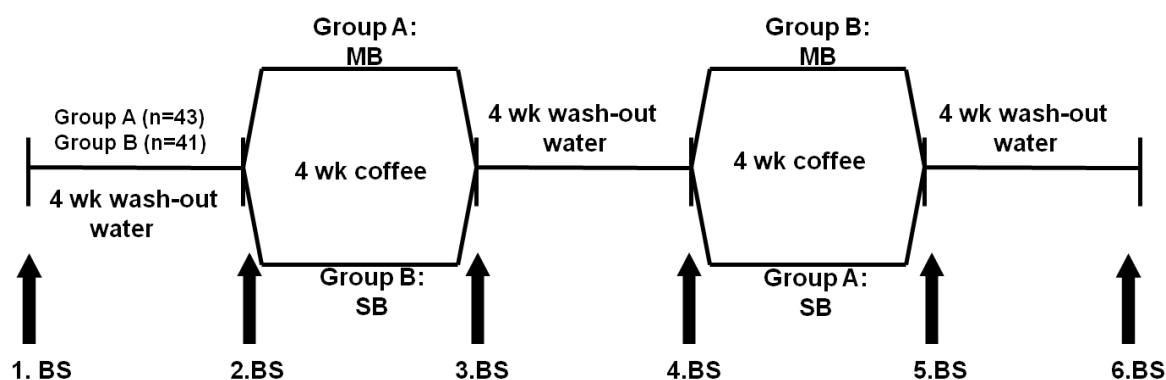


Figure 1 Study design; MB: market blend coffee; SB: study blend coffee; BS: blood sampling.

Coffees were distributed in coded, neutral packages. Thus, the respective coffee type was blinded to volunteers and supervisors. During the intervention periods, volunteers consumed daily 750 mL of black coffee (with/without sugar, no milk) in three equal portions (morning, noontime, afternoon), prepared using commercially available coffee pad machines (6 coffee pads à 7.5 g/day). During the wash-out phases coffee was replaced by similar volumes of water (750 mL) also consumed in three equal portions per day. The volunteers were instructed to keep their usual dietary habits during the study and to avoid the additional intake of coffee, caffeine containing products, dietary supplements and foods rich in polyphenols, such as cocoa and red berries. Furthermore, volunteers were instructed to provide food records of all food items consumed within a seven day period prior to the beginning of the 20 weeks study and within the last week of

each individual study phase to document individual dietary habits and to ascertain their compliance with the instructions.

At the first day of the study and at the last day of each study phase at fasting state in the morning, volunteers collected urine (50 mL) and were asked to empty the bladder prior to anthropometric measurements. Subsequently, body height, and body weight, body mass index (BMI) and body composition were determined. Thereafter, venous blood samples were taken. Additionally, women were asked to perform a pregnancy test.

2.2 Preparation and analysis of the coffee brew studied

The SB coffee was a 100 % blend of Arabica (*Coffea arabica*) roasts, consisting of light roast of, rich in green bean constituents and a major proportion of dark roast, particularly rich in roast products, as evidenced by its higher NMP content. MB coffee was blended from equal portions of five major commercially available regular coffee brands. The latter represented a typical medium roast filter coffee blend. Four of the five coffees proved to be pure Arabicas, the fifth contained some Robusta (*Coffea canephora*), as demonstrated by the presence of significant amounts of 16-*O*-methylcafesitol (Speer & Kölling-Speer, 2006). Both ground coffee blends were portioned into standard coffee soft pads (Tchibo GmbH, Hamburg, Germany) and packed into appropriate plastic bags, under inert gas. The bags were clearly distinguishable (red and silver, respectively) and carried a code. Color and code were not disclosed to volunteers and the staff conducting the intervention study (double blinding). The assignment of the two coffees was disclosed to the scientific staff only after all data associated with the intervention study had been collected. Invariably, coffee brews were freshly prepared immediately before consumption, using a pad machine (125 mL, 1 pad corresponding to 7.5 g coffee).

The coffee samples were analytically characterized using HPLC-DAD and HPLC-MS/MS methods previously described: Caffeine and caffeoylquinic acids (3-, 4-, and 5-CQA) were quantified by HPLC-DAD (324 nm, 272 nm) with external calibration (Weiss et al.). NMP and trigonelline were quantified by stable isotope dilution analysis with synthetic d₃-NMP after sample clean-up via solid phase extraction (SPE) on RP18 (Lang et al., 2012).

2.3 Anthropometric measurements

Body height and weight (digital precision scale, Seca delta 707, Hamburg, Germany) of the volunteers in underwear were measured and the BMI (kg per m²) was calculated. Body composition total body water (TBW), fat mass (FM) and fat free mass (FFM) were monitored in volunteers at horizontal position with empty bladder using three compartment bioelectrical impedance analysis with BIA 101 (SMT medical GmbH, Wuerzburg, Germany). Electrodes were placed at the right hand and foot (dry skin), following the manufacturer's instructions.

2.4 Processing and storage of blood, plasma and urine

Venous blood samples were collected in EDTA containing serum tubes and stored at 37 °C until the individual sampling period was completed. Samples were immediately submitted to the Comet assay procedure (see 2.6). For serotonin determination, whole blood was centrifuged (at 1,800xg at 4 °C /15 min) and plasma aliquots were stored at -80 °C. For determination of ghrelin, a protease inhibitor cocktail (SigmaFast™ protein inhibitor cocktail, Sigma Aldrich, Vienna, Austria) was added to whole blood samples before centrifugation (1,800xg at 4 °C for 15 min) according to the supplier's instructions. The resulting blood plasma was mixed with hydrochloric acid to a final concentration of 0.1 M to stabilize acylghrelin. Aliquots were stored at -80 °C.

In order to isolate peripheral blood mononuclear cells (PBMC) 10 mL of freshly collected human blood was layered on 10 mL of Histopaque-1077 (Sigma Aldrich, Vienna, Austria) and centrifuged at room temperature at 400xg for 20 minutes without brake. Lymphocytes were withdrawn from the cloudy middle layer and transferred into 12 mL RPMI 1640 medium (Invitrogen Life Technologies, Vienna, Austria) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin and tempered at 37°C. This cell suspension was centrifuged for 10 minutes at 250xg, the supernatant discarded and the pellet redissolved in 8 mL RPMI 1640 medium. After centrifugation cells were transferred into 700 µL ice cold PBS and again centrifuged. The pellet was resuspended in 1 mL RNeasy lysis buffer (QIAGEN, Hilden, Germany) and stored at -80°C. Urine samples were immediately frozen at -80°C.

2.5 Determination of plasma serotonin (5-HT) and ghrelin concentrations

Plasma serotonin and ghrelin were determined by means of ELISA kits (serotonin ELISA Kit, DLD Diagnostika GmbH, Germany and ghrelin ELISA Kit, Sceti K.K., Japan), and analyses carried out according to the manufacturer's protocols.

Total RNA was extracted according to the manufacturer's handbook using the RNeasy mini kit (QIAGEN, Hilden, Germany). In order to obtain cDNA out of 1 µg RNA, the Quantitect Reverse Transcription Kit (QIAGEN, Hilden, Germany) was used and quantitative real-time PCR was performed according to the manual of Quantitect, SYBR Green PCR and Quantitect Primer Assays (both QIAGEN, Hilden, Germany). The applied primers were Hs_GHRL_1_SG QT00041377 (ghrelin), Hs_GAPDH_1_SG QT00079247 (GAPDH), Hs_RPL13A_1_SG QT00089915 (RPL13a). All samples were determined at least in duplicates and controls without templates were included. PCR reaction protocol: 15 min at 95°C, 40 cycles of 15 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C. Relative transcription was calculated using the $2^{-\Delta\Delta C_t}$ method with normalised C_t -values on endogenous control genes GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and RPL13a (ribosomal protein L13a).

2.6 Determination of spontaneous and total DNA strand breaks (Comet Assay)

Alkaline single cell gel electrophoresis was performed to determine spontaneous and total (additional treatment with formamidopyrimidine glycosylase, FPG) DNA strand breaks according to Collins et al., 1996 (Collins et al., 1996), with slight modifications, as reported elsewhere (Bakuradze, et al., 2011).

2.7 Determination of NMP and trigonelline in human spot urine with LC-MS/MS

For compliance within the long term intervention study NMP, trigonelline and creatinine were monitored in human spot urine samples by HILIC-HPLC-MS/MS as reported recently (Lang et al., 2011). Briefly, the spot urine sample (10 µl) was mixed with an aliquot (1 ml) of a solution of the internal standards d₃-creatinine (10 µM), d₃-trigonelline (1µM) and d₃-NMP (1µM) in acetonitrile/water (95/5, v/v). After centrifugation, the clear supernatant (1µL) was subjected to HPLC-MS/MS analysis. The concentrations of the analytes NMP and trigonelline were normalized to the individual creatinine concentrations and are given as nmol analyte per µmol creatinine.

2.8 Dietary analysis

Nutrient intake (kcal, carbohydrates, fat and proteins) was assessed on the basis of seven day food records (five intervals, see chapter 2.1), using the nutrition software package PRODI 5 (Nutri-Science GmbH, Hausach, Germany).

2.9 Statistics

Data analysis involved testing for, potential confounding effects associated with group, treatment, and time, interaction and carry-over (Jones & Kenward, 1988). Normality was verified by applying the Shapiro-Wilk test and, if not rejected, *t*-tests were performed to test for significant effects. In case of rejection, data were transformed logarithmically prior *t*-tests. If normality could not be achieved by log transformation, data were analyzed nonparametrically by Wilcoxon tests. In cases of significant interactions or carry over effects, data of the second intervention period were excluded from calculation of treatment related effects. Two sample *t*-test (or Wilcoxon's rank sum test) were employed to compare the effectiveness of both coffees. One-sample *t*-tests (or Wilcoxon's signed rank tests) were applied to compare data before and after consumption of either MB or SB coffee. The statistical model allows for estimation of product-effect differences when comparing the two blends, although the effects of individual coffee blends cannot be distinguished from time effects in the cross-over design.

Spearman correlation coefficients were calculated based on the changes of the first wash-out period to the first intervention period and significance was estimated by means of *t*-tests.

3. Results

In the present study changes in body weight and composition and food intake as well as the influence on biomarkers associated with satiety and with maintenance of DNA integrity were monitored. Additionally, parameters related to metabolism of fat/ fatty acids, and biotransformation of certain coffee constituents, including chlorogenic acids were monitored and effects on energy metabolism determined within this intervention study. These are to be published in accompanying papers (Riedel et al., 2014; Winkler et al., 2014).

3.1 Constituent profiles in the coffee brews

The study coffees were characterized by determination of the key constituents caffeine, NMP, trigonelline and chlorogenic acids (sum of 3-, 4- and 5-caffeoylquinic acid) (Table 1). The content of caffeine did not differ between the two coffees. In contrast, contents of chlorogenic acids, NMP and trigonelline differed, reflecting the differential roasting/blending process. Thus, SB, contained about 50% the chlorogenic acids contents and 54% the trigonelline contents of MB, whereas in SB the NMP content was higher by a factor of three compared to MB.

Table 1 Concentration of selected constituents in the market blend (MB) and study blend (SB).

Compound	MB	SB
	[mg/g]	[mg/g]
Caffeine	12.39 ± 0.1	12.83 ± 0.2
Chlorogenic acids ^{a)}	19.31 ± 0.3	10.01 ± 0.3
N-methylpyridinium (NMP)	0.39 ± 0.0	1.20 ± 0.0
Trigonelline	6.27 ± 0.1	3.42 ± 0.2

Values are expressed as mean ± SD. ^{a)} sum of 3-, 4- and 5-caffeoylquinic acid;

3.2 NMP and trigonelline in urine

Due to their negligible metabolic breakdown and high water solubility, NMP and trigonelline are excreted preferentially and rapidly via the urine. Thus, monitoring urinary excretion provided a valuable short term biomarker for compliance in this human intervention study. Within the wash-out phases NMP as well as trigonelline were practically undetectable in urine (Figure 2). In contrast, all urine samples collected within the intervention periods contained NMP and trigonelline in concentrations indicative for the consumption of the respective brew in the required amount, providing compelling evidence for good compliance (Figure 2).

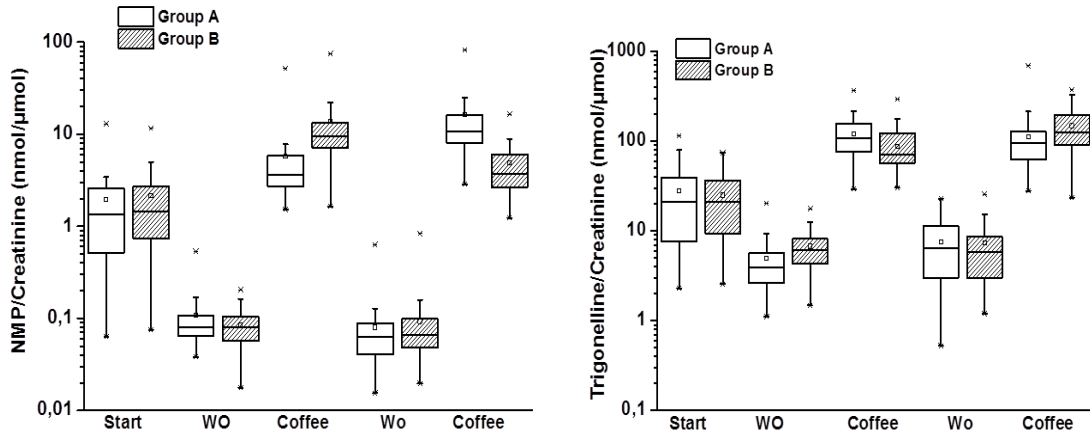


Figure 2 N-methylpyridinium (NMP) (nmol/ μ mol creatinine) (left) and trigonelline (nmol/ μ mol creatinine) (right) in urine samples of the group A and group B after each study phases.

3.3 Nutrient intake

On the basis of seven day food records prior the beginning of the study and at the end of each study phase, daily nutrient and energy intakes of the volunteers were calculated (Table 2). The statistical analysis of total energy intake, protein, fat, and carbohydrates comprised data from both intervention periods. The parameters of energy and nutrient intake were not significantly different during interventions with both coffees. In contrast, comparison of the results of the wash-out periods with the respective data after coffee ingestion showed a significant reduction of caloric ($p=0.026$), protein ($p=0.022$), and fat intake ($p=0.046$) associated with consumption of SB coffee. No distinct influence of MB on energy and nutrient intake was apparent over the whole study period. Values are expressed as mean $\pm$ SD. A statistical cross-over model was applied, that includes effects of group, treatment and time as well as interaction of treatment and time. Differences between interventions were analyzed by Student's *t*-test for normally distributed data. If neither carry over nor interaction was significant, data of both groups were pooled for comparisons of WO vs. SB and WO vs. MB.

Table 2 Average daily nutrient intake of healthy volunteers during the intervention study (on the basis of seven day food records)

	Group	WO	Market blend	MB vs WO <i>p</i> -value	WO	Study blend	SB vs WO <i>p</i> -value	MB vs SB <i>p</i> -value	Inter-action <i>p</i> -value	Carry-over <i>p</i> -value
Total energy intake [Kcal]	A	2049 ± 517	1939 ± 467		1968 ± 485	1997 ± 420				
	B	2075 ± 520	2106 ± 495		2199 ± 473	1990 ± 483				
	Both groups	2061 ± 516	2021 ± 486	ns	2081 ± 490	1994 ± 449	0.026	ns	ns	ns
Protein [g]	A	78.4 ± 19.8	73.8 ± 16.5		76.3 ± 17.1	75.1 ± 17.0				
	B	76.0 ± 22.2	77.8 ± 24.1		80.7 ± 18.4	74.1 ± 19.5				
	Both groups	77.2 ± 20.9	75.7 ± 20.5	ns	78.4 ± 17.4	74.6 ± 18.2	0.022	ns	ns	ns
Fat [g]	A	77.3 ± 24.5	71.6 ± 21.7		73.2 ± 21.9	73.9 ± 20.8				
	B	79.1 ± 24.0	78.9 ± 21.4		86.9 ± 23.7	77.5 ± 21.6				
	Both groups	78.2 ± 24.1	75.1 ± 21.8	ns	79.9 ± 23.6	75.7 ± 21.1	0.046	ns	ns	ns
Carbohydrates [g]	A	238.2 ± 62.6	230.8 ± 53.4		225.5 ± 58.9	233.0 ± 0.0				
	B	238.3 ± 62.8	242.0 ± 56.0		253.3 ± 55.6	231.7 ± 62.2				
	Both groups	238.0 ± 62.3	236.2 ± 54.6	ns	239.1 ± 58.6	232.0 ± 55.9	ns	ns	ns	ns

3.4 Body weight and body composition

A three compartment bioelectrical impedance analysis was used for anthropometric measurements. Results are summarized in Table 3. The statistical analysis of body weight/composition included data from both intervention periods of the study. Body weight of the volunteers was slightly reduced during both interventions; an effect, just reaching statistical significance after SB consumption ($p=0.037$). Of note, loss of body fat was more pronounced after MB and SB consumption (both $p < 0.001$). Thus, ingestion of the MB coffee was associated with diminished body fat (-0.64 ± 1.75 kg; $p=0.001$), whereas after SB ingestion body fat was found decreased even -0.90 ± 1.65 kg, ($p < 0.001$). The difference in reduction between SB and MB was significant ($p=0.02$). Of note, fat reduction was found significantly more pronounced ($p < 0.001$) in males (SB: -1.7 ± 1.7 kg; MB -1.15 ± 2.04 kg). FFM of volunteers as well as TBW were found increased during both intervention phases, compared to the preceding wash-outs: FFM: for MB $+0.57 \pm 2.08$ kg; for SB $+0.44 \pm 1.93$ kg; TBW: for MB $+0.33 \pm 1.4$ L; for SB $+0.39 \pm 1.7$ L; ($p < 0.05$, for both parameters). The FFM increase was more pronounced in male subjects (data not shown). Values are expressed as mean $\pm$ SD. A statistical cross-over model was applied, that includes effects of group, treatment and time as well as interaction of treatment and time. Differences between interventions were analyzed by Student's *t*-test for normally distributed data. If neither carry over nor interaction was significant, data of both groups were pooled for comparisons of WO vs. SB and WO vs. MB

Results

Table 3 Body weight and body composition of healthy volunteers during the intervention study (WO - wash-out; MB - market blend coffee; SB - study blend coffee)

	Group	WO	Market blend	MB vs WO <i>p</i> -value	WO	Study blend	SB vs WO <i>p</i> -value	MB vs SB <i>p</i> -value	Inter-action <i>p</i> -value	Carry-over <i>p</i> -value
Body weight t [kg]	A	70.79 ±10.7	70.62 ±10.5		70.85±10.6	70.54 ±10.7				
	B	69.86 ±12.0	69.6 ± 11.8		69.62±11.8	69.51 ±11.9				
	Both groups	70.34 ±11.3	70.12±11.1	ns	70.25±11.2	70.04 ±11.3	0.037	ns	ns	ns
Body fat [g]	A	16.50 ± 4.7	16.62 ± 4.5		16.52 ±4.7	15.21 ±5.04				
	B	16.17 ± 4.5	14.68 ±4.25		15.68 ± 4.1	15.24 ±4.43				
	Both groups	16.34 ± 4.6	15.70 ±4.48	0.001	16.12±4.39	15.22 ±4.73	<0.001	0.02	ns	ns
TBW [l]	A	39.74 ± 7.2	39.54 ± 7.3		39.77 ± 7.4	40.10 ± 8.1				
	B	39.15 ± 7.4	40.2 ± 8.1		39.76 ± 7.5	39.76 ± 8.0				
	Both groups	39.75 ± 7.3	39.88 ± 7.6	0.011	39.77 ± 7.4	40.17 ± 8.0	0.034	ns	ns	ns
FFM [kg]	A	54.29 ± 9.9	54.01± 9.9		54.33±10.1	55.32± 11				
	B	53.03 ± 9.9	54.26 ±10.6		53.67±10.5	53.70 ±10.6				
	Both groups	53.7 ± 9.9	54.1±10.4	0.015	54.00±10.1	54.51± 10.9	0.036	ns	ns	ns

3.5 Effects on plasma serotonin and ghrelin

Plasma serotonin and ghrelin concentrations as well as transcript levels of ghrelin in human lymphocytes are summarized in Table 4. Consumption of MB coffee was associated with a significant increase in plasma serotonin levels in comparison to the preceding wash-out period ($p < 0.001$), whereas after the intake of SB coffee no significant modulation was observed. Due to significant carry-over effects ($p < 0.001$) for plasma ghrelin levels the second intervention period was excluded from statistical analyses (Table 4). Values are expressed as mean ± SD. Relative Ghrelin transcription was calculated as an average from normalization of two housekeeping genes (GAPDH, RPL13a). Cross over analysis of ghrelin transcription was based on the delta cT values whereas comparisons to WO relied on relative transcriptions. Differences between interventions were analyzed by Student's *t*-test for normally distributed data sets or Wilcoxon rank sum test if data sets were not normally distributed. If neither carry over nor interaction was significant, data of both groups were pooled for comparisons of WO vs. SB and WO vs. MB. Otherwise only data from the first period were considered.

During the first intervention phase, plasma ghrelin levels were significantly diminished after consumption of MB coffee (-16 ± 16.51 pg/ml, $p < 0.001$) in comparison to the preceding wash-out period. In contrast, no modulation was observed after SB coffee.

Table 4 Plasma serotonin and ghrelin concentrations as well as relative ghrelin expression in healthy volunteers during the intervention study (WO - wash-out; MB - market blend coffee; SB - study blend coffee)

	Group	WO	Market blend	MB vs WO <i>p</i> -value	WO	Study blend	SB vs WO <i>p</i> -value	MB vs SB <i>p</i> -value	Inter-action <i>p</i> -value	Carry-over <i>p</i> -value
Serotonin [ng/ml]	A	64.0 ± 45.8	89.6 ± 47.6		64.9 ± 48.0	65.1 ± 44.9				
	B	54.7 ± 35.4	72.4 ± 47.6		67.9 ± 41.2	60.5 ± 39.1				
	Both groups	59.3 ± 40.6	81.0 ± 46.1	<0.001	66.4 ± 44.7	62.8 ± 42.0	ns	0.003	ns	ns
Ghrelin [pg/mL]	A	74.6 ± 33.2	54.6 ± 28.2	0.001	-	-		0.001	ns	0.001
	B	-	-		73.0 ± 53.7	77.4 ± 59.8	ns			
Ghrelin relative transcription	A	1.00	0.59 ± 0.3		1.00	1.19 ± 0.6				
	B	1.00	1.14 ± 0.8		1.00	0.62 ± 0.3				
	Both groups	1.00	0.86 ± 0.7	<0.001	1.00	0.91 ± 0.6	<0.001	ns	ns	ns

Transcript levels of ghrelin in human lymphocytes, normalized to two housekeeping genes (GAPDH and RPL13a) are given in Table 4. Since no significant carry-over or time effects were detected, both intervention periods were evaluated. Consumption of both coffees was associated with a decrease of ghrelin transcripts, compared to the preceding wash-out (both $p < 0.001$). Thus, whereas MB consumption was found associated with reduced ghrelin transcript and plasma ghrelin levels, this was not the case for SB consumption, where only a reducing effect on transcript levels, but not on plasma protein was observed.

Our findings thus indicate ingestion of MB coffee to be associated with increased serotonin level in plasma, decreased plasma ghrelin and ghrelin mRNA in lymphocytes.

3.6 Modulation of DNA strand breaks

The influence of coffee consumption on spontaneous and total (background plus FPG sensitive) DNA strand breaks in WBC was also investigated. Since the statistical analysis revealed significant interactions ($p < 0.001$) and/or carry-over effects ($p < 0.05$) on spontaneous and total DNA strand breaks, the second cross-over period had to be excluded from evaluation (data not shown). Within the first intervention period spontaneous and total DNA strand breaks were significantly decreased by comparison with the data of the respective preceding wash-out period (spontaneous: MB $p < 0.0001$; SB $p = 0.027$; total: MB $p < 0.0001$; SB $p < 0.0001$).

3.7 Correlation analysis

About 80 analytical parameters were investigated within this comprehensive human intervention study. In this paper, parameters of nutrient intake, body composition, and satiety are presented. Further, accompanying publications report about effects on energy metabolism and methylxanthines (Riedel, et al., 2014) and on transcriptional activity of selected genes (Winkler, et al., 2014).

The large amount of data collected allowed an insight into correlation of pairs or related groups of parameters by correlation analysis. Spearman correlations were calculated to detect possible linear or monotone relations. Pairwise correlations do not necessarily describe causal relations, because also other variables, potentially indirectly related to the respective parameters in focus may be of influence. The HEAT map with Spearman correlations of all parameters determined in this study is given in Figure 3.

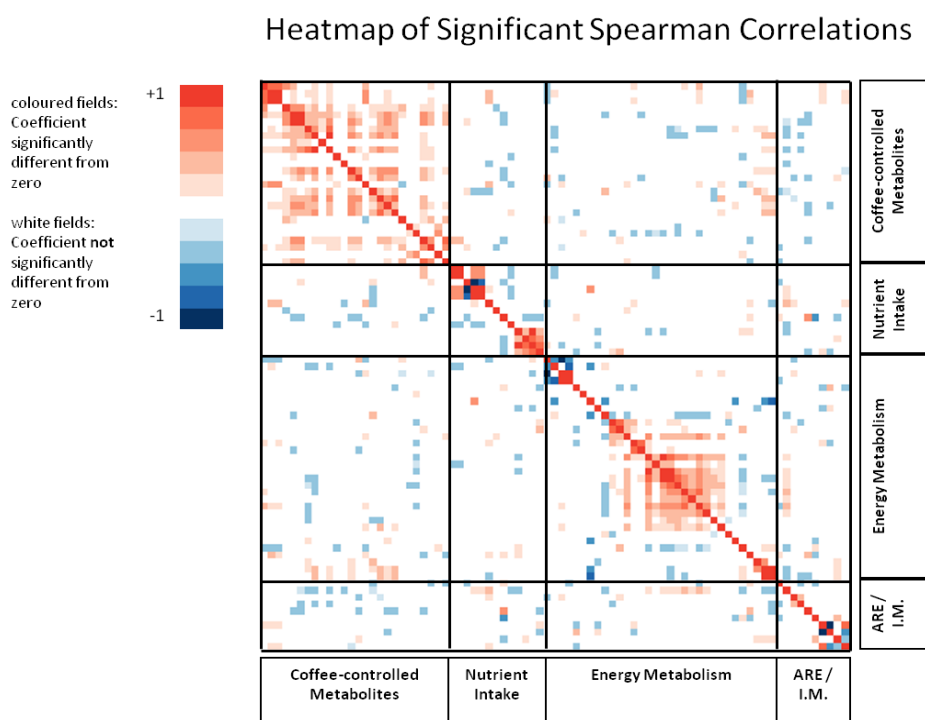


Figure 3 HEAT map correlation of all parameters investigated in the term of the human intervention study. Coloured fields: red: coefficient significantly different from zero (0 to +1); blue: coefficient significantly different from zero (0 to -1).

As an example, body fat was negatively correlated with the inflammation marker IL-6 ($p = -0.25$, $p < 0.001$). This correlation was more pronounced in male subjects (coefficient $p = -0.36$) than in females. IL-6 was also found negatively correlated with spontaneous DNA strand breaks ($p = -0.24$). Further negative correlations became apparent between body weight and the AMP/total adenosine nucleotide ratio and oleoyl-carnitine ($p = -0.28$ and $p = -0.33$, respectively). A marked positive correlation was apparent between the satiety marker leptin and body fat of female ($p = 0.37$) as well as male subjects ($p = 0.38$). Although correlation coefficients are quite low, still they suggest certain correlations between physiological parameters associated with coffee consumption, without necessarily implying a causal effect. The accompanying papers (Riedel, et al., 2014; Winkler, et al., 2014) provide a more in depth analysis and causal discussion of the respective individual parameters.

4. Discussion

Coffee consumption is associated with reduced risk of various degenerative diseases such as, diabetes type 2, cardiovascular disease and cancer. These disease risks appear associated with increased BMI, overweight or adiposity, and are often also found associated with moderate inflammation and oxidative stress (Otani, 2013; K. Stadler, 2012). The aim of our study was to evaluate whether a special coffee blend (SB), rich in both, antioxidant native constituents (such as chlorogenic acids) and roast products (such as NMP) might reduce body weight and body fat, reveal an influence on regulation of hunger and satiety and contribute to DNA integrity in healthy volunteers in comparison to the reference MB coffee. The SB coffee was produced by blending dark-roast coffee with significant portions of light-roast pure Arabica coffee (*Coffea arabica*). MB coffee was blended from five commercial coffees containing an unknown quantity of Robusta (*Coffea canephora*) coffee. The analytical data showed that both coffees contained comparable amounts of caffeine (about 12 mg/g coffee powder), in line with literature data (Maier, 1981). This translates into an approximate concentration of about 500-600 mg/L coffee in the brew. The coffees differed in chlorogenic acids, NMP and trigonelline contents by factors of two and higher (Table 1). It is known that trigonelline decomposes during coffee roasting, giving rise to pyridine related compounds like nicotinic acid, nicotinamide, and NMP (Lang, et al., 2008; R. H. Stadler, Varga, Hau, et al., 2002; R. H. Stadler, Varga, Milo, et al., 2002). Roasting of coffee likewise degrades chlorogenic acids and therefore lower amounts of chlorogenic acids are expected in dark roasts compared to light roasts.

In the course of our study, we investigated urinary excretion of NMP and trigonelline during the wash-out and the intervention periods. Dietary intake of NMP is largely restricted to coffee due to its roasting-induced formation. Therefore, it presents an excellent biomarker for compliance control. While urinary NMP and trigonelline excretion was low during the wash-out periods, it was significantly elevated by coffee intake pointing to good compliance (Figure 2).

At the end of the each intervention by SB, respectively MB coffee, a slight reduction of body weight, together with significant reduction of body fat ($p < 0.001$) compared to the preceding wash-out period was observed. These effects associated with consumption of SB were found to be more pronounced. Intake of energy and nutrient (protein and fat) were found significantly diminished only after consumption of SB coffee. Altogether, similar effects have been reported also in other studies. In our previous study consumption of a coffee blend similar to SB, was found associated with significant reduction of body weight/fat and energy/nutrient intake (Bakuradze, et al., 2011). Likewise, it also has been reported that a dark roast coffee characterized by a high concentration of NMP was more effective than light roast in reducing body weight and energy/nutrient intake in healthy volunteers (Kotyczka et al., 2011). However, coffee constituents other than those associated with roasting also appear of importance. For instance, a significant reducing effect of body fat found in the present study was also observed for MB, although not to the same extent. These findings may be reconciled with similar findings reported for consumption of chlorogenic acid-enriched coffee and green coffee bean extracts (Thom, 2007; Vinson, et al., 2012). In addition, in the present study we also found an increase in plasma serotonin together with a concomitant decrease in plasma ghrelin associated with MB coffee. These findings appear to suggest an increased satiation effect, however, the energy intake remained unchanged. Data from further experimental and human intervention studies likewise suggest intake of coffee constituents such as chlorogenic acids to modulate fat metabolism, entailing a reduction of body fat and body weight (Cho et al., 2010; Li et al., 2009; Thom, 2007; Vinson, et al., 2012). In addition, further, potentially as yet unknown constituents may as well be of biological relevance and contribute to a modulation of food intake, body composition, energy metabolism, or satiety. With respect to the presumed relation between coffee consumption and serotonin level, up to now no data is available to base firm conclusions on and further studies are required.

In the present study we also investigated the influence of coffee consumption on DNA integrity in WBC. Since significant interactions and/or carry-over effects on spontaneous

and total (background plus FPG sensitive) DNA strand breaks became apparent, the second study period was excluded from statistical analysis. Nevertheless, distinct DNA protective effects were exerted by both coffees during the first study period in comparison to the preceding wash-out phase. Therefore, although the above confounding effects do not allow conclusions on the second part of this cross-over study, still the results support DNA protective effectiveness of coffee in healthy humans. Likewise, in previous human intervention trials with coffee brews spontaneous and total DNA strand breaks were affected significantly (Bakuradze, et al., 2011; Bichler et al., 2007; Misik et al., 2010).

In conclusion, the present results allow to conclude consumption of both coffees to be associated with reduced energy and food intake, reduced body weight and body fat. They also indicate influence on satiety regulation in healthy subjects. These effects appear to depend on the constituent profile of coffees. In addition significant DNA protective effects were observed after consumption of both coffees. Altogether these findings support beneficial effects on human health associated with coffee consumption.

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All authors have approved the final manuscript.

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3 Conclusions and perspectives

Coffee consumption is proposed to exhibit beneficial effects on body weight development in humans. Since body weight maintenance presents a mass balance between energy intake and energy expenditure, variations result from a shift to one side or the other. The here presented PhD thesis comprises cell culture studies on the coffee constituents caffeine, trigonelline and its roasting-derived degradation product N-methylpyridinium, and nicotinic acid, for their ability to modify energy supply and energy expenditure and hence, for their potential to contribute to coffee's effect on energy homeostasis. The human intervention study aimed to link coffee-mediated alterations in energy metabolism and satiety to the body mass development of healthy volunteers.

Caffeine presents the most widely studied coffee constituent and has previously been hypothesized to increase energy expenditure in humans by elevating the basal metabolic rate. Several *in vitro* studies have been conducted to evaluate the effect of caffeine on mitochondrial respiratory activity in various tissues, but results are partly opposed and strongly dose-dependent. This work characterized the effect of moderate caffeine concentrations on cellular energy production in the human liver carcinoma cell line HepG2. Respiratory activity was decreased by direct addition of caffeine and after 24 hour incubation pointing to a reduction in cellular ATP production. Caffeine concentrations of 30 μ M, representing a physiological plasma concentration in humans that is reached after consumption of 3 – 4 cups of coffee, increased the mitochondrial membrane depolarization, presumably by an induction of proton leaks, immediately after addition to the cell culture medium and after long-term incubation for 24 hours. Moreover, the UCP-2 protein expression was augmented after 24 hours of incubation. However, it has to be taken into account that high non-physiological caffeine concentrations had to be applied to achieve this effect. Since uncoupling proteins and proton leaks in the mitochondrial membrane contribute to mitochondrial thermogenesis, this work proved the thermogenic effect of caffeine on the cellular level. Although compensation of the increased energy expenditure did neither result in enhanced fatty acid oxidation nor elevated mitochondrial respiratory activity. Caffeine administration to HepG2 cells rather caused energy restoration, possibly by attenuating energy consuming pathways. However, caffeine exposure seems to evoke distinct effects on energy production pathways in various tissues. In 3T3-L1 adipocytes, treatment with caffeine promoted fatty acid uptake, but no effect on the long-term fat storage was observed. Therefore, enhanced energy expenditure accompanied by increased mitochondrial activity is imaginable. At present, poor data rec-

ords are available to confirm this hypothesis, but further studies should be conducted to elucidate the effect of caffeine on the metabolic activity of the adipose tissue.

Based on the hypoglycaemic effect of trigonelline proposed in the literature, this work further investigated its capability to modify cellular energy production pathways to reveal the underlying molecular mechanism. The effect of N-methylpyridinium (NMP), the thermal degradation product of trigonelline that is highly present in dark-roast coffee, was also studied since currently no knowledge exists on its impact on cellular metabolic activity, substrate supply and thermogenesis. HepG2 cells, the model system that was applied for the examination of caffeine, were also employed for these studies. Exposure of HepG2 cells to NMP at a nanomolar concentration increased the cellular oxygen consumption over a time span of 24 hours, while the respiratory stimulation by trigonelline was manifested only at higher concentrations after pro-longed incubation time. Moreover, treatment with NMP augmented the cellular ATP content and enhanced glucose uptake. It might be concluded that NMP promotes cellular metabolic activity by initiating the utilization of glucose as energy substrate for ATP provision by oxidative phosphorylation. Although treatment with trigonelline also elevated the cellular glucose uptake of HepG2 cells, a shift to anaerobic ATP production can be assumed since ATP contents remained unchanged and extracellular acidification was increased. In order to assess the impact of NMP and trigonelline on thermogenesis, UCP-2 protein expression and the mitochondrial membrane potential were determined. Both compounds stimulated thermogenesis in HepG2 cells; however, while administration of NMP increased UCP-2 protein expression, trigonelline decreased the mitochondrial membrane potential.

Taken together, caffeine, trigonelline and NMP are coffee constituents that effectively alter cellular energy metabolism. Caffeine exhibits thermogenic effects on the cellular level that are reflected in the modification of uncoupling protein expression as well as mitochondrial membrane depolarization. However, it has to be considered that elevated non-physiological caffeine concentrations are needed to induce UCP-2 expression. In contrast, reductions in the mitochondrial membrane potential were already monitored at physiological concentrations, pointing to a mild uncoupling effect exhibited by caffeine immediately after addition to HepG2 cells in culture. NMP and trigonelline have been identified as further coffee constituents that induce mitochondrial thermogenesis. Augmented UCP-2 protein expression was ascertained after treatment with NMP at physiological concentrations that are achievable by regular coffee consumption. On the contrary, exposure to trigonelline diminished the mitochondrial membrane potential, but likewise when applied in physiological concentrations. Therefore, it might be concluded that,

besides caffeine, other compounds like NMP and trigonelline contribute to coffee's stimulatory effect on energy expenditure.

Regarding the coffee-induced changes in energy supply, the coffee constituents analyzed in this study exert different effects. While caffeine decreased respiratory activity and presumably caused energy restoration, NMP enhanced oxygen consumption rates and promoted the utilization of glucose as energetic substrate. In both cases, cellular ATP provision increased. Moreover, a shift from aerobic to anaerobic energy production was observed after trigonelline administration. Hence, individual coffee constituents tested exhibit discriminative effects on the metabolic activity *in vitro*. At present, it remains unclear which cell signaling cascades are activated or inhibited to obtain these metabolic changes and future studies should focus on exploring them. Certainly, assessment of the effect of individual compounds is necessary to identify the active compounds and to clarify their mode of action. In order to evaluate the overall effect of coffee, combinatory studies to uncover synergistic or antagonistic effects as well as *in vivo* intervention trials are essential.

Part of this dissertation was focused on the impact of various pyridine derivatives such as nicotinic acid, trigonelline and NMP, on intestinal fatty acid uptake. Cell culture studies were performed in enterocyte-like Caco-2 cells to identify the most important structural determinants. Next to an additional methyl group, the carboxylic group and the N-methylation presented important structural characteristics to predict the fatty acid uptake. Results indicated that incubation with 1 mM nicotinic acid alters lipid metabolism by activation of the nicotinic acid receptor HM74, resulting in augmented fatty acid uptake in Caco-2 cells. Nicotinic acid is known to inhibit lipolysis in adipocytes. Therefore it is applied in pharmacological doses to treat hyperlipidaemia. However, findings suggest a modification of lipid metabolism by nicotinic acid already in the intestine. Moreover, treatment with 1mM N-methyl-4-phenylpyridinium, a further pyridine derivative tested, inhibited fatty acid uptake of Caco-2 cells. The trans-epithelial electrical resistance of the cells was increased after exposure N-methyl-4-phenylpyridinium, pointing to an action on paracellular permeability and membrane integrity. According to these findings, it can be concluded that pyridine derivatives sharing the same core motif differently affect intestinal fatty acid uptake.

The human intervention study compared the effect of a 4-week consumption of two differently processed coffee beverages on parameters of energy metabolism and thermogenesis as well as on the regulation of satiation in healthy normal-weight subjects. The optimized study blend (SB) coffee contained higher amounts of NMP, but lower amounts

of chlorogenic acid and trigonelline than the market blend (MB) coffee. However, both coffees possessed similar caffeine concentrations. The overall aim of this long-term cross-over study was to prove a body weight modulating effect and to clarify whether differences in the coffee ingredient profile are reflected in body mass development and changes in energy metabolism or food intake of the participants.

Independent of the type of coffee consumed, plasma free fatty acid concentrations of the volunteers increased after 4 weeks of coffee intervention in comparison to the preceding wash-out period. In the short-term, elevation of plasma free fatty acids in healthy subjects after ingestion of caffeine and regular coffee has already been suggested in the literature, but data of this study demonstrate that a regular consumption of caffeinated coffee prolongs this effect. Since both coffees contained comparable amounts of caffeine, the results of this human intervention study, in line with evidence from the literature, indicate that caffeine is one of the active compounds in coffee that promotes the utilization of fatty acids as energy substrate. Correlation analyses support this hypothesis and revealed a positive association between plasma free fatty acids and levels of ATP normalized to the total adenosine nucleotides (TAN) in lymphocytes. In addition, a reduction in body fat was observed after administration of both coffees and for male subjects, a significant negative correlation between body fat and the ATP/TAN ratio was ascertained, allowing the conclusion that endogenous body fat was utilized to maintain energy supply. However, body fat reduction was more pronounced after consumption of the NMP-rich SB coffee than after consumption of the MB coffee, rich in chlorogenic acid and trigonelline, and a significant decrease in body weight was only ascertained after administration of the SB coffee, suggesting the beneficial effect on body composition to depend on the coffee constituent profile.

Glucose presents a further energetic source for humans and intact glucose metabolism is essential to prevent human disease, especially the development of type 2 diabetes mellitus. Although modifications in glucose metabolism after acute coffee consumption were reported in humans, prolonged effects have rarely been monitored. In the here presented study, plasma glucose concentrations remained unchanged after consumption of either SB or MB coffee, and no association between plasma glucose concentrations and the energetic status could be determined. Fasting serum insulin concentrations were slightly increased in subjects after consumption of the MB coffee compared to the preceding wash-out period, while no alterations were observed after consumption of the SB coffee. Consequently, the calculated HOMA-IR (homeostatic model assessment of insulin resistance) rose after MB coffee consumption. Although the MB coffee contained

higher amounts of chlorogenic acids and trigonelline than the SB coffee, no long-term beneficial effect of the MB coffee on insulin sensitivity was verified within this study. Moreover, plasma levels of osteopontin, a pro-inflammatory cytokine, were augmented in volunteers after ingestion of the SB coffee. Previous studies ascertained increased osteopontin concentrations upon obesity, and demonstrated improved insulin sensitivity after neutralization of the osteopontin action. So far, no investigations have been dedicated to the effect of coffee consumption on osteopontin concentrations in humans. The results presented in this study provide insights to coffee-derived modifications in osteopontin concentrations in humans. To explain these variations, further studies are necessary to identify the responsible coffee constituents and their mechanism of action. Considering this knowledge, it might be valuable to produce an optimized coffee beverage that beneficially restricts the osteopontin action and improves symptoms of metabolic disorders related to obesity.

In order to estimate the impact of coffee consumption on energy expenditure, the uncoupling protein-2 was determined as a marker for mitochondrial thermogenesis in isolated lymphocytes, employed as surrogate biomarker. UCP-2 protein expression was only augmented after consumption of the NMP-rich SB coffee. It can be hypothesized that NMP plays a role in the induction of thermogenesis, since *in vitro* studies also showed increased UCP-2 contents in HepG2 cells after NMP treatment. Thus, increased energy expenditure evoked by administration of the SB coffee might partly have contributed to the reduction in body weight and body fat monitored after SB intervention.

Discriminative effects of the two coffee beverages were also observed for selected parameters of satiety. After 4 weeks of MB consumption, plasma ghrelin concentrations were reduced and plasma serotonin concentrations augmented when compared to the preceding wash-out period. In contrast, no alterations were observed after administration of the SB coffee. The orexigenic peptide ghrelin is secreted from the stomach to signalize hunger sensation and stimulate food intake. Serotonin release mediates a satiation signal. The reduction in ghrelin and the increase in serotonin in the plasma of the volunteers after consumption of the MB coffee, rich in chlorogenic acids and trigonelline, point to a reduced hunger feeling after over-night fasting. Beside their action on lipid and glucose metabolism, the hormones leptin and insulin have to be considered as satiety signals. The increase of both hormones was more pronounced after MB than after SB coffee consumption providing further support for a stimulation of satiety by the MB coffee. In addition, total energy intake was reduced only by SB ingestion. These findings suggest

that the coffee beverages administered in this intervention study differently affect satiety feelings and food intake in healthy normal-weight humans.

However, some restrictions have to be taken into account when interpreting the data of the presented intervention study. This cross-over study was designed to compare the effectiveness of two coffee beverages to modify body weight development. Since a parallel water control group is missing, evaluation of the individual coffee effects can only be accomplished by incorporation of the preceding wash-out period. Here, time effects have to be considered and the changes observed in some parameters cannot exclusively be attributed to coffee consumption alone. Moreover, the physical activity of the participants was not determined and hence, no conclusion can be drawn about the contribution of exercise to energy expenditure and proportional changes over the study period.

In summary, long-term consumption of both coffees stimulated energy metabolism. Caffeine might be a major coffee component contributing to the utilization of endogenous fat storages for energy provision in humans. In addition, results from the cell culture studies point to NMP as a further potential coffee constituent that promotes thermogenesis and cellular glucose utilization. In the long-term, a beneficial effect of the NMP-rich SB coffee on UCP-2 protein expression could also be ascertained. Biochemical parameters of satiety were differently affected by MB and SB coffee intervention. Results indicate that the MB coffee, rich in chlorogenic acid and trigonelline, induces biomarkers of satiety, suggesting coffee constituents, others than caffeine to play a role in mechanisms regulating satiety. Hence, consideration of the coffee constituent profile is essential for evaluating coffee's biological effects. Future studies should verify the coffee constituents responsible for mediation of satiety feelings and elucidate their role in the regulation of food intake. Considering this knowledge, it might be promising to develop a functional coffee beverage that effectively contributes to a reduction in food intake and facilitates to slim down. It might be also worth to focus on overweight subjects. Especially with regard to the high prevalence of obesity and thereof related diseases, promotion of a well-balanced energy metabolism in conjunction with a moderate body weight could help to maintain human health and well-being.

4 Abstract

Coffee consumption is supposed to promote body weight maintenance in humans. This work was focused on the effects of coffee and coffee constituents on markers of energy metabolism and satiety in cells in culture and healthy subjects. Among the coffee constituents, caffeine, trigonelline and its roasting-derived degradation product N-methylpyridinium (NMP) were analyzed for their effects on mitochondrial respiratory activity, thermogenesis and substrate utilization in the human liver carcinoma cell line HepG2. On the cellular level, caffeine treatment increased the uncoupling protein-2 (UCP-2) and the mitochondrial membrane depolarization, both mechanisms that enhance mitochondrial thermogenesis. NMP and trigonelline also stimulated thermogenesis in HepG2 cells. While caffeine restored cellular ATP as a mechanism to compensate increased energy expenditure, NMP exhibited a stimulating effect on the respiratory activity, and promoted the utilization of glucose as energy substrate. In addition, investigations on the impact of pyridine derivatives such as nicotinic acid, trigonelline and NMP, also present in coffee, on intestinal fatty acid uptake were made. Cell culture studies in enterocyte-like Caco-2 cells revealed that nicotinic acid modifies lipid metabolism in the intestine by activation of the nicotinic acid receptor HM74 resulting in augmented fatty acid uptake. A human intervention trial was conducted to study the impact of two differently processed coffees on parameters of energy metabolism and satiety. In healthy, normal-weight volunteers, a 4-week consumption of both coffee beverages increased plasma free fatty acid concentration that were positively correlated to ATP levels in lymphocytes. Since body fat was reduced by coffee consumption, these findings point to an intensified utilization of endogenous body fat as energy substrates. Protein expression of UCP-2 was increased in lymphocytes after ingestion of a study blend (SB) coffee that contained higher amounts of NMP but lower amounts of chlorogenic acid and trigonelline than a market blend (MB) coffee tested. Discriminative effects of the two coffee beverages were also observed for satiety parameters. Consumption of the MB coffee augmented plasma serotonin and decreased plasma ghrelin concentrations, pointing to increased satiation. No alterations were observed after consumption of the SB coffee. These results indicate that caffeine might be a major coffee component stimulating energy metabolism. However, results from the cell culture experiments support the hypothesis that NMP may also present a potential constituent in coffee that promotes thermogenesis and cellular glucose utilization. Future studies should elucidate whether other coffee constituents, next to caffeine and NMP, play a role mechanisms regulating energy metabolism.

5 Zusammenfassung

Kaffeeconsum ist mit einer geringeren Prävalenz an Übergewicht und Adipositas assoziiert. Diese Forschungsarbeit untersuchte den Einfluss von Kaffee und Kaffeeinhaltsstoffen auf Mechanismen des Energiestoffwechsels und der Sättigungsregulation im Rahmen von Zellkulturstudien und einer Humanstudie. Koffein, Trigonellin und das aus diesem bei der Kaffeeröstung entstehende N-methylpyridinium (NMP) wurden hinsichtlich ihrer Wirkung auf die mitochondriale Atmungsaktivität und Thermogenese sowie die Substratbereitstellung für die ATP Produktion in der humanen Leberkrebszelllinie HepG2 untersucht. Die Behandlung der Zellen mit Koffein führte zu einem Anstieg des entkoppelnden Proteins-2 (UCP-2) und der mitochondrialen Membrandepolarisierung, welche Merkmale mitochondrialer Thermogenese sind. NMP und Trigonellin stimulierten ebenfalls die Thermogenese. Während Koffein eine Verminderung von energieverbrauchenden Stoffwechselvorgängen hervorrief, um den erhöhten Energieverbrauch durch gesteigerten Thermogenese zu kompensieren, übte NMP einen stimulierenden Effekt auf die Atmungsaktivität aus und förderte die Verwendung von Glukose als Energiesubstrat. Die Zellkulturstudien zum Einfluss von Pyridinderivaten, wie Nikotinsäure, Trigonellin und NMP, auf die Fettsäureaufnahme von enterozyten-ähnlichen Caco-2 Zellen zeigten, dass es durch Nikotinsäure bereits in Darmzellen zur Aktivierung des Nikotinsäurerezeptors HM74 und hierdurch zur Erhöhung der Fettsäureaufnahme kommt. Im Rahmen der Humanstudie erfolgte der Vergleich zweier Kaffeegetränke mit unterschiedlichem Inhaltsstoffprofil. Bei den gesunden normalgewichtigen Probanden erhöhte der 4-wöchige Konsum beider Kaffees die Plasmakonzentration an freien Fettsäuren, welche positiv mit dem ATP-Gehalt der Lymphozyten korrelierte. Da das Körperfett durch den Kaffeeconsum vermindert wurde, lässt sich auf einen verstärkten Verbrauch endogener Fettmasse zur Energiebereitstellung schließen. Der Konsum des Studienkaffees (SB), welcher höhere Gehalte an NMP aber niedrigere Gehalte an Chlorogensäure und Trigonellin als der im Vergleich getestete Referenzkaffee (MB) aufwies, erhöhte die UCP-2 Konzentration in den Lymphozyten der Probanden. Unterschiedliche Effekte der beiden Kaffees wurden auch auf die Parameter der Sättigungsregulation beobachtet. Die Verabreichung des MB Kaffees führte zu einem Anstieg der Plasmaserotonin- und zur Verminderung der Plasmaghrelinkonzentration, wodurch auf eine verstärkte Sättigung geschlossen werden kann. Diese Ergebnisse deuten auf Koffein als eine der Hauptverbindungen im Kaffee, die einen stimulierenden Effekt auf den Energiestoffwechsel hervorruft. Allerdings stellt NMP ebenfalls einen potentiellen Kaffeeinhaltsstoff dar, welcher die Thermogenese und die Glukoseverwertung fördert. Zukünftige Studien sollten klären ob weitere Kaffeeinhaltsstoffe eine Rolle in der Regulation des Energiestoffwechsels spielen.

6 Curriculum Vitae

Work experience

11/2009 - 04/2013	Research associate Department of Nutritional and Physiological Chemistry, University of Vienna, Austria - Laboratory work in the field of molecular nutrition Teaching
06/2004 - 07/2004	DreiPlus Planungsgruppe Architekten und Ingenieure GmbH, Berlin, Germany - Secretary

Education

Since 11/2009	PhD program Natural Sciences (Chemistry) Department of Nutritional and Physiological Chemistry, University of Vienna, Austria Title: "Impact of coffee consumption on mechanisms of en- ergy metabolism and satiety <i>in vitro</i> and <i>in vivo</i> "
10/2004 - 11/2009	Academic studies of Nutrition Friedrich Schiller University of Jena, Germany Diploma thesis at the Department of Nutrition, Food Sci- ence, Physiology and Toxicology, University of Navarra, Pamplona, Spain Title: "Influence of the Torrefacto roast and the storage conditions on the antioxidant capacity of coffee brew"
09/2008 - 03/2009	Research student (ERASMUS program) University of Navarra, Pamplona Spain
07/2004	University-entrance degree Friedrich-Fröbel-Gymnasium, Bad Blankenburg, Germany

Internships

08/2011	Institute of Inorganic Chemistry, Cell Culture Facility, Uni- versity of Vienna, Vienna, Austria
10/2007	Medizinische Fachschule Georgius Agricola, Saalfeld, Germany
09/2007	Milchwerke Thüringen GmbH, Erfurt, Germany

04/2007	Hofmann & Sommer GmbH & Co. KG Chemisch-Pharmazeutische Fabrik, Königsee, Germany
03/2007	Department of Veterinary and Food Control, Landratsamt Saalfeld-Rudolstadt, Rudolstadt, Germany
09/2006	Geflügelhof Teichweiden GmbH, Teichweiden, Germany
09/2005	Arbeiterwohlfahrt Seniorenheim, Rudolstadt, Germany

Conferences and trainings

09 - 11/10/2013	CoCoTea, Second International Congress on Cocoa, Coffee and Tea, Naples, Italy Oral presentation: "Long-term effects of regular coffee: 4-week consumption of medium roast and dark roast coffee differentially affects parameters of satiety and energy status in humans"
20 - 22/03/2013	50 th Congress of the German Nutrition Society (DGE), Bonn, Germany Poster presentation: "Strukturabhängige Wirkung von Pyridinderivaten auf die Fettsäureaufnahme von differenzierten Caco-2 Zellen"
11 - 16/11/2012	ASIC Costa Rica 2012, The 24 th International Conference on Coffee Science, San José, Costa Rica Oral presentation: "Potential effect of coffee and coffee constituents for promoting body weight maintenance in healthy subjects"
14 - 16/03/2012	49 th Congress of the German Nutrition Society (DGE), Freising, Germany Poster presentation: "Veränderung der mitochondrialen Energie-und Wärmeproduktion durch Koffein <i>in vitro</i> "
13 - 16/09/2011	CoCoTea, First International Congress on Cocoa, Coffee and Tea, Novara, Italy Poster presentation: "Dose-dependent effect of caffeine on mitochondrial energy production and thermogenesis in HepG2 cells"
09 - 13/03/2009	Scholarship European Cooperation in Science and Technology COST Action 927 Training School, Granada, Spain "Assessing the generation and bioactivity of neo-formed compounds in thermally treated foods"
05 - 06/11/2008	II International Congress of Food Science, Technology and Safety, Pamplona, Spain Participation

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- 1) Riedel A., Pignitter M., Hochkogler C.M., Rohm R., Walker J., Bytof G., Lantz I., Somoza V. *Caffeine dose-dependently induces thermogenesis but restores ATP in HepG2 cells in culture.* Food and Function 2012, 3, 955-964
- 2) Riedel A., Hochkogler C.M., Lang R., Bytof G., Lantz I., Hofmann T., Somoza V. *N-methylpyridinium, a degradation product of trigonelline upon coffee roasting, stimulates respiratory activity and promotes glucose utilization in HepG2 cells.* Food and Function 2014 (DOI: 10.1039/C3FO60320B)
- 3) Riedel A., Lang R., Rohm B., Rubach M., Hofmann T., Somoza V. *Structure-dependent effects of pyridine derivatives on intestinal fatty acid uptake: Regulation of nicotinic acid receptor and fatty acid transporter expression.* The Journal of Nutritional Biochemistry 2014 (in revision)
- 4) Riedel A., Dieminger N., Bakuradze T., Lang R., Montoya G., Hochkogler C.M., Winkler S., Bytof G., Lantz I., Stiebitz H., Hofmann T., Richling E., Marko D., Schipp D., Raedle J., Somoza V. *A 4-week consumption of medium roast and dark roast coffee affects parameters of energy status in healthy subjects.* Food Research International 2014 (in revision)
- 5) Bakuradze T., Montoya Parra G.A., Riedel A., Somoza V., Lang R., Dieminger N., Hofmann T., Winkler S., Hassmann U., Marko D., Schipp D., Raedle J., Bytof G., Lantz I., Stiebitz H., Richling E. *Influence of a four week coffee intervention on nutrient intake, body composition, biomarkers associated with satiety and DNA integrity in humans.* Food Research International (submitted December 13th, 2013)
- 6) Winkler S., Dieminger N., Blust V., Riedel A., Bakuradze T., Montoya G., Hassmann U., Lang R., Hofmann T., Somoza V., Richling E., Bytof G., Stiebitz H., Lantz I., Schipp D., Raedle J., Marko D. *Modulation of inflammatory gene transcription after coffee consumption.* Food Research International (submitted January 7th, 2014)
- 7) Rohm B., Riedel A., Widder S., Ley J.P., Kramer G.E., Somoza V. *Capsaicin and its analogs nonivamide and trans-pellitorine effectively inhibit free fatty acid uptake without TRPV1 activation and increase acetyl-coenzyme A synthase activity in Caco-2 cells.* Journal of Lipid Research (manuscript in preparation)