University of Veterinary Medicine Hannover
Institute for Food Toxicology

Studies on adverse effects of L-carnitine on gastrointestinal and cardiovascular parameters as well as on the metabolome in Fischer 344 rats

THESIS
Submitted in partial fulfilment of the requirements for the degree
DOCTOR OF PHILOSOPHY
(PhD)
awarded by the University of Veterinary Medicine Hannover

by
Michael Telamon Empl
Brussels

Hannover, Germany, 2018
Supervisor: Prof. Dr. Pablo Steinberg (until 07.02.2017)  
Prof. Dr. Gerhard Breves (from 08.02.2017)  
Supervision group: Prof. Dr. Pablo Steinberg/Prof. Dr. Gerhard Breves  
Prof. Dr. Maren von Köckritz-Blickwede  
Prof. Dr. Ingo Just  

1st evaluation: Prof. Dr. Gerhard Breves  
Department of Physiology  
University of Veterinary Medicine Hannover  
Hannover, Germany  
Prof. Dr. Maren von Köckritz-Blickwede  
Department of Physiological Chemistry and Research Center for Emerging Infections and Zoonoses (RIZ)  
University of Veterinary Medicine Hannover  
Hannover, Germany  
Prof. Dr. Ingo Just  
Institute of Toxicology  
Hannover Medical School  
Hannover, Germany  

2nd evaluation: Prof. Dr. Klaus Eder  
Institute of Animal Nutrition and Nutritional Physiology  
Justus Liebig University Giessen  
Giessen, Germany  

Date of final exam: 30.10.2018  

Sponsorship: The present study was partly funded by the German Federal Institute for Drugs and Medical Devices (Bundesinstitut für Arzneimittel und Medizinprodukte [BfArM]; grant no.: V-14999/68502/2012-2013)
Parts of the present thesis have been published previously in the following peer-reviewed scientific journals:


* These authors contributed equally to this work

Parts of the present thesis have been presented previously as posters at the following national and international conferences:


51st Congress of the European Societies of Toxicology (EUROTOX 2015). 13–16.09.2015, Porto, Portugal
To Christina and my family
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List of abbreviations

ACF  Aberrant crypt foci
ACS  Acyl-CoA synthetase
ATP  Adenosine triphosphate
BfR  German Federal Institute for Risk Assessment
     (German: Bundesinstitut für Risikobewertung)
BW   Body weight
CACT Carnitine acylcarnitine translocase
CID  PubChem Compound Identifier
CoASH Coenzyme A
CPT1 Carnitine palmitoyl transferase 1
CPT2 Carnitine palmitoyl transferase 2
CRC  Colorectal cancer
CVD  Cardiovascular disease
CYP  Cytochrome P450 monooxigenase
CYP2E1 CYP isoform 2E1
DEN  Diethylnitrosamine
DMA  Dimethylamine
DMN  Dimethylnitrosamine
EFSA  European Food Safety Authority
EFSA AFC Panel  EFSA Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food
EFSA NDA Panel  EFSA Panel on Dietetic Products, Nutrition and Allergies
EMA  European Medicines Agency
EU   European Union
F344 rat  Fischer 344 rat
FA   Fatty acid
FABPpm Plasma membrane fatty acid-binding protein
FAT  Fatty acid translocase
FATP Fatty acid transport protein
FMO3 Flavin-containing monooxygenase 3
GC-MS Gas chromatography-mass spectrometry
HED  Human equivalent dose
IARC International Agency for Research on Cancer
LCFA Long-chain fatty acid
LC-MS Liquid chromatography-mass spectrometry
LDL  Low-density lipoprotein
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>MA</td>
<td>Methylamine</td>
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<tr>
<td>NCD</td>
<td>Non-communicable disease</td>
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<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>NOCs</td>
<td>N-Nitroso compounds</td>
</tr>
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<td>NTP</td>
<td>National Toxicology Program</td>
</tr>
<tr>
<td>OCTN</td>
<td>Carnitine/organic cation transporter</td>
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<tr>
<td>PCD</td>
<td>Primary carnitine deficiency</td>
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<td>RYR</td>
<td>Red yeast rice</td>
</tr>
<tr>
<td>SCD</td>
<td>Secondary carnitine deficiency</td>
</tr>
<tr>
<td>SDAV</td>
<td>Sialodacryoadenitis virus</td>
</tr>
<tr>
<td>SLC</td>
<td>Solute carrier</td>
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<tr>
<td>TMA</td>
<td>Trimethylamine</td>
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<tr>
<td>TMAO</td>
<td>Trimethylamine N-oxide</td>
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<tr>
<td>USA/US</td>
<td>United States of America</td>
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<tr>
<td>VKM</td>
<td>Norwegian Scientific Committee for Food and Environment (Norwegian: Vitenskapskomiteen for mat og miljø)</td>
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<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cells</td>
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<tr>
<td>VZ NRW</td>
<td>Consumer Association of North Rhine-Westphalia (German: Verbraucherzentrale Nordrhein-Westfalen)</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>γBB</td>
<td>γ-Butyrobetaine</td>
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Studies on adverse effects of L-carnitine on gastrointestinal and cardiovascular parameters as well as on the metabolome in Fischer 344 rats

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L-Carnitine is a naturally occurring quaternary ammonium compound mainly found in muscle tissue. Its physiological function is primarily to enable the transport of activated long-chain fatty acids to the inner compartment of the mitochondrion, where they are oxidized to produce energy for the cell. Because of its involvement in this so-called “carnitine shuttle”, it is often falsely assumed that the exogenous administration of L-carnitine will enhance fatty acid degradation, especially in muscle tissue. In fact, this assumption is the reason why this nutrient is heavily advertised as “fat-burning” or “performance-boosting” dietary supplement. However, up to today, there are no convincing scientific data supporting these claims and the marketing of carnitine-containing dietary products signifying the contrary is consequently prohibited in the European Union (EU). In spite of the European Food Safety Authority (EFSA) having reviewed and dismissed all hitherto submitted carnitine-related health claims, dietary supplements containing up to several grams of L-carnitine and promising a fast weight loss or a higher sports performance are readily available.

The healthy mammalian organism upkeeps L-carnitine homeostasis by dietary ingestion, endogenous synthesis and renal reabsorption. Cellular L-carnitine uptake (e.g. intestinal and muscular absorption as well as renal reabsorption) is for the most part an active transporter-mediated process, which obeys saturable Michaelis-Menten kinetics. Therefore, L-carnitine quantities above the carrier’s (carnitine/organic cation transporter 2; OCTN2) threshold are not transported and would consequently either remain in the gut or be excreted in the urine. This principle generally allows the mammalian organism to keep bodily L-carnitine levels steady and is the main reason why the muscle carnitine content can only hardly—if at all—be increased. It can also be deduced from this that the more L-carnitine is orally administered, the less is absorbed and the lower the bioavailability will be. L-carnitine not absorbed in the gut is subjected to extensive gut microbial metabolism, which leads to the formation of trimethylamine (TMA) as primary metabolite. TMA can then be further transformed by gut bacteria to dimethylamine (DMA) or be metabolized, mostly in the liver, to trimethylamine N-oxide (TMAO). Once formed, all of these molecules may be nitrosated in the gastrointestinal tract in the presence of dietary or endogenous nitrosating agents such as nitrite and thus yield dimethylnitrosamine (DMN). DMN is a highly carcinogenic compound mainly inducing hepatic tumors in experimental animals.

The primary aim of the present project was therefore to investigate whether non-absorbable (i.e. high) amounts of L-carnitine administered over the course of one year to experimental animals would induce the formation of precancerous lesions (aberrant crypt foci; ACF) in the colon of these animals and thus indicate a possible carcinogenic potential emanating from L-carnitine supplementation and metabolism. The Fischer 344 (F344) rat, a strain commonly used in cancer research, was thereby chosen as model and the quantities administered as well as the duration of administration were selected to reflect a regular consumption of dietary supplements containing several grams of L-carnitine. Since a publication linking TMAO to the onset of atherosclerosis was published during the course of the study, the initial aim was expanded to additionally investigate whether L-carnitine supplementation might enhance the incidence of atherosclerotic lesions in the aorta of the F344 rats. Moreover, using targeted as
well as untargeted metabolomics techniques, the influence of L-carnitine on the plasma metabolome was analyzed.

The chronic supplementation of up to 5 g/L L-carnitine via drinking water did neither lead to a significant formation of ACFs nor to an increased occurrence of atherosclerotic lesions in the experimental animals. In the course of the metabolomics analyses, 359 metabolites were detected in total, with 29 being significantly influenced by the L-carnitine supplementation. However, with the exception of a tenfold increase of the plasma TMAO concentration in the group receiving the highest carnitine concentration, changes in the (relative) abundance of those metabolites was quite small and therefore most probably biologically irrelevant.

In summary, the present study shows that a chronic supplementation of up to 5 g/L L-carnitine via drinking water does not lead to major adverse effects in F344 rats. However, since TMAO has been associated with the emergence of cardiovascular diseases, the increase in plasma TMAO levels might indicate a possible health risk for the consumer and therefore the chronic intake of carnitine-containing dietary supplements is not recommended, even more so as they are ineffective in promoting weight loss or enhancing physical performance.
Zusammenfassung

Untersuchungen zu adversen Effekten von L-Carnitin auf gastrointestinale und kardiovaskuläre Parameter sowie auf das Metabolom in Fischer 344-Ratten

Michael T. Empl


Trotz der Tatsache, dass die Europäische Behörde für Lebensmittelsicherheit (EFSA) alle bis heute zur Genehmigung eingereichten gesundheitsbezogenen Angaben zu L-Carnitin abgelehnt hat, sind Nahrungsergänzungsmittel oder andere Produkte mit einem L-Carnitin-Gehalt bis zu mehreren Gramm, welche einen schnellen Körpergewichtsverlust sowie eine gesteigerte sportliche Leistung versprechen, leicht erhältlich.


Daraus kann ferner geschlossen werden, dass je mehr L-Carnitin oral zugeführt wird, desto weniger wird im Darm resorbiert und desto niedriger ist folglich auch die Bioverfügbarkeit. Nicht absorbiertes L-Carnitin unterliegt im Darm einem ausgiebigen mikrobiellen Abbau, welcher zur Bildung des primären Metaboliten Trimethylamin (TMA) führt. TMA kann dann weiter durch enterale Bakterien zu Dimethylamin (DMA) oder, hauptsächlich in der Leber, zu Trimethylamin-N-oxid (TMAO) verstoffwechselt werden. Sind diese Stoffe einmal gebildet, können sie im Magen-Darm-Trakt durch aus der Nahrung stammende oder endogen gebildete Nitrosierungsagenzien, beispielsweise Nitrit, zu Dimethylnitrosamin (DMN) nitrosiert werden. DMN ist eine stark kanzerogene Substanz, welche im Tierversuch hauptsächlich die Entstehung von Lebertumoren induziert.

Es war daher das primäre Ziel des vorliegenden Projekts zu untersuchen, ob nicht resorbierbare (d. h. hohe) Mengen an L-Carnitin, welche über einen Zeitraum von einem Jahr Versuchstieren verabreicht wurden, zur Bildung von präkanzerogenen Läsionen (aberrante Kryptenfoci; ACF) im Dickdarm dieser Tiere führen. So sollte ein Hinweis darauf gewonnen werden, ob von einer L-Carnitin-Supplementierung und der damit assoziierten Metabolisierung

Die chronische Supplementierung von bis zu 5 g/L L-Carnitin über das Trinkwasser hat weder zur Bildung einer nennenswerten Anzahl an ACFs geführt, noch die Inzidenz atherosklerotischer Läsionen in den Versuchstieren erhöht. Im Rahmen der Metabolomics-Analyse wurden insgesamt 359 Metaboliten erfasst, von denen 29 durch die L-Carnitin-Gabe signifikant beeinflusst wurden. Allerdings waren die relativen Mengenveränderungen dieser Metaboliten, mit Ausnahme eines zehnfachen Anstiegs der TMAO-Konzentration, stets relativ klein und damit höchstwahrscheinlich biologisch nicht relevant.

Zusammenfassend lässt sich aus den Ergebnissen der vorliegenden Studie schliessfolgern, dass eine chronische Supplementierung von bis zu 5 g/L L-Carnitin über das Trinkwasser zu keinen bedeutsamen adversen Effekten in F344-Ratten führt. Da TMAO allerdings mit der Entstehung von kardiovaskulären Erkrankungen in Verbindung gebracht wird, könnte die Erhöhung der Plasmakonzentration dieses Metaboliten auf ein mögliches Gesundheitsrisiko für den Verbraucher hinweisen. Daher kann die chronische Einnahme von Carnitin-haltigen Nahrungsergänzungsmitteln nicht empfohlen werden, insbesondere aufgrund der Tatsache, dass sie nicht wirksam sind, also weder die Gewichtsabnahme noch die sportliche Leistungsfähigkeit erhöhen.
1. Introduction

Amidst rising international tensions as well as political and economic insecurity, governments, authorities and populations all over the world increasingly have to face the so-called “non-communicable diseases (NCD) pandemic” (Allen 2017). The World Health Organization (WHO) defines NCDs as non-contagious, chronic and slow-developing illnesses, with cardiovascular diseases (CVDs), cancers, chronic respiratory diseases and diabetes representing the four main NCD categories (WHO 2018b). In fact, according to newest available estimates, CVDs and malignant cancers are the two most common causes of death on a worldwide scale (WHO 2018a). Although both conditions fundamentally differ regarding underlying disease mechanisms, symptoms, outcomes and therapy, it is now sufficiently recognized that their occurrence is majorly influenced by a common set of lifestyle-associated risk factors such as smoking, physical inactivity or the consumption of certain dietary ingredients and/or patterns (reviewed by Ezzati and Riboli 2013). Especially a nutrition characterized as “unhealthy” (i.e. a diet low in fruits and vegetables and high in trans fatty acids, processed meat, salt or alcohol) has been strongly associated with the worldwide CVD and cancer burden (Lim et al. 2012). Accordingly, changes in lifestyle and especially nutritional behavior might reduce the incidence of NCDs and some authors even propose “[... ] that if the major risk factors for chronic disease were eliminated, around three-quarters of heart disease, stroke and type 2 diabetes would be prevented along with 40 % of cancers [...]” (Lenoir-Wijnkoop et al. 2013). In addition, preventing the development of these diseases would not only save 15 million people from an early passing each year (WHO 2018c), but also lift the enormous financial weight NCDs put on many countries’ healthcare systems (reviewed by Muka et al. 2015). It is therefore not surprising that the United Nations General Assembly adopted a resolution in 2011 aiming at reducing NCD occurrence (General Assembly resolution 66/2). Up to now, however, numerous countries have yet to implement many of the agreed-upon measures (WHO 2017b, 2018d).

Diet is evidently an important modifiable risk factor contributing to NCD development (Melaku et al. 2018). However, most people do not necessarily change their eating habits or lifestyle accordingly (reviewed by de Ridder et al. 2017). The reasons for this are manifold and range, for example, from a lack of knowledge about the implications a certain type of diet has on health (Sanderson et al. 2009), to a lack of motivation due to factors such as unavailable financial resources or slow health improvement in spite of dietary regimen changes (Nagelkerk et al. 2006; Linmans et al. 2015). On the other hand, as further reviewed by de Ridder et al. (2017), it is not necessarily a lack of information on healthy eating patterns and ingredients that frequently hampers a dietary change, but, in addition to various other psychological and (social) environmental factors, the consumer’s belief that his diet is already sufficiently healthy, his socio-economic status (e.g. income and education) as well as confusion and/or unresponsiveness resulting from overly complex or vague public information on nutrition. Irrespective of the actual reasons, a change of diet and lifestyle is generally perceived as hard to achieve (Kelly and Barker 2016). Expectedly, many people not willing to or not capable of significantly changing their diet might resort to the consumption of certain putatively health-promoting dietary products—comprehensively summarized as “health supplements” by Patwardhan et al. (2015) and elucidated in more detail in chapter 2.1—which seemingly promise an improvement of their health status in an effortless way (Goetzke and Spiller 2014; reviewed by van Buul and Brouns 2015). In addition, health supplements have become increasingly attractive to health-conscious consumers, who, for instance, seek to enhance their health in an ostensibly more natural and cheaper way when compared to standard medications (Sax 2015; Sauer and Plauth 2017), or use them to boost mental or physical performance...
Introduction

(Frey et al. 2017). Interestingly, the use of such products seems particularly common among people that physically exercise, especially professional athletes (reviewed by Knapik et al. 2016 as well as Garthe and Maughan 2018). The most frequently used health supplements thereby include, for example, protein and carbohydrate-containing products, various amino acids, minerals and vitamins as well as different ergogenic substances (i.e. physical performance enhancers) such as caffeine and creatine (reviewed by Parr et al. 2017). The rationale behind the use of such compounds, which, of course, is not only limited to a sportive environment, is often related to the physiological or pharmacological properties they exert.

A case in point is the main subject of the present work, a quaternary ammonium compound and conditionally essential nutrient termed L-carnitine (\(\text{L-}\gamma\text{-trimethylamino-}\beta\text{-hydroxybutyrate;}\) \textbf{Fig. 1}; Odle et al. 2014). Described in more detail in chapter 2.2, it has been advertised and marketed for almost forty years as a supplement to enhance physical performance and to decrease body weight (BW) due to its role in fatty acid catabolism (see \textbf{Fig. 2}; reviewed by Grunewald and Bailey 1993 as well as Jeukendrup et al. 1998; VZ NRW 2018).

\textbf{Fig. 1: The chemical structure of L-carnitine.} Structure adapted from the “PubChem Compound” database entry with the PubChem Compound Identifier (CID) 10917 (Kim et al. 2016).

The food industry has reacted to the popular demand (Skeie et al. 2009; Kantor et al. 2016; Knopf 2017) for health supplements by supplying an ever-growing global market (estimated at 114 billion € in 2016 [Grand View Research, Inc. 2018]) with a vast array of health-oriented foodstuffs (Biglardi and Galati 2013), as is impressively affirmed by over 85,000 dietary supplement products marketed currently in the United States of America (USA; Dwyer et al. 2018). However, albeit various supplements have been sold for roughly 100 years (reviewed by Swann 2016) and are essentially perceived as safe and effective (Ten Hoeve 2011; Sax 2015; Dodge 2016), concerns pertaining to their safety, quality, effectiveness, legal definition and regulation as well as marketing have been constantly raised (Katz 2013; Schmitt and Ferro 2013; Cohen 2014; Starr 2015; reviewed by Rocha et al. 2016, Aronson 2017, Končić 2018 and Ronis et al. 2018). For example, in the case of L-carnitine, it is still a matter of debate whether the above-mentioned claims regarding its ergogenic and weight-reducing properties are actually supported by scientific evidence (e.g. reviewed by Jeukendrup and Randell 2011 as well as Peeling et al. 2018; Del Vecchio et al. 2017). Moreover, on a larger scale, experts and governmental risk assessment agencies such as the German Federal Institute for Risk Assessment (BfR; German: \textit{Bundesinstitut für Risikobewertung}) even argue that the intake of health supplements is not only unnecessary when a balanced diet is consumed and a person does not suffer from a specific nutrient deficiency, but may actually be harmful to health (reviewed by McCormick 2010 as well as Kamangar and Emadi 2012; BfR 2018).

In view of the discussion surrounding L-carnitine and other health supplements, the following introductory chapters will firstly give a brief overview of supplement classifications and definitions as well as their legal regulation, with a special emphasis on the legislation in the European Union (EU). Later, L-carnitine and its physiological functions will be presented in more detail. Finally, information on potential adverse effects possibly related to a prolonged and high-dose L-carnitine intake investigated in the frame of the present work—i.e. colorectal cancer (CRC), atherosclerosis and (toxic) metabolite formation (e.g. dimethylnitrosamine
[DMN] and trimethylamine N-oxide [TMAO])—as well as details on the animal model and distinctive methods used herein (the Fischer 344 rat and metabolomics) will be given.
2. Literature review*

2.1. “Health supplements”

2.1.1. Definition(s)

The term “health supplements” is used by Patwardhan et al. (2015) to describe a rather large and heterogeneous group of foodstuffs encompassing, for example, products referred to as functional foods, nutraceuticals, phytochemicals, sports supplements or food (dietary) supplements. The consumption of such products is generally associated with an additional dietary value, allegedly providing benefits for health and/or an enhancement of physical performance as well as body functionality (Aggett et al. 2012). However, with the exception of the term “food supplements”, which is legally but rather broadly defined in the EU under Directive 2002/46/EC (see below for details), there is no legal and unanimously accepted designation for any of these food commodities (reviewed by Santini et al. 2018). Consequently, the distinctions between different kinds of health supplements are blurry and dealing with this topic is frequently accompanied by confusion (reviewed by Aronson 2017). For example, Humpf et al. (2014) concisely characterize functional foods as “[...] whole food[s], food supplement[s], enriched or fortified food[s] with beneficial health effects when consumed as part of a regular diet [...]”, while the term nutraceutical (a blend of the words “nutrition” and “pharmaceutical” penned by Stephen L. DeFelice, M.D.) quite similarly denotes “[...] ‘a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of a disease.’ [...]” (Brower 1998 as cited by Kalra 2003). To make matters worse, the latter sentence emphasizes that there is not only confusion, overlap and difficulties concerning the definition of various health supplement categories but also regarding their delimitation from medicinal products (i.e. drugs), whose purpose it is, in contrast to foodstuffs (Noble 2017; Stephan 2017), to “treat[...] or prevent[...] disease in human beings [...]” and/or “[...] to restor[e], correct[...] or modify[...] physiological functions by exerting a pharmacological, immunological or metabolic action [...]” (Article 1[1][b] of Directive 2004/27/EC). However, as further elaborated by Stephan (2017), there are food commodities that do contain compounds (e.g. caffeine or alcohol) exerting such (pharmacological) effects, without being inevitably classified as medicinal products and (stringently) regulated according to general medicinal law (Directive 2001/83/EC).

An interesting example showcasing the extent of this conundrum is the case of a food supplement named red yeast rice (RYR), which contains monakolin K, a compound identical to the active principle (lovastatin) of the first cholesterol-lowering statin drug Mevacor® (reviewed by Steffen 2017). Yet, in a ruling from 2009 (case C-140/07), the European Court of Justice did not classify a certain RYR supplement as a drug, even though RYR products may generally contain considerable levels of lovastatin inducing similar adverse effects as the prescription-only medicines—up to 19 mg/day when following the manufacturer’s intake recommendations (Gordon et al. 2010)—and some European regulatory bodies have either issued warnings over their consumption or legally consider them drugs above a daily statin dose of 5 mg (reviewed by Steffen 2017 and Santini et al. 2018). A similar disparity also exists in the case of L-carnitine-containing supplements, as courts in EU member states, for instance in Germany, have issued conflicting judgments as to whether certain products are to be

* Note: In order to keep the overall number of references low, reviews and textbooks were primarily used as sources of information throughout the literature review.
considered as drugs, which require a specific marketing authorization, or as dietary supplements (reviewed by Chopra et al. 2010).

Interestingly, it is clearly stated in Article 1(2) of Directive 2004/27/EC that in cases in which it is not clear whether medicinal or another set of laws (e.g. food law) applies to a certain product, such product has to be dealt with under the provisions of Directive 2001/83/EC, i.e. general medicinal law. Essentially, apart from the example of the RYR product mentioned above, this latter approach seems to be the practice generally followed by the European Court of Justice in case of doubt (Coppens et al. 2006).

### 2.1.2. Legal regulation in the European Union

In spite of certain issues regarding their definition and classification, health supplements are generally considered to be foodstuffs in the EU (Noble 2017). They are therefore governed by a transnational set of rules (e.g. summarized by Bragazzi et al. 2017), for which Regulation (EC) No 178/2002 (general food law) sets the basis, although national member state legislation still plays an important role as well (see e.g. Martinez-Sanz et al. 2017 and Noble 2017 for examples of national legislation in Spain and Germany, respectively). In Article 2 of Regulation (EC) No 178/2002, food is defined as “[...] any substance or product, whether processed, partially processed or unprocessed, intended to be, or reasonably expected to be ingested by humans [...]”. Also, according to Articles 14(1), 16, 17(1) and 19 of said regulation, all foodstuffs ought to be safe for the consumer, their “[...] labelling, advertising and presentation [...]” shall not be deceptive and food business operators are responsible for the compliance of their products with all legal provisions.

So far, the only statute specifically governing certain health supplement products in the EU is the so-called food supplements directive (Directive 2002/46/EC), in which food supplements are defined as “[...] foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients [i.e. vitamins and minerals] or other substances with a nutritional or physiological effect [...] marketed in dose form [...]” (Article 2a and b) and in which the use and labeling of certain vitamins and minerals as ingredients for food supplements is regulated. In contrast to these compounds (specified in the annexes of Directive 2002/46/EC as amended), the use and marketing of other health supplements or “other substances” (as mentioned in the definition of food supplements in Directive 2002/46/EC) is not precisely regulated in the EU and therefore fall under the jurisdiction of a diverse and rather intricate set of EU and/or national laws valid for all foodstuffs (Breitweg-Lehmann 2017; Noble 2017). This includes, apart from legislation generally governing the hygiene, safety and labeling of foods (e.g. Regulation [EC] No 852/2004, Commission Regulation [EC] No 2073/2005, Commission Regulation [EC] No 1881/2006 or Regulation [EU] No 1169/2011), the traditional herbal medicinal products directive (Directive 2004/24/EC), the health claims regulation (Regulation [EC] No 1924/2006), the fortified foods regulation (Regulation [EC] No 1925/2006), the food additives regulation (Regulation [EC] No 1333/2008), the foods for specific groups regulation (Regulation [EU] No 609/2013), the novel foods regulation (Regulation [EU] No 2015/2283) or a plethora of applicable legal provisions in each EU member state. However, a comprehensive discussion of all implicated European and national legal acts would go beyond the scope of the present overview and therefore only legislation with particular relevance to L-carnitine supplements will be presented in the following paragraph.

What sets health supplements largely apart from “regular” foodstuffs are the purported claims of a specific health or performance advantage manufacturers use to advertise and sell these products. Often these claims can be exaggerated or misleading (Covolo et al. 2013; Wang et al. 2014a; Avery et al. 2017). In order to protect consumers from such practices, the EU has
implemented legislative acts aiming at regulating the labelling and information accompanying foodstuffs. Generally, according to Article 7 of the food information for consumers regulation (Regulation [EU] No 1169/2011), “food information shall not be misleading [...]” (Article 7[1]), “[...] shall be accurate, clear and easy to understand for the consumer [...]” (Article 7[2]) and “[...] shall not attribute to any food the property of preventing, treating or curing a human disease, nor refer to such properties [...]” (Article 7[3]). Notwithstanding these provisions, the EU allows a food business operator to tag his product with so-called nutrition and health claims, provided these are made in accordance with the health claims regulation (see e.g. Gilsenan 2011 or Verhagen and van Loveren 2016 for a comprehensive procedural and regulatory overview). Health claims are defined in this statute as “[...] any claim that states, suggests or implies that a relationship exists between a food category, a food or one of its constituents and health [...]” (Article 2[5]) and are classified into so-called “general function claims” (Article 13; e.g. claims regarding functions of the body or weight control), “reduction of disease risk claims” (Article 14[1][a]) as well as “claims referring to children’s development and health” (Article 14[1][b]; Article 1[1] of Regulation [EC] No 109/2008 amending Regulation [EC] No 1924/2006). Most importantly, the health claims regulation establishes that any health claim made must to be scientifically substantiated (Article 6) before it can be formally authorized by the European Commission. The European Food Safety Authority (EFSA) thereby plays an important role in the frame of this process, as its Panel on Dietetic Products, Nutrition and Allergies (EFSA NDA Panel) scientifically evaluates the numerous claims before approval (Gilsenan 2011).

According to the EU Register of Nutrition and Health Claims (European Commission 2016), as of the 20th of October 2016 (date of last update), 260 out of a total of 2326 processed claims have been authorized—228 according to Article 13(1), 6 according to Article 13(5) (“[...] claims [...] based on newly developed scientific evidence and/or which include a request for the protection of proprietary data [...]”), 14 according to Article 14(1)(a) and 12 according to Article 14(1)(b) of Regulation [EC] No 1924/2006—which corresponds to a rejection rate of approximately 87 % and only supports the assertion that many of the claims made prior to the adoption of the health claims regulation were not appropriate. Remarkably enough, all health or function claims concerning food supplements comprising L-carnitine or one of its derivatives (e.g. “increase in endurance capacity” or “contribution to normal lipid metabolism”) submitted for evaluation to EFSA’s NDA panel were rejected as scientifically not tenable (EFSA NDA Panel 2011a, b, c; EFSA NDA Panel et al. 2018). Consequently, in the EU, it is not permitted to commercialize L-carnitine-containing dietary supplements in conjunction with any claim that promises positive effects on health, physical performance or weight reduction emanating from this compound. Nevertheless, L-carnitine supplements advertised with exactly such claims are very well available for sale in Europe, as underpinned by the results of a search performed on the 10th of September 2018 using Google as well as the keyword “L-carnitine”. That being said, L-carnitine is authorized EU-wide as an ingredient for use in food intended for special purposes according to Regulation (EU) No 609/2013 as well as a medicinal product for the treatment of specific medical conditions (see chapter 2.2.4 for details) in some EU countries (e.g. Germany; PharmNet.Bund 2018) and the USA (Winter 2003).

In summary, there is no legislative act that single-handedly regulates all types of health supplements in the EU, although the food supplements directive (Directive 2002/46/EC) as well as the health claims regulation (Regulation [EC] No 1924/2006) apply to a number of food commodities and the latter provides an acceptable level of consumer protection from fraudulent claims. Nevertheless, the European regulatory framework is complicated and contains numerous loopholes (e.g. lacking or unclear definitions), which not only leave room
for conflict and potential harm for the consumer, but may also impede growth and innovation in the health supplements sector (Gilsenan 2011; Moors 2012; Noble 2017; reviewed by Bröring et al. 2017).

2.2. L-carnitine

2.2.1. Physiological functions

The mammalian organism can oxidize macronutrients (carbohydrates, proteins and lipids) to CO\textsubscript{2} and H\textsubscript{2}O, which, in the process, leads to the production of adenosine triphosphate (ATP) necessary to fulfill its energetic needs. What kind of cellular fuel is used depends on the tissue, its function as well as its energy expenditure and requirements: for example, under non-fasting conditions, most tissues (the brain exclusively) use glucose to produce ATP, whereas fatty acids (FA) and ketone bodies—produced from FAs in the liver—are predominantly used in case of prolonged food shortage (Berg et al. 2007b, p. 855ff.; reviewed by Houten and Wanders 2010). Conversely, in muscle tissue, the consumption of a particular substrate is dependent on the degree of physical effort (i.e. glucose during high-intensity muscle work and FAs during extended low to moderate intensity muscle work; reviewed by Lundsgaard et al. 2018), with the exception of heart muscle, which relies mostly on FAs for energy production (Berg et al. 2007b, p. 856). FAs are primarily stored in adipose tissue, yield the most ATP per molecule out of all three types of macronutrients (reviewed by Melzer 2011) and are degraded through a process designated as β-oxidation, which mainly takes place in the mitochondrial matrix (Berg et al. 2007a, p. 692ff.). However, the majority of intracellular FAs are too long (so-called long-chain fatty acids [LCFA] with > 12 carbon atoms) to freely enter this compartment (Lehner and Quiroga 2016). Hence, as detailed step by step in Fig. 2, the mitochondrial uptake of activated LCFA needs to be assisted by a specialized transport system of which L-carnitine is an essential part and which is therefore appropriately termed “carnitine shuttle” (reviewed by Houten and Wanders 2010).

Apart from being indispensable for the transfer of activated LCFA into the mitochondrion, L-carnitine serves several “secondary” physiological functions, such as modulating LCFA use in biological membrane formation and remodeling Rebouche (2010, p. 108), acting as a possible antioxidant (Gülçin 2006) or as scavenger of (surplus) acyl residues (reviewed by Steiber et al. 2004). This last process regulates the coenzyme A (CoASH) to acyl-conjugated CoA ratio and therefore the amount of free CoASH available for other metabolic pathways as well as the inhibitory and/or toxic effects these acyl moieties might possibly exert on glycolysis, mitochondria or other bodily processes and compartments (reviewed by Mitchell et al. 2008 and Schooneman et al. 2013; Rebouche 2012, p. 443).

Importantly, only the L-enantiomer of carnitine occurs naturally and exerts biological functions, while D-carnitine potentially causes serious adverse effects (see chapter 2.2.2 for details; reviewed by Bieber 1988). Consequently, unless otherwise stated, the term “carnitine” refers solely to L-carnitine throughout the present work.
Fig. 2: Graphical depiction of the “carnitine shuttle”. Long-chain fatty acids (LCFA; brown-colored parts of the figure) destined for energetic catabolism in the mitochondrial matrix (β-oxidation) are transported from the bloodstream to the interior of the cell mostly via specialized transport proteins such as the “fatty acid translocase” (FAT; also termed CD36), various “fatty acid transport proteins” (FATP) or the “plasma membrane fatty acid-binding protein” (FABPpm; reviewed by Kazantzis and Stahl 2012). Carnitine enters the cell through a sodium-dependent transporter termed “carnitine/organic cation transporter 2” (OCTN2; reviewed by Tamai 2013). In order to trap the LCFA inside the cell and “activate” them for β-oxidation, “acyl-CoA synthetases” (ACS) firstly adjoint each fatty acid with a “coenzyme A” moiety (CoASH; red-colored parts of the figure), yielding so-called long-chain “acyl-CoA thioesters” (LC acyl-CoA; reviewed by Grevengoed et al. 2014). However, unless they are conjugated to L-carnitine (blue-colored parts of the figure), LC acyl-CoAs cannot penetrate the mitochondrion. Therefore, as reviewed by Houten and Wanders (2010) as well as Longo et al. (2016), they must be transferred across the organelle’s membrane using the so-called “carnitine shuttle”, a process encompassing several enzymes catalyzing three sequential reactions: 1. “carnitine palmitoyl transferase 1” (CPT1) transfers the acyl moiety from LC acyl-CoAs to carnitine, releasing CoASH and yielding long-chain acylcarnitines (LC acylcarnitines); 2. “carnitine acylcarnitine translocase” (CACT) shuttles the newly created acylcarnitines into the mitochondrial matrix in exchange for unbound carnitine generated in the frame of the third reaction; 3. “carnitine palmitoyl transferase 2” (CPT2) transforms LC acylcarnitines back to LC acyl-CoAs. These are subsequently broken down to acetyl-CoA molecules during the ensuing β-oxidation processes, which are then used by the cell to fuel the ATP-generating “Krebs cycle” (reviewed by Houten and Wanders 2010 as well as Knothnerus et al. 2018). The figure is an adaptation of Fig. 1 (“Schematic representation of mitochondrial fatty acid oxidation in humans”) from Knothnerus et al. (2018), used under the Creative Commons Attribution 4.0 International License (CC BY 4.0; http://creativecommons.org/licenses/by/4.0/). © The Author(s) 2018.
2.2.2. Homeostasis

L-carnitine was first isolated in 1905 by Gulewitsch and Krimberg from “Liebig’s Extract of Meat”, hence the name derived from the Latin word for “meat” or “flesh” (caro, carnis). According to Rebouche (2004), the biological processes determining L-carnitine homeostasis can be summarized as follows: “[c]arnitine homeostasis is maintained by absorption from diet, a modest rate of synthesis, and efficient renal reabsorption [...].” L-carnitine intake from food differs according to the daily dietary pattern and ranges from < 1 μmol/kg BW in individuals consuming a strictly vegan diet, up to 15 μmol/kg BW in people adhering to an omnivorous food regimen abundant in red meat (reviewed by Rebouche 2004). Omnivores thereby acquire most L-carnitine through their diet, especially meat products (Demarquoy et al. 2004). In contrast, people consuming vegetarian or vegan diets, which contain only little or minute amounts of this nutrient (Demarquoy et al. 2004), respectively, rely mostly on endogenous synthesis as well as increased renal reabsorption to upkeep L-carnitine homeostasis (reviewed by Reuter and Evans 2012 as well as El-Hattab and Scaglia 2015). Endogenous L-carnitine is synthesized in mammals by four successive enzymatic reactions from protein-derived lysine and methionine (Fig. 3), with the enzyme necessary for the last reaction—the hydroxylation of γ-butyrobetaine (γBB) to L-carnitine catalyzed by “γ-butyrobetaine dioxygenase” (EC 1.14.11.1)—only being expressed in selected tissues depending on the species (reviewed by Vaz and Wanders 2002). For example, in humans, the highest γ-butyrobetaine dioxygenase activity is observed in the kidney, although this enzyme is also functional in the brain and liver (Rebouche and Engel 1980). In contrast, in the rat, carnitine synthesis essentially occurs in the liver (Tanphaichitr and Broquist 1974). Remarkably, the tissues relying most on fatty acid oxidation for energy supply, namely heart and skeletal muscle, are not capable of de novo L-carnitine synthesis due to a lack of γ-butyrobetaine dioxygenase expression (reviewed by Rebouche 2004).

The majority of the total bodily L-carnitine pool—estimated to be ≈ 130 mmol in a healthy man weighing 70 kg and comprising free as well as acyl-conjugated L-carnitine—is stored in skeletal muscle tissue (Brass 1995). This fact is reflected by a large concentration difference between plasma (free L-carnitine: 40–50 μmol/L; total amount including acylcarnitines: 50–60 μmol/L) and muscle (2,000–4,000 μmol/kg), but also between plasma and other organs such as heart, kidney and liver (300–1,000 μmol/kg; reviewed by Rebouche and Paulson 1986 as well as Evans and Fornasini 2003). This “[...] steep concentration gradient [...]” implies that L-carnitine uptake must be governed by active energy-consuming processes (reviewed by Angelini et al. 1992). Indeed, L-carnitine is actively transported with variable affinity across the cellular membrane by a group of so-called “carnitine/organic cation transporters” (OCTN), which belong to the family of “solute carriers” (SLC), and which are expressed in a variety of tissues such as intestine, kidney, liver and muscle (reviewed by Tamai 2013). Although the mechanisms governing cellular carnitine uptake are not yet fully understood, it is well established that among all OCTNs, the high-affinity sodium-dependent Na+/carnitine symporter “OCTN2” (encoded by the SLC22A5 gene) plays the most prominent role in regulating L-carnitine homeostasis and transport across cellular membranes (reviewed by Strijbis et al. 2010 and Tamai 2013). As comprehensively summarized by Volk (2014) from the above-cited review by Tamai (2013), “[...] in the kidney, OCTN2 is involved in the reabsorption of carnitine in the proximal tubule, [...] it contributes to the resorption of carnitine [in small intestine], [...] it is needed for the intracellular accumulation of carnitine [in heart and skeletal muscle] [...] [and] impairment of OCTN2 function leads to systemic [or primary] carnitine deficiency [...] a disease that might cause cardiomyopathy and progressive skeletal weakness [...]” (see section 2.2.4 for details). Nonetheless, other, less specific active transporters (e.g. OCTN1 [encoded by the...
SLC22A4 gene [encoded by the SLC6A14 gene]) as well as passive diffusion seem to be additionally involved in L-carnitine transport across cellular membranes (reviewed by Reuter and Evans 2012).

Active cellular transport is generally a saturable process, which can be (simply) described by “Michaelis-Menten kinetics” (Vivian and Polli 2014; Alberts et al. 2015, p. 600ff.). “Michaelis-Menten constants” (K\textsubscript{m} = substrate concentration at which an enzymatic reaction proceeds at half-maximal velocity) for L-carnitine uptake in human, rat and murine OCTN2 have been reported as being in the low micromolar range (K\textsubscript{m} = 4–25 \textmu M; summarized by Kobayashi et al. 2005), while, in humans, general K\textsubscript{m} values for carnitine transport—i.e. values not specific for a particular carrier protein—vary greatly among different tissues and range from 2–60 \textmu M in heart and skeletal muscle, to 10–200 \textmu M as well as 500 \textmu M in kidney and liver, respectively, up to more than 1,000 \textmu M in the brain (reviewed by Reuter and Evans 2012). On the basis of these K\textsubscript{m} values, OCTN2 and possibly involved additional transporters in muscle and renal tissue are likely on the verge of saturation at physiological plasma carnitine concentrations and certainly saturated when high supradietary doses are administered (reviewed by Evans and Fornasini 2003, Stephens et al. 2007, Krähenbühl 2010 as well as Reuter and Evans 2012). This allows the organism to uphold L-carnitine homeostasis within narrow limits and has wide-ranging implications for this compound’s suitability as dietary supplement with added functionality. For example, the kidney’s reabsorption threshold for L-carnitine closely matches its plasma concentration (40–50 \textmu mol/L in healthy adults), for which reason this organ is able to retain up to 99 % of the circulating L-carnitine under normal physiological conditions and to excrete any superfluous carnitine in case it is administered in doses exceeding this limit (Rebouche et al. 1993; Bain et al. 2006; reviewed by Evans and Fornasini 2003). Likewise, in the intestine, the more L-carnitine is ingested, the less is absorbed, with the bioavailability of dietary doses being as high as 86 % and bioavailability of high supradietary doses (up to several grams or 100 mg/kg BW) ranging only from 4–25 % (reviewed by Rebouche 2004). This reduction in bioavailability is attributable to the saturation of the involved carrier proteins and the rather low membrane passage of the highly polar L-carnitine via passive diffusion, which seems to be the major mechanism of intestinal uptake once active transport is working at maximum capacity (Li et al. 1992; Claus 2014). The grounds on which L-carnitine is recommended and sold as ergogenic or weight-reducing supplement hinges on the assumption that exogenous supply will primarily increase the L-carnitine content in muscle tissue—thereby seemingly increasing the rate of fatty acid oxidation—and consequently spare “[... endogenous carbohydrate stores [...]” (Jeukendrup et al. 1998), reduce muscle fatigue and “[... improve endurance performance [...]” (Jeukendrup et al. 1998) as well as lower (excess) fat stores in the body (reviewed by Cerretelli and Marconi 1990, Jeukendrup et al. 1998, Karlic and Lohninger 2004, as well as Jeukendrup and Randell 2011). However, due to the effective elimination of surplus L-carnitine, the saturable nature of muscular uptake and because the muscle, regarding L-carnitine, constitutes a compartment largely disconnected from the rest of the body with a slow turnover of up to 8 days (Rebouche and Engel 1984; reviewed by Brass 1995), increasing the amount of this compound in muscle tissue seems to be virtually impossible (Wagenmakers 1999 as cited by Jeukendrup and Randell 2011; reviewed by Brass 2000 and Stephens et al. 2007). In fact, many studies demonstrate that there is only minor evidence—if any—that supplemental L-carnitine increases muscle carnitine content and would therefore significantly enhance physical performance or be of help in losing weight (reviewed by Brass 2000, Brouns et al. 2002, Karlic and Lohninger 2004 as well as Jeukendrup and Randell 2011; Burrus et al. 2018). Furthermore, even if the muscle carnitine content could be increased, FA oxidation would still not be enhanced, as its rate limiting steps,
i.e. fat store mobilization by the hormone-sensitive lipase (EC 3.1.1.79) and mitochondrial FA transport by CPT1, are either not influenced by L-carnitine at all (the former) or already saturated at physiological plasma carnitine concentrations (the latter; Ströhle et al. 2004).

Apart from the reversible conjugation to acyl moieties, L-carnitine is not metabolically transformed once absorbed by the mammalian organism and, in case it is not retained by the kidney, predominantly excreted via the urine (reviewed by Rebouche and Seim 1998 as well as Reuter and Evans 2012). Adult omnivores thereby eliminate $\approx 5 \mu\text{mol total L-carnitine/kg BW}$ each day, while grown-up individuals consuming a vegetarian or vegan diet excrete a daily amount of $\approx 1–2 \mu\text{mol total L-carnitine/kg BW}$ (Lombard et al. 1989), which nicely illustrates the kidney’s capacity to adjust its reabsorption capacity to a lower dietary intake in order to maintain bodily carnitine levels at physiological concentrations.

It has to be noted that the D-enantiomer of carnitine inhibits its cellular uptake, which not only leads to a decrease in tissue concentrations but also impedes fatty acid oxidation and causes muscle weakness and cardiac arrhythmias (reviewed by Kendler 1986 and Fuhrmann 2000, p. 80). This is of importance, as D-carnitine was detected in commonly available health supplements as part of a racemic mixture, i.e. in fractions up to $\approx 50\%$ of total carnitine content (Sánchez-Hernández et al. 2010). Although the majority of analyzed products (20 out of 22) in that specific study contained $\leq 3\%$ D-carnitine, the forbidden marketing of impure or racemic mixtures of carnitine—which are cheaper than pure L-carnitine (Benardot 2012, p. 121)—might still pose a health risk for the consumer (Sánchez-Hernández et al. 2010).

In conclusion, it can be deduced that the healthy human organism adapts intestinal uptake as well as renal reabsorption and excretion of L-carnitine to the ingested or circulating amount. Thus, the more L-carnitine is administered, the less is absorbed or retained, which keeps amounts in blood and tissues at constant physiological levels. L-carnitine ingested in high quantities as part of a health supplement will therefore not be absorbed for the most part and either be excreted unchanged via the feces or metabolized by the gut microbiota (see chapter 2.2.3 for details).

### 2.2.3. Bacterial metabolism

As discussed in the preceding section, even dietary amounts of L-carnitine are not fully taken up by the intestine, let alone high doses of several grams. Unabsorbed L-carnitine remaining in the bowel can be metabolized by microorganisms present in the mammalian gut, a process experimentally described in humans for the first time by Rebouche and Chenard (1991) and detailed in Fig. 3. In bacteria, L-carnitine generally functions as osmoprotectant and may be used as “[...] sole carbon, nitrogen and energy source” (reviewed by Rebouche and Seim 1998) as well as terminal electron acceptor under anaerobic conditions (reviewed by Meadows and Wargo 2015).
Fig. 3: Graphical depiction of L-carnitine catabolism and synthesis in the mammalian gut and liver. Ingested and unabsorbed L-carnitine as well as other dietary quaternary amines (e.g. choline) are degraded by certain types of gut bacteria to trimethylamine (TMA), which can then be further oxidized and demethylated to trimethylamine N-oxide (TMAO) as well as dimethylamine (DMA), respectively. DMA may subsequently be demethylated to yield methylamine (MA) and ammonia (NH\textsubscript{3}). After their formation in the bowel, TMA and the other bacterial carnitine metabolites can enter the circulation and reach the liver, where especially TMA is oxidized to TMAO by “flavin-containing monooxygenase 3” (depicted as FMO). As detailed in chapter 2.2.2, L-carnitine can be endogenously synthesized from N\textsuperscript{6}-trimethyllysine via \(\gamma\)-butyrobetaine (\(\gamma\BB\)). The methyl groups needed for the formation of the former molecule are basically derived from the one-carbon cycle, which involves the demethylation of methionine (methionine \(\rightarrow\) S-adenosylmethionine [SAM] \(\rightarrow\) S-adenosylhomocysteine [SAH] \(\rightarrow\) homocysteine) followed by the remethylation of homocysteine (homocysteine \(\rightarrow\) methionine), with the methyl group being derived from the folate cycle (5-methyl-THF [THF-\(\mathrm{CH}_3\) \(\rightarrow\) tetrahydrofollic acid [THF]) or other molecules such as betaine (trimethylglycine; reaction catalyzed by “betaine homocysteine S-methyltransferase” [BHMT]). The figure caption is summarized from Claus (2014) as well as review articles by Vaz and Wanders (2002), Blom and Smulders (2011) as well as Ducker and Rabinowitz (2017). Figure reprinted from Cell Metabolism, Volume 20, S. P. Claus, Mammalian-microbial cometabolism of L-carnitine in the context of atherosclerosis, Pages 699–700, Copyright (2014), with permission from Elsevier.
Several bacterial catabolic pathways for L-carnitine in the rat and human gut have been described over the years, mainly establishing that various species—some, such as Serratia marcescens, Salmonella Typhimurium or Pseudomonas aeruginosa, not necessarily part of a healthy gut microbiota—can degrade this compound to trimethylamine (TMA; Fig. 4A), γBB (Fig. 4B) or glycine via the sequential demethylation of trimethylglycine (reviewed by Kleber 1997, Rebouche and Seim 1998, Meadows and Wargo 2015 as well as Fennema et al. 2016; Uanschou et al. 2005). In fact, in mammals, TMA is entirely of bacterial origin (Seim et al. 1985; reviewed by Tang and Hazen 2014) and several works have shown that human and murine intestinal bacteria catabolize L-carnitine and structurally related quaternary amines such as betaine and choline to TMA and γBB—the “[...] immediate biosynthetic precursor [...]” (Koeth et al. 2014) of L-carnitine—in distinct gut segments, with intestinal bacteria also degrading the latter molecule to TMA (Fig. 3; Zhang et al. 1999; Wang et al. 2011; Koeth et al. 2013; Koeth et al. 2014).

Fig. 4: The chemical structure of trimethylamine (A; TMA) and γ-butyrobetaine (B; γBB), both bacterial metabolites of L-carnitine. Structures adapted from the “PubChem Compound” database entries with the CIDs 1146 (TMA) and 725 (γBB; Kim et al. 2016).

After its enteral formation, most TMA—a gaseous and fishy-smelling compound—crosses the intestinal barrier by passive diffusion and reaches the liver through the hepatic portal circulation, where up to 99 % are oxidized to the odorless trimethylamine N-oxide (TMAO; Fig. 5A) by “flavin-containing monoxygenase 3” (FMO3; Fig. 3; Zhang et al. 1995; reviewed by Mackay et al. 2011). This latter compound is then subject to an almost complete urinary elimination in less than 24 h (Al-Waiz et al. 1987). In humans, a defective FMO3—an enzyme also expressed in other mammalian species (reviewed by Krueger and Williams 2005)—is the primary cause of trimethylaminuria, a medical condition accompanied by a characteristic bodily malodor (“fish odor syndrome”) due to non-functioning TMA oxidation and its subsequent excretion “[...] in the urine, sweat, expired air, and other bodily secretions [...]” (reviewed by Mitchell and Smith 2001). In addition to being oxidized to TMAO, TMA may be demethylated by “cytochrome P450 monooxygenases” (CYP) in the liver of rodents and humans to form dimethylamine (DMA; Fig. 5B) and methylamine (MA; Gut and Conney 1993; reviewed by Russell et al. 2013).

Fig. 5: The chemical structure of trimethylamine N-oxide (A; TMAO) and dimethylamine (B; DMA), hepatic and bacterial metabolites of TMA. Structures adapted from the “PubChem Compound” database entries with the CIDs 1145 (TMAO) and 674 (DMA; Kim et al. 2016).
However, the mammalian liver is not the only place of TMA transformation, as gut bacteria are also able to metabolize this amine to TMAO and DMA (Fig. 3; Smith et al. 1994; reviewed by Chhibber-Goel et al. 2016 and Fennema et al. 2016). Interestingly, a microbiota-mediated conversion of TMAO back to DMA (Zhang et al. 1993) and TMA (Hoyle et al. 2018) has also been described (Fig. 3).

The occurrence of the above-mentioned gut flora-dependent L-carnitine metabolites has potentially important implications for human health, as they have been either associated with the formation of carcinogenic nitrosamines—especially DMN (reviewed by Bain et al. 2005)—or as being involved in atherosclerogenesis (reviewed by Tang and Hazen 2014). Both of these potentially health-impeding associations will be discussed in more detail in chapter 2.3.

2.2.4. Deficiencies and supplementation in disease

According to Pons and De Vivo (1995), “[c]arnitine deficiency can be defined as a state of carnitine concentration in plasma or tissues that is below the requirement for the normal function of the organism [...]”. Two types of carnitine deficiency are commonly distinguished, namely “primary carnitine deficiency” (PCD) and “secondary carnitine deficiency” (SCD), although differences between both types may not always be clear-cut (Angelini et al. 1987).

PCD results from a variety of rare (incidence ranging from ≈ 1:40,000 to 1:100,000) autosomal recessive mutations in the gene coding for OCTN2, which results in an impaired cellular uptake as well as increased renal loss of L-carnitine (reviewed by Longo et al. 2006 and Fu et al. 2013). The clinical appearance of PCD is heterogeneous as well as age-dependent and mainly encompasses a hepatic form in infants (≈ 2 years of age; clinical findings include hypoketotic hypoglycemia, hyperammonemia and hepatic encephalopathy), a myopathic form in children (≈ 4 years of age; clinical findings include progressive cardiomyopathy, hypotonia and muscle weakness) and a comparably mild form in adults, either manifesting with no symptoms at all or merely through decreased physical fitness (reviewed by Magoulas and El-Hattab 2012). The therapy of this disorder consists principally of high-dose oral L-carnitine supplementation (e.g. 50–400 mg/kg BW/day; reviewed by Magoulas and El-Hattab 2012). As such, PCD is, according to Stanley (2004) and Odle et al. (2014), the only carnitine-related disease in which oral supplementation of L-carnitine has been shown to be conclusively effective and clinically validated. Notwithstanding this, the United States Food and Drug Administration has permitted the use of L-carnitine for the mitigation of SCD induced by hemodialysis (Winter 2003).

The causes underlying SCD are more diverse than those that determine PCD and comprise congenital errors of metabolism (reviewed by Vernon 2015)—i.e. mutations in genes encoding for proteins involved in various metabolic pathways—as well as a plethora of various other factors, including chronic renal and liver diseases—resulting in decreased L-carnitine synthesis or increased L-carnitine loss—malnutrition and malabsorption—resulting in decreased L-carnitine uptake—or therapeutic interventions such as hemodialysis and treatment with certain drugs (e.g. valproic acid; reviewed by Pons and De Vivo 1995). Depending on the underlying condition, the clinical symptoms of SCD may be either similar or less serious to those caused by PCD and may be initiated by stressful events such as fasting, exercise or infection (Duran et al. 1990; reviewed by Pons and De Vivo 1995 as well as Reuter et al. 2008). Still, whether L-carnitine supplementation is warranted as therapy of SCD is a matter of debate. On the one hand, several studies show that L-carnitine and some of its acyl derivatives may indeed have beneficial effects when supplemented in case of SCD or even other conditions which are not primarily related to L-carnitine metabolism, for example chronic CVDs, type 2 diabetes, chemotherapy-induced fatigue, infertility or Alzheimer’s disease (reviewed by Ahmad 2001; Ferrari et al. 2004, Bain et al. 2006, Reuter et al. 2008, Flanagan et al. 2010 and Wang
et al. 2018; Winter 2003; Matsui et al. 2018). However, just as many scientists argue that L-carnitine supplementation is not warranted under all circumstances, that there is no conclusive proof of efficacy of carnitine/acylcarnitine supplementation in SCD as well as in other disease states and that supplementation would only be necessary if body stores drop to a very low level (reviewed by Kerner and Hoppel 1998, Ahmad 2001, Bain et al. 2006, Flanagan et al. 2010, Mingorance et al. 2011, Shang et al. 2014 and Fernandez-Prado et al. 2017; Stanley 2004; Odle et al. 2014; Liepinsh et al. 2015).

2.3. Bacterial metabolites: a possible link between L-carnitine intake and adverse effects?

2.3.1. L-carnitine-derived amines and potential N-nitrosamine formation

In contrast to CVDs (see chapter 2.3.2), cancerous diseases only account for ≈ 15 % of all estimated worldwide death cases in 2016, although this number still translates to about 9 million people (WHO 2018a). Global predictions compiled by the International Agency for Research on Cancer (IARC) for the year 2018 suggest that, taken together, malignant neoplasms of the gastrointestinal tract—including cancers of the mouth and oropharynx, esophageal cancer, stomach cancer as well as CRC—are the most common types of cancer in both men and women (≈ 3.9 million new cases), with stomach cancer (≈ 1 million new cases) and CRC (≈ 1.8 million new cases) being diagnosed the most (Ferlay et al. 2018). Already in 1981 Doll and Peto estimated in a pioneering article that diet probably accounted for 35 % of all cancer deaths. While this value might have been too high—as suggested by Blot and Tarone (2015) and evidenced by the newest cancer incidence estimates from Germany (Behrens et al. 2018)—diet still appears to play an important role in the development of gastrointestinal cancers, especially risk factors such as the rather controversially discussed consumption of red and processed meat (reviewed by Brenner et al. 2014, Abnet et al. 2015, Zhao et al. 2017a, Zhao et al. 2017b as well as Kruger and Zhou 2018; Bouvard et al. 2015; Vineis and Stewart 2016).

Several carcinogenic compounds such as aflatoxins or polycyclic aromatic hydrocarbons can either contaminate various foodstuffs or be formed during their preparation and/or preservation (reviewed by Abnet 2007). Of particular interest in this context are N-nitrosamines, which are regularly identified in some food commodities and, along with N-nitrosamides, constitute the group of N-nitroso compounds (NOCs; Fig. 6A; Pfundstein and Spiegelhalder 2007, p. 931 and 939ff.). Depending on the detection method used for their identification, N-nitrosamines can be further divided into volatile compounds such as DMN (Fig. 6B) or diethylnitrosamine (DEN) and non-volatile compounds such as N-nitrosoprolin (Pfundstein and Spiegelhalder 2007, p. 932 and 936).

![Fig. 6: The general chemical structure of N-nitroso compounds (A; NOCs; R1 and R2 denote various possible organic substituents such as acyl or aryl groups) and the chemical structure of dimethylnitrosamine (B; DMN). Structures adapted from Pfundstein and Spiegelhalder (2007, p. 931; NOCs) and the “PubChem Compound” database entry with the CID 6124 (DMN; Kim et al. 2016).](image-url)
Generally, N-nitrosamines result from the reaction of amines with nitrosating agents such as nitrite (NO$_2^-$) or oxides of nitrogen (NO$_x$; Fig. 7; Eisenbrand and Habermeyer 2013, p. 775). Strictly speaking, in the human stomach, the actual nitrosating agent is not nitrite, but dinitrogen trioxide (N$_2$O$_3$) formed by NO$_2$ via nitrous acid (HNO$_2$) in aqueous solutions (Fig. 7A; reviewed by Habermeyer et al. 2015). They are commonly derived from the nitrosation of secondary or, to a lesser degree, tertiary amines (Fig. 7B; reviewed by Tricker and Kubacki 1992). Nonetheless, the formaldehyde-catalyzed reaction of the primary amine MA with NO$_2$ also yields nitrosamines such as DMN (Obiedzinski et al. 1980). Depending on the basicity of the amine, the nitrosation reaction occurs preferably in an acidic environment (pH < 5), although nitrosamine formation occurring under less acidic as well as alkaline conditions or even in physiological matrices (e.g. blood) has been described (Lijinsky et al. 1972; Keefer and Roller 1973; Challis and Kyrtopoulos 1977; Eisenbrand and Habermeyer 2013, p. 775).

![Fig. 7: General mechanism of secondary amine nitrosation.](image)

Humans can be exposed to NOCs preformed exogenously or through their endogenous formation at various body sites (e.g. the stomach or bladder; reviewed by Tricker and Preussmann 1991, Mirvish 1995 as well as Hill 1996). Exogenous formation of NOCs may occur for instance in tobacco, certain dietary (e.g. cured meats, smoked fish, cheese and beer) and medicinal products (e.g. valsartan; EMA 2018) as well as during industrial activity (e.g. rubber production; (Scanlan 2003, p. 4143ff.; Eisenbrand and Habermeyer 2013, p. 776ff.). Endogenous formation is mainly attributable to the ingestion or presence of certain (dietary) precursor molecules (e.g. DMA and other amines or NO$_2$ derived from NO$_3$ reduced in the saliva or stomach), the endogenous production of nitric oxide (e.g. during inflammatory processes) or to bacterial redox metabolism of various nitrogen species (Tricker et al. 1992; Scanlan 2003, p. 4143ff.; reviewed by Habermeyer et al. 2015). Since these processes can either take place under acidic conditions or physiological pH, “[... NOC formation may consequently] occur at a number of sites in the body [...]” (reviewed by Hughes and Rowland 2000 as well as Habermeyer et al. 2015). Interestingly, endogenous formation of NOCs—estimated to range from 2.5–4.4 µg/day in women and men, respectively—exceeds exogenously assimilated nitrosamine amounts by ≈ 10-fold (Pfundstein and Spiegelhalder 2007, p. 951f.), inferring that endogenous formation is the main route of NOC exposure in humans (reviewed by Tricker 1997).

The carcinogenic potential of NOCs was firstly reported by Magee and Barnes in 1956, who showed that DMN induced hepatic neoplasms in a non-specified albino rat strain. Since then,
> 300 NOCs have been investigated regarding their tumor-inducing potential and were found to constitute one of the most potent class of carcinogens, inducing tumors in all 39 animal species used up to that point (reviewed by Bogovski and Bogovski 1981 as well as Tricker and Preussmann 1991). More specifically, DEN was carcinogenic in all 26 animal species examined, ranging from amphibians and fishes up to primates, whereas both DMN as well as methylnitrosourea—a nitrosamide—were tumorigenic in the 16 species used to investigate their carcinogenic effects (reviewed by Bogovski and Bogovski 1981). A characteristic feature of NOC carcinogenicity in animals is a pronounced organotropism highly dependent on the chemical structure, the administered dose, the method as well as the interval of NOC application and the animal species used to investigate tumorigenic effects (Lijinsky 1987). For example, as summarized by Pfundstein and Spiegelhalder (2007, p. 947), symmetrically substituted alkyl or aryl N-nitrosamines—whose carcinogenic potency diminishes with increasing molecular weight—predominantly induce liver tumors in animals, while unsymmetrically substituted molecules rather lead to the formation of esophageal cancers. In contrast, owing to their chemical instability, N-nitrosamides mainly exert local effects at the place of application or formation (Pfundstein and Spiegelhalder 2007, p. 932f. and 947).

In order to exert their carcinogenic activity, N-nitrosamines—but not spontaneously decomposing N-nitrosamides—need to firstly be metabolized by CYPs expressed in various tissues (DMN is e.g. metabolized in buccal mucosa, esophagus, colon, liver and bladder), a process yielding highly reactive metabolites, which are able to alkylate DNA and thus to induce tumor-forming mutations (Fig. 8; reviewed by Tricker and Preussmann 1991).

**Fig. 8: Graphical depiction of the toxicogenation of NOCs.** Based on book chapters written by Yang et al. (1994) as well as Eisenbrand and Habermeyer (2013), the metabolic activation of NOCs can be outlined as follows: N-nitrosamines are hydroxylated at the α-carbon atom by CYPs to yield an “α-hydroxynitrosamine” derivative. In contrast, the nitrosated amide “alkylnitrosourea” is not subjected to enzymatic hydroxylation, as it spontaneously forms the above-mentioned “α-hydroxynitrosamine” in aqueous solutions at physiological pH. Due to its chemical instability, the α-hydroxylated derivative—the proximal carcinogen—decomposes and eventually gives rise to a carbonyl metabolite (R–CHO) as...
well as to “alkyl diazohydroxide” or the corresponding “diazonium ion”—the ultimate carcinogens—whose electrophilic “carbenium” (R–H₂C⁺) moiety may covalently methylate nucleophilic sites of nucleobases (especially guanine) and proteins. Nitrosamines containing short-chain alkyl groups (e.g. dimethylnitrosamine; DMN) are predominantly metabolized by CYP isof orm 2E1 (CYP2E1), although large differences between species, specific nitrosamines and involved metabolizing CYP isoforms exist.

Figure reprinted from Mutation Research, Volume 259, A. R. Tricker and R. Preussmann, Carcinogenic N-nitrosamines in the diet: occurrence, formation, mechanisms and carcinogenic potential, Pages 277–89, Copyright (1991), with permission from Elsevier.

Although the overwhelming amount of animal carcinogenicity data as well as occupational, dietary or leisure exposure of humans strongly suggests that NOCs may also be carcinogenic in humans and several sites at which NOCs may predominantly induce cancer have been proposed—especially in the gastrointestinal tract—no definitive conclusion as to the causality of NOC exposure in the formation of such cancers has been established over the years (reviewed by Bartsch and Montesano 1984, Mirvish 1995, Eichholzer and Gutzwiller 1998, Lijinsky 1999, Jakšyzn and González 2006 as well as Song et al. 2015; Scanlan 2003, p. 4146; Keszei et al. 2013). This is also reflected by the IARC classification of certain NOCs such as DMN, which, as of the 30th of July 2018 (date of last update), are not classified as carcinogenic to humans (group 1) but merely as probably carcinogenic to humans (group 2A; IARC 1987; IARC 2018). Nevertheless, a limited number of studies have found a significant association between DMN intake and CRC (Knekt et al. 1999; Loh et al. 2011).

As noted by Bae et al. (2014), “the association between gut microbiota-dependent [...] [L-carnitine] metabolites and [...] [CRC] [...] is unknown [...], although they found an association between elevated plasma TMAO levels and CRC in postmenopausal women. Since the bacterial degradation of L-carnitine in the gut leads to the formation of nitrosatable precursors in the form of secondary (DMA) and tertiary amines (TMA and TMAO; see 2.2.3 for details), the ingestion of this nutrient may result—in the presence of dietary or endogenous nitrite as well as, optionally, bacterial species harboring specific enzymes—in the formation of N-nitrosamines (especially DMN) and consequently induce CRC or other gastrointestinal cancers (reviewed by Bain et al. 2005; Ufnal et al. 2015 and Subramaniam and Fletcher 2018). Indeed, as further reviewed by Bain et al. (2005), processes leading to DMN formation from L-carnitine metabolites have been described as taking place under conditions prevailing in certain areas of the human gastrointestinal tract, although these studies were performed mostly in vitro.

2.3.2. TMAO and atherosclerosis

By far, CVDs account for the most deaths globally, with an estimated ≈ 18 million cases (≈ 31 % of all deaths) in the year 2016 (WHO 2018a). CVD is a term generally referring to various medical conditions of the cardiovascular system, encompassing disorders such as “coronary heart disease” (also known “ischemic heart disease”), “cerebrovascular disease” (i.e. “stroke”), “peripheral arterial disease”, “rheumatic heart disease”, “congenital heart disease”, “deep vein thrombosis” and “pulmonary embolism” (WHO 2017a). Coronary heart disease and ischemic stroke—which are both projected to account for approximately 70 % of all CVD-related deaths (WHO 2018a)—but also many other CVDs are largely due to a pathological process termed “atherosclerosis” (reviewed by Frostegård 2013). Commonly, this process is defined by the gradual build-up of so-called “atherosclerotic plaques” in the innermost layer of an artery (i.e. the tunica intima), initiating narrowing as well as “sclerosis” (i.e. hardening; derived from the Greek word for “hard” [σκληρός]) of the vessel and subsequent “thrombosis” (i.e. interruption of the blood flow due to the formation of coagulates) induced mainly by
ruptured plaques (reviewed by Sakakura et al. 2013 and Rafieian-Kopaei et al. 2014). The result of this obstruction are pathologies more commonly known as “heart attack” or “stroke” (PubMed Health 2014), which are characterized by “ischemia”, a term delineating the “[...] occlusion of the arterial blood supply [...] resulting in a severe imbalance of metabolic supply and demand, causing tissue hypoxia [...]” (Eltzschig and Eckle 2011). If appropriate measures are not taken in time, the ensuing “infarction” (i.e. cellular necrosis) of the affected tissues can lead to a patient’s death (Caplan 2009; Calvert 2014).

Atherosclerosis is considered a chronic inflammatory disorder, instigated by the deposition and retention of “[...] cholesterol-containing low-density lipoprotein (LDL) particles [to proteoglycans] in the arterial wall [...]” (Bäck and Hansson 2018, p. 45). In order for these particles to enter the intima and cause an initial atherosclerotic lesion termed “(pathologic) intimal thickening”, endothelial function must be disturbed and its permeability increased, a process predominantly occurring in the parts of a vessel where the blood flow is altered (e.g. at bifurcations) and which is promoted by “irritative stimuli” (e.g. oxidative stress) stemming from lifestyle-dependent factors such as smoking, dyslipidemia, diabetes or hypertension (reviewed by Libby et al. 2011, Bergheanu et al. 2017 and Kattoo et al. 2017; Zaromitidou et al. 2016). Once in the subendothelial compartment of the intima, LDL undergoes chemical changes (e.g. oxidation), eliciting endothelial cells to draft leukocytes (primarily monocytes but later also T lymphocytes) to the emerging atherosclerotic lesion and thus initiate an inflammatory process largely responsible for promoting the progression from initial “(pathologic) intimal thickening” to a lesion termed “fibroatheroma” up to final plaque and thrombus formation (Zaromitidou et al. 2016; Bäck and Hansson 2018).

Based on reviews by Libby et al. (2011), Moore and Tabas (2011), Bergheanu et al. (2017) and Durham et al. (2018), atherosclerotic plaque formation can be briefly summarized as follows: firstly, after LDL retention stimulates monocytes to enter the vessel wall, they differentiate to macrophages, start taking up these modified LDL molecules and form so-called “foam cells”. All the while vascular smooth muscle cells (VSMC)—either originating from the tunica intima or migrated from the tunica media—begin to multiply and produce extracellular matrix molecules (e.g. collagen and proteoglycans). As lesion formation advances, the extracellular matrix components form a so-called “fibrous cap” around it, effectively shielding it from coagulation factors in the blood. Furthermore, owing to various cellular stress factors, foam cells start decaying inside the encapsulated lesion (e.g. through apoptosis), eventually releasing the previously accumulated intracellular lipids as well as other cellular remains into the interstitial compartment and thereby inducing the creation of the so-called “necrotic core of the plaque”—a typical feature of fibroatheromas. Finally, macrophage-triggered death of VSMCs as well as macrophage-derived proteases lead to the weakening of the fibrous cap—creating a so-called “vulnerable plaque”—which may ultimately rupture and consequently induce the formation of a thrombus impeding blood flow. In addition, plaque rupture as well as vessel stiffness (i.e. sclerosis) is promoted by intimal calcification sustained by VSMCs differentiated to an osteochondrogenic phenotype.

In the midst of the currently ongoing discussion on the role of LDL cholesterol in atherosclerosis development (see e.g. DuBroff 2017, Ference et al. 2017 and Okuyama et al. 2018), gut flora-derived TMAO quite suddenly appeared in the public eye as potentially causative agent of atherosclerotic CVD, providing a possible explanation for the association of meat-heavy diets with this disorder (Koeth et al. 2013), or for the fact that significantly lowering serum cholesterol levels (e.g. through statin-containing drugs) does not seem to influence atherosclerosis development much (reviewed by Spector 2016 and Spence 2016). In fact, recent studies—mainly from Dr. Stanley L. Hazen’s lab—suggest that TMAO derived from bacterial metabolism...
of dietary quaternary ammonium compounds (e.g. L-carnitine; see chapter 2.2.3 for details) very possibly induces and correlates concentration-wise with atherosclerosis as well as ischemic CVD in humans (Wang et al. 2011; Koeth et al. 2013; Tang et al. 2013; Koeth et al. 2014; Tang et al. 2014; Wang et al. 2014b; Zhu et al. 2016; Li et al. 2017; Zhu et al. 2017; reviewed by Qi et al. 2018). Yet, the exact molecular processes by which TMAO presumably promotes atherosclerosis still remain elusive, although TMAO-related increase in foam cell formation and inflammation as well as alterations in cholesterol metabolism/transport have been brought forward as putative mechanisms (reviewed by Zeisel and Warrier 2017). Moreover, it has to be noted that not all scientists agree with this “TMAO-promotes-CVD-hypothesis” (e.g. Landfald et al. 2017) and that several studies have either not found a significant association between TMAO and CVD or even attribute (cardio)protective effects to this compound (Mueller et al. 2015; Yin et al. 2015; Collins et al. 2016; reviewed by Zeisel and Warrier 2017 as well as Nowiński and Ufnal 2018).

2.4. The animal model as well as distinct analytical methods used in present work

2.4.1. The Fischer 344 rat

The Fischer 344 (F344) rat is an inbred strain originally developed in September 1920 at Columbia University (New York, USA) by mating breeding pair number 344 obtained from a local rat breeder named Fischer, hence the name (Lindsey and Baker 2006, p. 28f.). Since its creation, this multipurpose (Siglin et al. 2014, p. 32) strain has predominantly been used in cancer research due to “[...] its sensitivity to various chemical classes, its relatively [sic] low spontaneous tumor rate (except for testicular Leydig cell tumors), [...] its small size, good breeding characteristics, [adequate lifespan], and relatively easy maintenance [...]” (Cameron et al. 1985; Foster and Frost 2018, p. 10f.). It has been consequently chosen since the 1970s as the strain of predilection used in chronic carcinogenicity studies performed by the US National Cancer Institute and National Toxicology Program (NTP; Foster and Frost 2018, p. 10).

Untreated F344 rats are known to develop certain spontaneous malignancies in a gender-dependent manner: male rats develop, in this order of frequency, testicular adenoma, mononuclear cell leukemia, adrenal gland pheochromocytoma and pituitary gland neoplasms, while female animals mostly develop pituitary gland neoplasms, followed by mammary gland fibroadenomas and mononuclear cell leukemia (Haseman et al. 1998). Indeed, it was the high incidence of some of these spontaneous neoplasms (e.g. Leydig cell tumors) as well as other disorders (e.g. occasional seizures), along with progressively decreasing fertility and the intent to prospectively use a common rat breed suited for all types of studies, which led the NTP to stop using its in-house F344 substrain (F344/N) in favor of Sprague-Dawley rats starting in 2006, in spite of extensive control and carcinogenicity data acquired over its decades-long use (reviewed by Maronpot et al. 2016). Nevertheless, as further reviewed by Maronpot et al. (2016), the tumors inherently found in F344(N) rats are most likely not of any relevance for human carcinogenesis, and thus this strain may still be a usable model to study cancers or other conditions. This would be especially true for animals not derived from the NTP stock and thus not necessarily affected by additional fertility problems, seizures or chylothorax (reviewed by King-Herbert et al. 2010).
2.4.2. Metabolomics

So-called “omics” techniques are high-throughput laboratory methods aiming at investigating an extensive set of cellular or bodily components at once—e.g. changes in the genome ("genomics") or changes in mRNA and protein expression ("transcriptomics" and "proteomics", respectively; reviewed by Altmäe et al. 2014). In contrast, "classical" laboratory methods (e.g. PCR or western blotting) usually only allow the detection of one single parameter per sample at a time, although they are valuable for the validation of results obtained using “omics” techniques (reviewed by Altmäe et al. 2014). As such, according to Riekeberg and Powers (2017), “[m]etabolomics is a rapidly growing field of study that endeavors to measure the complete set of metabolites (generally considered to be the intermediates and products of cellular metabolism less than 1 kDa in size) within a biological sample (that is, the metabolome) in order to achieve a global view of the state of the system [...].” This is accomplished by subjecting biological samples—which may be all kinds of body fluids and excretions (e.g. blood, urine or sweat) as well as tissues or cells (reviewed by Trivedi et al. 2017)—to examination by advanced analytical chemistry techniques such as liquid or gas chromatography coupled with mass spectrometry (LC-MS and GC-MS, respectively) or nuclear magnetic resonance (NMR; reviewed by Smolinska et al. 2012). Based on review articles published by Johnson et al. (2016) and Newgard (2017), two metabolomics approaches are generally distinguished: firstly, “untargeted” or “shotgun metabolomics”, a method best performed using high-resolution mass spectrometry and mostly applied to analyze the change in relative abundance of a large and previously unknown set of metabolites (≈ 3,000) in a single experimental entity or between two or more test groups (e.g. control vs. treated). On the other hand, “targeted metabolomics”, are utilized to measure absolute concentrations of a predefined and rather small group of (interrelated) compounds, for instance quaternary ammonium derivatives such as L-carnitine, betaine and choline (see chapter 5 for details). In contrast to a targeted approach—where the metabolites analyzed are known and quantified via the above-mentioned techniques using (commercially available) standards—the reliable detection of metabolites as well as possibly related metabolite clusters is not as easy in the frame of an untargeted approach. A case in point is metabolite identification, which usually occurs by comparing the recorded spectra to data contained in far-from-complete-databases (reviewed by Riekeberg and Powers 2017). Thus, many metabolites contained in a sample may remain undetected, in addition to other critical factors possibly affecting metabolite discovery such as sample preparation or the chemistry of the compound(s) in question (reviewed by Johnson et al. 2016). In addition to metabolite identification, which is considered the most laborious step encountered during an untargeted metabolomics analysis, the large datasets obtained from such a study necessitate the application of several processing steps (e.g. filtering, imputation of missing data or transformation) before rather sophisticated statistical analyses (e.g. principal components analysis or various univariate analyses such as the t-test or an analysis of variance)—allowing the actual biological interpretation of the data—can be applied (reviewed by Spicer et al. 2017). Even though untargeted metabolomics is not as accurate, more time-consuming and its data often more difficult to analyze and interpret than targeted analytical methods, their use allows the objective investigation of changes possibly occurring in a very large set of small molecules and thus the uncovering of potential new and unexpected disease-related biomarkers or alterations of cellular pathways (reviewed by Johnson et al. 2016). In fact, untargeted metabolomics have been successfully used to identify and validate more than 100 metabolic biomarkers linked to various diseases, including the relationship between plasma TMAO and CVD (see chapter 2.3.2 for details; reviewed by Newgard 2017 and López-López et al. 2018).
3. Aims of the present work

As discussed in the preceding sections, L-carnitine-containing health supplements are readily available for sale and advertised with all sorts of scientifically non-substantiated claims regarding their performance-enhancing as well weight-reducing properties. Many of these products contain L-carnitine in considerable quantities of up to several grams. In view of the inverse relationship between orally supplemented amounts and bioavailability, it has to be expected that a significant proportion of a high oral L-carnitine dose would not be absorbed by the intestine and therefore become accessible to gut microbial metabolism prior to fecal excretion.

The occurrence and chemical transformation of bacterial L-carnitine metabolites are possibly associated with the onset of adverse effects. The general aim of the present study was therefore to investigate whether oral L-carnitine doses up to 5 g/L, administered for the duration of one year to experimental animals (rats) and reflecting a high “recreational” human consumption pattern, might induce specific adverse effects (see objectives below) in said animals. As such, the data obtained herein could be useful in the assessment of the potential risk stemming from the regular and/or excessive consumption of freely available L-carnitine supplements. Overall, the general aim of the present study can be divided into primary and secondary objectives.

3.1. Primary objective

Intestinal bacterial metabolism of L-carnitine results in the formation of metabolites containing an amine moiety such as DMA, TMA and TMAO (see chapter 2.2.3 for details), which could, under conditions prevalent for instance in the stomach or the colon, be transformed to potentially carcinogenic N-nitrosamines, especially DMN (see chapter 2.3.1 for details). The primary objective of the present study was thus to investigate whether a chronic high-dose administration of L-carnitine would induce the emergence of preneoplastic lesions in the rat gut. The F344 rat (strain DuCrI) was chosen as an animal model for this task, as it is a well-accepted and established model used for decades in carcinogenicity testing.

3.2. Secondary objectives

In the course of the aforementioned carcinogenicity study, an article published by Koeth et al. (2013) reported that gut bacterial metabolism of L-carnitine—TMAO to be precise—increased atherosclerotic lesions in mice. This finding prompted the conduct of additional analyses, aiming at examining whether a chronic high-dose administration of L-carnitine might induce similar effects in the animals used in the primary carcinogenicity experiment, i.e. increase the incidence of atherosclerotic lesions in the aorta of F344 rats.

Finally, targeted and untargeted metabolomics techniques were applied to plasma samples collected at the end of the carcinogenicity study in order to identify potential alterations of the entire (measurable) metabolome emanating from the feeding of L-carnitine to the above-mentioned animals. As a result, these data could provide insight into changes in (relative) abundance of carnitine-related metabolites such as TMAO, but also of compounds not primarily associated with L-carnitine and its metabolism, tentatively even unmasking previously unknown metabolic variations or disturbances.
4. Publication 1

The influence of chronic L-carnitine supplementation on the formation of preneoplastic and atherosclerotic lesions in the colon and aorta of male F344 rats

Michael T. Empl¹, Patricia Kammeyer², Reiner Ulrich², Jan F. Joseph³, Maria K. Parr³, Ina Willenberg¹, Nils H. Schebb¹, Wolfgang Baumgärtner², Elke Röhrdanz⁴, Christian Steffen⁴, Pablo Steinberg¹

¹ Institute for Food Toxicology and Analytical Chemistry, University of Veterinary Medicine Hannover, Hannover, Germany
² Department of Pathology, University of Veterinary Medicine Hannover, Hannover, Germany
³ Institute of Pharmacy, Free University of Berlin, Berlin, Germany
⁴ Federal Institute for Drugs and Medical Devices, Bonn, Germany

Published in:
© Springer-Verlag Berlin Heidelberg 2014

Contribution to the manuscript:
MTE was involved in the planning and design of the study, in addition to being responsible for its organization, coordination and actual performance (animal care, L-carnitine exposure, physiological parameter and urine sample collection). MTE collected all organ and blood samples as well as post-mortem physiological organ data, statistically analyzed and interpreted the dataset and wrote the manuscript.
5. Publication 2

The influence of a chronic L-carnitine administration on the plasma metabolome of male Fischer 344 rats*

Christoph H. Weinert1, **, Michael T. Empl2, **, Ralf Krüger3, **, Lara Frommherz1, Björn Egert1, Pablo Steinberg2, Sabine E. Kulling1

1 Department of Safety and Quality of Fruit and Vegetables, Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Karlsruhe, Germany
2 Institute for Food Toxicology and Analytical Chemistry, University of Veterinary Medicine Hannover, Hannover, Germany
3 Department of Physiology and Biochemistry of Nutrition, Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Karlsruhe, Germany

* Parts of the data shown in the present publication have been presented as poster at the 51st Congress of the European Societies of Toxicology (EUROTOX 2015) in Porto, Portugal (Empl et al. 2015b)

** These authors contributed equally to this work

Published in:
© 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

A correction to this publication—rectifying wrongly reported L-carnitine dosage units (correct dosages/units: 0, 0.1, 0.2 and 0.5 % or 0, 1, 2 and 5 g/L)—will be published in an upcoming issue of Molecular Nutrition & Food Research. The citation is as follows: Mol Nutr Food Res. 2018;62(23):1870096. doi:10.1002/mnfr.201870096. © 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Contribution to the manuscript (as stated in the publication):
MTE and PS designed and conducted the initial animal experiment (Empl et al. 2015a). CHW, RK, and LF performed analytical measurements and data quality control. BE processed the GC × GC-MS raw data. CHW carried out the statistical evaluations. CHW, MTE, RK, LF, PS, and SEK wrote or contributed to writing of the manuscript.
6. Discussion and conclusion

6.1. General considerations

In the frame of the present project, the questions whether a chronic and high-dose (up to 5 g/L) L-carnitine supplementation induces preneoplastic and atherosclerotic lesions as well as changes in the metabolome were addressed using F344 rats, a rodent bioassay model often used in cancer research (see chapter 2.4.1 for details). The rationale behind performing these analyses was based on the fact that bacterial metabolism of L-carnitine in the gut results in the formation of certain metabolites containing an amine moiety, which may be further subjected to metabolic or chemical transformation and thus yield carcinogenic or proatherogenic compounds such as DMN and TMAO (see chapters 2.2.3, 2.3.1 and 2.3.2 for details). Due to this nutrient being heavily advertised as “fat burner” and “sports performance enhancer”, L-carnitine or health supplements containing L-carnitine may seem quite attractive to many consumers. With some supplement manufacturers recommending physiologically non-absorbable daily intake doses up to several grams, a fairly large number of people would therefore be hypothetically at risk of suffering adverse health effects resulting from “recreational” L-carnitine intake. The data presented herein thus provides evidence potentially helpful in assessing whether a long-term intake of high doses of L-carnitine may pose a health risk to the general population consuming health supplements containing this substance.

Neither the primary hypothesis of the present project—i.e. that L-carnitine enhances the formation of DMN and preneoplastic lesions (so-called “aberrant crypt foci”; ACF) in the colon of the experimental animals—nor both secondary hypotheses—i.e. that L-carnitine exerts proatherogenic effects and causes alterations in the plasma metabolome—could be confirmed, with the exception of slight shifts in the abundance of a few metabolites and a tenfold increase in plasma TMAO content when compared to control animals (Empl et al. 2015a; Weinert et al. 2017).

6.2. Carcinogenicity study: critical aspects

Although the above-mentioned results might suggest that a chronic daily intake of a human equivalent dose (HED) of up to \( \approx 57 \) mg/kg BW L-carnitine (Empl et al. 2015a) does not induce CRC, and thus refute our primary hypothesis previously also set up by others (e.g. Bain et al. 2005), a few points possibly affecting ACF formation have to be taken critically into account before a final (robust) appraisal of this study outcome can be made.

Firstly, it has to be acknowledged that the experimental animals suffered from a sialodacryoadenitis virus (SDAV) infection at the very beginning of the one-year carcinogenicity study (Empl et al. 2015a). Although this rat-specific coronavirus does not affect the gastrointestinal tract and only potentially “[…] interfere[s] with research involving the lacrimal and salivary glands […] [], the respiratory, immune, nervous, and ophthalmic systems […] [as well as] fetal and neonatal development […]” (Otto et al. 2015, p. 177ff.; Baker and Lipman 2015, p. 1465), an influence of this infectious agent on the endpoints investigated in the present study cannot be excluded beyond doubt.

A second point majorly determining ACF formation are the conditions prevailing in the animal’s gut, which should ideally permit the efficient nitrosation of the bacterial L-carnitine metabolites DMA, TMA and TMAO. The hypothesis that NO\(_2\) in combination with secondary amines causes tumors in vivo—doubtlessly through \( N \)-nitrosamine formation—was confirmed as early as 1969 in Sprague-Dawley rats by Sander and Bürkle and subsequently verified by Dr. William Lijinsky and others using a variety of secondary and tertiary amines as well as rat models, including
Discussion

F344 rats (Newberne and Shank 1973; Taylor and Lijinsky 1975; Lijinsky and Taylor 1977; Lijinsky and Reuber 1980; Meier-Bratschi et al. 1983; Lijinsky 1984). Since the formation of carcinogenic N-nitrosamines does in fact occur in vivo when certain conditions are met (see chapter 2.3.1 and below for details), the question arises whether these were fulfilled in the colon of the F344 rats used herein. The following paragraphs will therefore successively discuss to what extent the design of the present study was favorable for such a nitrosation reaction to occur.

The gut bacterial metabolites originating from L-carnitine—i.e. DMA, TMA and TMAO (see chapter 2.2.3 for details)—all form DMN when nitrosated (Lijinsky et al. 1972; reviewed by Mirvish 1975b). That at least some of the above-mentioned carnitine metabolites were formed in the gut of the F344 rats—and hence could have been available for nitrosation—is demonstrated by the carnitine concentration-dependent increase in plasma TMAO (Weinert et al. 2017), which most likely results from gut microbiota-derived TMA. Nonetheless, L-carnitine treatment did neither increase nor majorly influence the DMN concentration in the urine of the F344 rats (Empl et al. 2015a), thereby generally suggesting that nitrosation of formed bacterial carnitine metabolites did not occur. However, measurement of urinary content might not have been the ideal—though the only experimentally feasible—method to accurately determine the total DMN burden of the animals, as approximately two thirds of an oral dose are exhaled as CO₂ after degradation to formaldehyde in the liver (see chapter 2.3.1; reviewed by Haggerty and Holsapple 1990), while only negligible amounts of that same dose are found in urine (= 6 %) or other body compartments (Heath and Dutton 1958; Phillips et al. 1975). Indeed, due to extensive first pass metabolism, small doses of DMN (< 40 µg/kg BW; Eisenbrand and Habermeyer 2013, p. 781) do not reach systemic circulation and other organs besides the liver at all and are therefore barely or not detectable in urine (Kraft et al. 1981; Swann et al. 1984). Consequently, measurement of the intracolonic or hepatic rather than urinary content might have given a better representation of the actual amount of DMN the F344 rats were actually exposed to. In any case, DMN measured in the present study (Empl et al. 2015a) is not related to the L-carnitine treatment and can therefore be considered of incidental nature from an unknown source. A possible though unproven explanation for the occurrence of these “basal” DMN levels (≈ 330 ng/ml on average), in addition to potential reasons given in publication 1 (Empl et al. 2015a), could be endogenous formation in the bladder resulting from infectious processes, as has been described by Hawksworth and Hill (1974) in Sprague-Dawley rats experimentally infected with Escherichia coli EB 555.

In mammals, the nitrosating agent precursors NO₃ and NO₂ can be formed interchangeably via NO—by action of commensal bacteria and various enzymes as well as a low pH in the stomach—in the frame of a cyclic process commonly denominated “nitrate-nitrite-nitric oxide pathway” (reviewed by Lundberg et al. 2008). However, neither the diet nor the drinking water fed to the F344 rats during the carcinogenicity study were additionally supplemented with NO₃ or NO₂ (Empl et al. 2015a). Thus, potential nitrosating agents for DMN formation in the present study must have been derived from environmental sources (e.g. NOₓS in air as well as NO₃ in animal feed and drinking water) or endogenous synthesis (e.g. from arginine; Green et al. 1981; Saul and Archer 1983; reviewed by Marletta 1988 and Gangolli et al. 1994). Consequently, although the actual NO₃ or NO₂ concentrations in drinking water and animal feed were not measured and are therefore unknown (Empl et al. 2015a), one might generally speculate whether the amounts of NO₂ ingested by the F344 rats were sufficient to induce significant DMN formation from bacterial L-carnitine metabolites. This is of importance, since
the formation rate of DMN from DMA and NO$_2$—under optimal *in vitro* conditions (e.g. pH ≈ 3)—has been reported to be expectedly proportional to DMA concentration but additionally also proportional to the square of the NO$_2$ concentration (Mirvish 1970; Cachaza et al. 1978). This implies that exponential amounts of NO$_2$ in relation to L-carnitine metabolites would have been needed to form appreciable amounts of DMN under the most likely less than optimal reaction conditions prevalent in the colon of the F344 rats (e.g. pH; see below). Hence, the absence of L-carnitine-dependent DMN (and possibly ACF) formation (Empl et al. 2015a) might have resulted from insufficient amounts of available nitrosating agents. In contrast, TMA, as precursor to the detected TMAO (Weinert et al. 2017), was most certainly formed and available for nitrosation, as was perhaps DMA.

Another important factor determining the nitrosation of amines is the basicity of the involved compound as well as the acidity (pH) of the reaction compartment. As noted by Pfundstein and Spiegelhalder (2007, p. 933), the lower the pH, the more the actual nitrosating agent N$_2$O$_3$ is produced from NO$_2$; but the less non-protonated secondary amine—i.e. the only form that is nitrosated—is available. Therefore, at a certain acidic pH, highly basic amines such as DMA ($pK_a = 10.7$) are not as readily nitrosated as less basic compounds (Pfundstein and Spiegelhalder 2007, p. 933). In fact, due to its high basicity, diethylamine did not form any tumors when administered to female Sprague-Dawley rats in conjunction with NO$_2$ via the diet, while less basic amines such as N-methylbenzylamine did (Sander et al. 1968; reviewed by Lijinsky 1980). Therefore, in addition to the other factors discussed (e.g. the pH in the colon), the basicity of DMA potentially derived from L-carnitine in the gut of the F344 used in the present study renders the onset of a nitrosation reaction unlikely. In contrast, the nitrosation of tertiary amines such as TMA is not clearly dependent on the $pK_a$ value of the compound and follows a different reaction mechanism (so-called “nitrosative dealkylation”) when compared to secondary amines (Gowenlock et al. 1979; Sun et al. 2010). Thus, the other two potential L-carnitine-related metabolites may still undergo nitrosation to form DMN in the rat gut, provided all other conditions for an optimal reaction are met (see below).

The importance of the pH value for DMN formation was confirmed by Lane and Bailey in 1973 under “physiologic” conditions, when they incubated equal amounts (100 mg/L) of NO$_2$ and DMA with human gastric juice at different pH values (1.7–4.5) and found that the highest yield was obtained at pH 2.5 (≈ 0.035 mg/L), whereas the lowest amount of DMN (< 0.01 mg/L) was formed at pH 4.5. In contrast, when diethylamine and NO$_2$ are incubated in more basic gastric juice such as that of the rat (≈ pH 4.5), 3.5–7.5 times less DEN is produced when compared to yields obtained in human gastric content (Sen et al. 1969). Moreover, the higher the pH (up to 8), the more NO$_2$ (one order of magnitude per pH value starting at pH 3) needs to be supplied to the reaction system in order to yield similar amounts of amine nitroso derivatives (Sander et al. 1968). These quantitative data—but also studies by others (e.g. Scanlan et al. 1974 or Meier-Bratschi et al. 1983)—additionally show that, even if optimal conditions are met *in vitro*, nitrosation reactions involving DMA and other dialkylamines generally proceed to yield only minute amounts (usually < 1 %) of *N*-nitrosamines (reviewed by Shephard and Lutz 1989). The pH in the gastrointestinal tract of the rat is markedly different from conditions prevailing in human gut and ranges from pH 3.2 in the stomach of fed animals (in humans: usually pH 1–2.5, but up pH 5 when fed) to an average ≈ 5.5 in all the other intestinal compartments (in humans: up to = 7 in the colon; McConnell et al. 2008; Khutoryanskiy 2015). Consequently, the pH conditions prevalent in the colon of the rat do not seem very favorable for optimal DMA
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nitrosation, especially when NO_2^- is not supplemented. Nonetheless, contrary to secondary amines, tertiary amines such as TMA or TMAO are more readily nitrosated to form DMN in a weakly acidic environment (pH 3.2–5.6), although yields at 37 °C are often orders of magnitude lower than the already low quantities of DMN formed from DMA (Fiddler et al. 1972; Lijinsky et al. 1972; Scanlan et al. 1974; Sun et al. 2010). Again, keeping this in mind, the emergence of noteworthy amounts of carcinogenic DMN from L-carnitine-derived TMA and TMAO seems highly unlikely and may explain why no ACFs or liver tumors were formed in the animals used in the present carcinogenicity study (Empl et al. 2015a). Interestingly, Fiddler et al. (1972) reported that L-carnitine and other quaternary ammonium compounds may also be a direct source of DMN, although in very low amounts produced under non-physiological circumstances. Evidently, neither this DMN formation pathway nor nitrosation reactions described as being catalyzed by (rat) gut bacteria at pH values of ≥ 7 in vitro (Sander 1968; Hawksworth and Hill 1971; Klubes et al. 1972) or in vivo (male Sprague-Dawley rats [Hashimoto et al. 1976] and male F344/N rats [Massey et al. 1988]) contributed in any way to the chemical conversion of L-carnitine metabolites in the frame of the present study.

An additional factor that may have had an influence on the non-occurred carnitine-dependent DMN formation in the colon of the F344 rats is the presence of potential nitrosation inhibitors. For example, ascorbic acid (reviewed by Mirvish 1975a), α-tocopherol or glutathione have all been described as being inhibitors of N-nitrosation (Pfundstein and Spiegelhalder 2007, p. 935). Since these compounds are, at least partly, components of the diet fed to the rats (see supplementary material accompanying publication 1; Empl et al. 2015a), they might have repressed DMN formation, as might have done other dietary components potentially reacting more readily with amines or the nitrosating agents (Pfundstein and Spiegelhalder 2007, p. 935). In this context, the dilution of the whole “nitrosation reaction system” resulting from food intake must also be taken into account, as a gastric content dilution by half has been discussed as “[...] reduc[ing] [...] DMN formation [by an] eightfold [...]” (Mirvish 1970).

Lastly, before hypothetically formed DMN could exert its carcinogenic effects locally in the colon of the F344 rats, it would need to be metabolically activated, predominantly by CYP2E1 (see Fig. 8). Since this enzyme is known to be expressed in the colon of rats (Hakkak et al. 1996), a metabolic activation of DMN could theoretically occur in the large intestine of this animal species.

With all of the above in mind, the negative result of the carcinogenicity study could be attributed to a number of factors majorly influencing the formation of ACFs from the L-carnitine metabolites DMA, TMA and TMAO. These factors include the less than favorable chemical properties of the compounds to be nitrosated, the less than optimal pH of ≈ 5.5 prevailing in the colon of the F344 rats and the low efficiency of the nitrosation reaction as such. It has to be mentioned though, that actual DMN levels in compartments other than urine were not measured (Empl et al. 2015a) and that it therefore cannot be conclusively established if a nitrosation reaction of carnitine-derived metabolites actually occurred, let alone which metabolites were exactly formed (with the exception of TMAO). Further, the animals did not receive any supplementary NO_3^- or NO_2^-, possibly resulting in the levels of available nitrosating agents not being sufficient. Since a supplementation of these agents would, in addition to potentially boosting DMN formation, more realistically reflect a human dietary intake pattern—as does the dietary presence of nitrosation inhibitors—one could hypothetically think of repeating this experiment with the above-mentioned compounds added to the diet. Moreover, although labor-intensive and stressful for the animals, chemical analysis of formed bacterial carnitine metabolites as well as DMN in other matrices and compartments than urine could be performed in the frame of such a novel study, in order to get a better perspective if the reactions
hypothesized in the above paragraphs actually occur in vivo. In practice however, it is very likely, due to the aforementioned and immutable chemical properties of the nitrosation reaction as well as of the compounds involved, that no DMN and consequently no ACFs or CRC would be formed from a chronic and high-dose administration of L-carnitine in combination with NO₃ or NO₂. Therefore, a reiteration of the present study with slight changes in the design would be unnecessary, as the probability of appreciable DMN formation from L-carnitine and its metabolites is very low from a chemical point of view. Indeed, this statement is supported by animal studies performed in rats, which consistently show that when NO₂ is administered in combination with various amines, only tumors in other sites than the colon are induced, provided enough nitrosation products were formed (e.g. summarized by Lijinsky and Taylor 1977; Lijinsky 1984). Also, DMN is rather known to induce liver neoplasms and only very rarely—if at all—leads to CRC formation in rats (Peto et al. 1991). In conclusion, the formation of CRC in humans resulting from L-carnitine supplementation is unlikely, even more so that the higher intraluminal pH found in the human gut when compared to the rat (McConnell et al. 2008; Khutoryanskiy 2015) is more disadvantageous for an efficient nitrosation reaction to actually occur. However, many factors affect amine nitrosation in the gut, including bacterial nitrosamine synthesis (reviewed by Macfarlane and Macfarlane 1997), and it can therefore not be conclusively excluded that metabolites of L-carnitine might promote the formation of carcinogenic N-nitrosamines such as DMN, especially since the present study has some shortcomings such as the SDAV infection of the animals (Empl et al. 2015a), a putatively too low level of nitrosating agents and a lack of quantitative data regarding metabolite and DMN formation from L-carnitine.

6.3. Analysis of the metabolome

Since the implications of the L-carnitine supplementation on the cardiovascular system as well as on the minor shifts in the abundance of certain metabolites have already been detailed in in publication 1 (Empl et al. 2015a) and 2 (Weinert et al. 2017), respectively, only the major finding of the metabolomics study—i.e. the about tenfold increase of plasma TMAO in rats receiving 5 g/L L-carnitine—will be discussed.

In addition to being a possible precursor to DMN (see chapter 2.3.1 for details), TMAO has been recently associated with CVD (see chapter 2.3.2 for details). Yet, ever since Koeth et al. (2013) published their pivotal finding that L-carnitine-derived TMAO promotes atherosclerosis, scientists have argued as to what extent—if at all—TMAO is involved in the development of such diseases (reviewed by Johri et al. 2014 as well as Cho and Caudill 2017). More specifically, the debate revolves around whether TMAO is actually a CVD-causing agent or merely a factor accompanying the onset of such an illness (reviewed by Zeisel and Warrier 2017). As summarized by Vallance et al. (2018), "[...a] growing body of evidence is accruing indicating that the gut microbe-generated metabolite, TMAO, is linked to CVD and thrombosis risks [...]." Several recent review articles have summarized the findings supporting this claim, which is mainly based on the epidemiological association of TMAO with CVD and elevated risk of death, the advancement of atherosclerosis in experimental animal models as well as mechanistic data inferring for instance that TMAO exerts pro-thrombotic effects or amplifies foam cell formation (Velasquez et al. 2016; Cho and Caudill 2017; Zeisel and Warrier 2017; Nowiński and Ufnal 2018). On the other hand, the causational link between CVD and TMAO is disputed by others, for example on the grounds that the consumption of fish—naturally rich in this amine—is not associated with CVD at all (Landfald et al. 2017). At the moment it is therefore probably safe to say that several questions need to be addressed before the role of
TMAO in CVD can be unequivocally elucidated and that drastic dietary changes limiting TMAO or TMAO precursor uptake are presently not warranted (reviewed by Cho and Caudill 2017). Nonetheless, as long as the risk originating from TMAO cannot be thoroughly assessed, consuming foodstuffs which largely and chronically increase the bodily TMAO content should be avoided. This includes L-carnitine, as it has been recently shown—similarly to the major finding described in publication 2 (Weinert et al. 2017)—that a daily L-carnitine supplementation of 1000 mg for over a year significantly increased TMAO plasma levels in patients suffering from mitochondrial disorders (Vallance et al. 2018).

Lastly, it has to be mentioned that the metabolic profile measured in the F344 rats (Weinert et al. 2017) only reflects the momentary metabolome composition on the day of sacrifice, as the study was originally not designed with metabolite kinetics in mind. In addition, due to limitations of the presently available analytics techniques and losses during sample preparation, only a fraction of the small molecules comprising the whole metabolome can be measured and detected at once, in spite of the fact that a fairly high number of 359 metabolites in total were identified herein (Weinert et al. 2017). It can therefore neither be finally excluded that shifts in metabolite abundance might have occurred over the course of the study (e.g. differences in the metabolite profile/abundance between the beginning and the end of the study), nor that the L-carnitine treatment did not have an influence on any other non-detected metabolites.

6.4. Conclusions

Aside from a tenfold increase in plasma TMAO, the one-year L-carnitine supplementation with HEDs of up to ≈ 57 mg/kg BW (≈ 4 g/day for an adult weighing 70 kg) did not induce any biologically relevant changes in male F344 rats (Empl et al. 2015a; Weinert et al. 2017). Further, of the initially 80 rats, 77 terminated the carcinogenicity study in a very good overall condition, previously published and discussed non carnitine-related pathological findings notwithstanding (i.e. acute lesions of unknown etiology in the liver as well as age-related lesions in the heart; Empl et al. 2015a). With this and chapters 6.2 and 6.3 in mind, one could thus conclude that L-carnitine consumption would not be detrimental to health when consumed in the long-term, at least up to the highest concentration tested herein. Indeed, this statement is in line with findings from a small quantity of rather large long-term studies (6–12 months) mainly performed in diseased human subjects, which were given L-carnitine doses as high as 100 mg/kg BW/day (≈ 7 g/day for an adult weighing 70 kg) and which suffered only from occasional minor adverse effects in the form of a “fishy body odor” or gastrointestinal symptoms such as nausea or diarrhea (reviewed by Hathcock and Shao 2006 as well as VKM 2015). This quite clearly indicates that chronic consumption of L-carnitine is most probably not carcinogenic, as also stated by Hathcock and Shao (2006). Since some of the patients enrolled in the above-mentioned studies suffered from CVDs (Hathcock and Shao 2006), a robust conclusion from these data regarding the possible CVD-inducing effects of high-dose L-carnitine intake cannot be drawn. Although daily doses as high as 100 mg/kg BW do not appear to be harmful, EFSA only considers a daily L-carnitine dose of 2 g (≈ 21–29 mg/kg BW for an adult weighing 70 kg) as well-tolerated and improbable to cause adverse health effects (EFSA AFC Panel 2003).

In conclusion, even though the present study has some shortcomings, the data generally allow to conclude that L-carnitine up to the highest daily dose administered (≈ 4 g for an adult weighing 70 kg) would generally be safe and not induce adverse effects in the form of cancerous lesions or changes in the metabolome. However, as the CVD-related implications and the risk emanating from a high TMAO plasma content are not yet fully elucidated, it is not advisable to ingest L-carnitine-containing health supplements in order to not unnecessarily
increase the risk of possibly contracting a CVD. This is all the more important, as the benefit associated with the consumption of such supplements in persons not attained by PCD is doubtful at best.
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8. Acknowledgements

First of all, I would like to thank Prof. Dr. Pablo Steinberg for enabling me to perform the present work in his research group as well as for his ongoing support and excellent supervision.

I am also very grateful to my supervision group, Prof. Dr. Gerhard Breves, Prof. Dr. Maren von Kockritz-Blickwede and Prof. Dr. Ingo Just for all their help and support during the course of my PhD studies. My special gratitude thereby goes to Prof. Breves, not only for assuming the supervision of my project when Prof. Steinberg left the TiHo to become president of the Max Rubner-Institut, but also for always backing and assisting me in so many ways.

The performance of such a study is never the work of a single person and I would therefore like to express my sincere gratitude to all co-authors of as well as persons acknowledged in the journal articles included in the present thesis. Without their help, the present work could not have been performed and completed as it was. In this context, I would like to especially thank my colleague Bettina Seeger for her valuable help during sample collection.

I would also like to thank all my current and former colleagues at the Institute for Food Toxicology as well as other institutions of the TiHo. Special thanks go to Jutta Barras-Akhnoukh, Julia Hausmann and Alexander Krybus for excellent technical assistance, to Pascal Hoffman for superb work, always being up for a joke and for being such an easy to work with PhD student and to Tina Kostka for her stunning work and dedication, for being the only other member of “Team Empl”, for bearing with my humor and for her non-stop supply with food and drinks.

An immense “thank you” also goes to my excellent and trusted friends Julia, Stephan, Amelie, Johannes, Steffi and Annika for all the good times we had—and hopefully will have for many years to come—their support and help as well as for always having a sympathetic ear for all my problems.

I also want to sincerely thank my parents and siblings for always supporting me through all the ups and downs of life.

Finally, the biggest “thank you” goes to my girlfriend Christina. Without her love and endless support the present thesis would have never seen the light of day.