Here, there and everywhere. A multi organ approach to acylcarnitine metabolism

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Citation for published version (APA):
Discussion: A multi organ perspective on acylcarnitine metabolism
Introduction

In the past few decades much of the metabolic research has focused on the relation between obesity and insulin resistance. Researchers strive after more understanding of, and control over the deranging body weight of the western population and all consequential diseases this problem brings along. Insulin resistance is one of the main derangements of overfeeding, and disturbed lipid metabolism is identified as one of the etiological factors of this derangement. Within the theory of lipotoxicity, where lipid intermediates such as ceramides, gangliosides and diacylglycerol accumulate in insulin sensitive tissues, acylcarnitines have also been suggested to interfere with insulin signalling. This idea was based on several studies, showing elevated acylcarnitine levels in obese and insulin resistant humans and rodents (1-4). Impaired FAO, either low FAO or high but incomplete FAO, could lead to an accumulation of acylcarnitines inside the cell which could eventually end up in the plasma compartment (2, 4). Therefore, alterations in the plasma profile were thought to reflect acylcarnitine metabolism on the tissue level. But interestingly, acylcarnitine profiles in plasma did not correlate with levels in muscle tissue of fasted men (5). This discrepancy is relevant, as insulin resistance occurs at the tissue level, whereas acylcarnitine profiles are often measured in plasma. These findings, together with the proposed hypotheses, illustrate difficulties in the interpretation of altered acylcarnitine profiles. The interpretation of the plasma acylcarnitine profile requires knowledge of the origin and the kinetic properties of acylcarnitines in general, and additionally understanding of the reciprocal relation between different organs participating in acylcarnitine metabolism. This perspective further explores acylcarnitine metabolism in physiological and pathological conditions to clarify its involvement in insulin resistance and glucose intolerance.

Acylcarnitine metabolism in different species

Acylcarnitine metabolism is extensively studied in humans and model organisms. However, important differences exist between species, including carnitine biosynthesis sites, but also acylcarnitine concentrations in general (6-10). Most important are the differences between humans and rodents, which are the main model organisms in acylcarnitine research (7). An example is carnitine synthesis, which mainly occurs in human kidney in contrast to rodents where it mainly takes place in liver (10). Additionally, the expression of the enzyme carnitine acetyltransferase (CrAT) is highest in human liver while in rodents it is highly expressed in skeletal muscle (11). Comparing measurements of insulin sensitivity and energy expenditure of different species can be challenging as well (12). Even within one species these differences can be found, such as the weight gain upon a HFD, the regulation of glucose tolerance and possibly acylcarnitine metabolism between mouse strains (9, 13-15). These differences hamper a direct translation of rodent data to the human setting and vice versa.
The origin and function of acylcarnitines

Acylcarnitines are intermediates of mitochondrial acyl-CoA oxidation, and comprise of an acyl group coupled to L-carnitine. L-carnitine enables transport of (mainly long chain) acyl-CoA over the mitochondrial membrane, which is otherwise impermeable to these acyl esters. Therefore, L-carnitine fulfils a crucial role in the supply of substrates to the mitochondrion for energy production (10, 16). The acyl groups esterified to L-carnitine can originate from different substrates. Fatty acids are the most abundant species coupling to L-carnitine in order to be oxidized, as carnitine is essential for FAO. Therefore, acylcarnitines are generally specified as fatty acid oxidation (FAO) intermediates. However, carbohydrates, amino acids and ketone bodies contribute to the formation of some acylcarnitines as well (3, 17-19). Acylcarnitines are often measured in a profile, and as such they are regarded as a family of metabolites. Historically, acylcarnitines were divided in acid-soluble and –insoluble species, later on specified as free carnitine, short chain- and long chain acylcarnitines (20). The introduction of tandem mass spectrometry allowed the identification of individual acylcarnitine species, based on the molecular mass determined by the length of the acyl group attached to the carnitine molecule (21).

Acylcarnitines can be divided based on their origin, plasma concentration or clinical relevance. As the origin of the acyl groups varies, they do not necessarily behave similarly or fulfil similar metabolic roles. Free carnitine is often decreased in obesity, possibly due to increased acylation of carnitine by the abundant amount of FFA present in plasma and tissues (3, 22). And as carnitine is mainly absorbed from the diet, specifically meat and dairy products, levels can be low in vegetarians and vegans (23). C2-carnitine mainly derives its acetyl group from either lipids or carbohydrates, and therefore its level can vary upon different metabolic states as well (17): in chow fed mice that were fasted, C2-carnitine concentrations increased (9) (chapter 5), which was not observed when HFD fed mice were fasted (chapter 5). Of the remaining acylcarnitines, the presence of the individual species can vary in different metabolic states. As carnitine is crucial for FAO, an increase in the full range of acylcarnitines can be seen in FAO-stimulating conditions such as fasting or HFD-induced insulin resistance, both known for increased FAO rates along with decreased rates of carbohydrate oxidation (CHO) (3, 9). But other species follow different patterns, which can be based on their origin. Amino acid derived species such as C3- and C5-carnitine tend to increase upon meals containing protein (18) (chapter 4). And C4OH-carnitine, which is primarily ketone body derived, is mainly seen in ketotic and insulin resistant states (9, 19).

As described above, we found substantial differences between C2- and C16-carnitine kinetics, which again can be explained by the origin of the substrate that is bound to the carnitine molecule. Here, parameters such as pool size, rate of appearance and elimination of C2-carnitine were affected by changes in metabolic state such as fasting or high fat diet feeding. This implies that C2-carnitine either is a transporter for carnitine towards tissues to meet increased carnitine requirements due to metabolic changes (chapter 4), or possibly as a carrier of the acetyl group. The pool size, rate of appearance or elimination of C16-carnitine did not change under different metabolic conditions, demonstrating that C16-carnitine may be a by-product of FAO rather than a...
carnitine carrier or energy substrate. These differences in kinetic properties of C2- and C16-carnitine illustrate that changes in acylcarnitine levels do not simply reflect overall metabolic changes such as alterations in FAO or insulin sensitivity. It also illustrates that acylcarnitine profiles reflect many different metabolic processes, and should be interpreted per individual species.

**Acylcarnitine profiles in plasma and tissues**

Plasma is often the main compartment used for the analysis of the acylcarnitine profile. This profile has been considered as a reflection of whole body acylcarnitine metabolism for a long time, as many studies report plasma acylcarnitine alterations in relation to the metabolic condition in tissue (1, 3, 24). However, we have shown that the plasma profile does not represent a specific organ or tissue (9)(chapter 3). Although we did not find correlations between plasma and tissue acylcarnitines, we did find groups of acylcarnitines forming clusters within the compartments, which do relate to each other in a topological overlap measure plot, a technique often used to see how large sets of genes relate to each other (chapter 3). Here we showed that despite lacking correlations between plasma and muscle acylcarnitines, long chain acylcarnitines in four different compartments of striated muscle tissue form a cluster under fed and fasted circumstances, suggesting muscle specific coordinated acylcarnitine metabolism. The fact that we found no relation between plasma and skeletal muscle corroborated with other studies, where plasma and muscle acylcarnitine levels did not correspond, acylcarnitine fluxes to and from muscle were low and administration of a carnitine precursor did not lead to changes in muscle acylcarnitine levels (5)(chapter 4 and 7). Long chain acylcarnitines in plasma did show some relation with long chain species in liver (9), which we recently confirmed in two studies where plasma acylcarnitines mostly resembled liver acylcarnitines as well (chapter 4 and 7). This relation between plasma and liver acylcarnitines was shown earlier in a study in fasted macaques, but here no individual chain lengths were measured (25). In conclusion, the finding that correlations between plasma and organs were absent, corresponds with the differences in acylcarnitine fluxes through different organs (chapter 4), pointing out that these compartments play different roles in whole body acylcarnitine metabolism.

**The interplay of different organs in acylcarnitine metabolism**

As concluded in the previous paragraph, various concentrations, fluxes and kinetics are found in the organ compartments involved in acylcarnitine metabolism (5, 9)(chapter 4, 5 and 7). To understand these characteristics per organ, we need to understand the organ-specific role in whole body acylcarnitine metabolism under different metabolic circumstances.

First, not every fatty acid oxidizing organ is capable of de novo carnitine synthesis (10, 26). This requires an efficient system of distribution and transportation, where organs can either supply or receive carnitine and where plasma facilitates the actual transport to and from organs (26). Within this system, the liver plays a central role in both the production and distribution of acylcarnitines. The capacity to synthesize carnitine in
Figure 1 provides an overview of different organs and their involvement in acylcarnitine metabolism under fed and fasted conditions. Here, the liver has a main role in the synthesis and distribution of carnitine (via acetylcarnitine) and other acylcarnitines. Plasma carnitine levels regulate hepatic carnitine production. Skeletal muscle is known for its high total carnitine content that depends on carnitine uptake via plasma acetylcarnitine. Muscle takes up little other acylcarnitines and hardly relates to other compartments. WAT showed a strong increase in all acylcarnitines upon fasting, possibly reflecting lipolysis, as acylcarnitines correlated with FFA upon weight loss. The kidney has a distinct function in the synthesis and reabsorption of carnitine. However, it takes up both carnitine and acylcarnitines for urinary excretion, thereby regulating the carnitine pool. A major part of the total carnitine pool is absorbed from the diet by the gut. The gut releases acylcarnitines as well, mainly upon feeding. It is unknown whether gut is capable of de novo carnitine synthesis. Finally, the plasma compartment contains a profile which is a mixture of acylcarnitines from all participating tissues. But no specific tissue is represented by the plasma acylcarnitine profile. However, it does have an important function in the exchange and distribution between all different organs involved in acylcarnitine metabolism.
the liver is well established in rodents, where liver contains high levels of the crucial enzyme for carnitine production, gamma-butyrobetaine dioxygenase (BBH) (26). This de novo hepatic carnitine production provides the liver with sufficient carnitine for higher FAO rates when needed, but also enables inter organ distribution of carnitine. As such, liver contains high concentrations of C2-carnitine, both in fed and fasted mice (9). Moreover, the fluxes of C2-carnitine to and from the liver are highest in fed and fasted pigs, when compared to fluxes over skeletal muscle, gut and kidney (chapter 4). We found a negative correlation between venous plasma concentrations of carnitine and its flux over the liver, suggesting a feedback mechanism where low plasma carnitine concentrations correlate with high production of carnitine by the liver and vice versa. This feedback mechanism may be mediated by the intestinal carnitine uptake, where the liver might sense low portal vein carnitine levels and synthesize carnitine when needed. Carnitine synthesis itself is regulated by PPAR-alpha, which is an important regulator of fatty acid oxidation and is therefore abundantly expressed in fatty acid oxidizing tissues (6, 26, 27). Accordingly, it has been demonstrated that PPAR-alpha activation results in enhanced carnitine production (26). However, carnitine concentrations are not known to influence PPAR-alpha activity. An interesting aspect is that the expression of the carnitine transporter OCTN2 is rather low in liver when compared to muscle and kidney in humans (28). OCTN2 supposedly transports carnitine mainly into the cells, against a gradient of high carnitine concentrations inside and low concentrations outside of the cell. This could explain the relatively low hepatic OCTN2 expression, as liver is able to synthesize carnitine and is therefore not depending on carnitine influx. It also suggests that efflux of carnitine does not occur via OCTN2 transport, but possibly by diffusion. In conclusion, the liver seems to play a key role in regulating the liver and plasma carnitine pool.

Another organ capable of de novo carnitine synthesis is the kidney, which also contains the enzyme BBH (10, 29). In humans, the kidney is actually the main organ for carnitine production as it highly expressed BBOX1, the gene encoding for BBH (10, 29). We have shown carnitine production by the kidney in fed and fasted pigs (chapter 4). However, uptake of acylcarnitines by the kidney might be of similar importance, as shown by the negative fluxes in kidney, meaning acylcarnitine uptake in both fed and fasted state. The uptake of carnitine and C2-carnitine by the kidney may be mainly for excretion purposes and subsequent regulation of the acylcarnitine pool, as carnitine and acylcarnitines appear in urine as well (30, 31). Alternatively, it might facilitate FAO in the kidney itself. It is unknown whether the gut is capable of de novo carnitine synthesis. One mouse study has suggested that carnitine was produced in mouse intestines, but to our knowledge no other study has reproduced this finding so far (32). In our study on acylcarnitine fluxes in pigs, we did find a modest postprandial peak of the portal vein carnitine flux. However we were unable to determine whether this was de novo carnitine synthesis or uptake from the diet. The gut released other acylcarnitines as well, suggesting postprandial distribution of dietary carnitine, lipids and protein.

The skeletal muscle compartment is not capable of carnitine synthesis, and is therefore dependent on carnitine distribution from other organs via plasma (10). The need for carnitine is high in muscle, as it relies heavily on FAO (5, 33). The acylcarnitine concentration
in skeletal muscle is the highest of all studied organs, and approximately 95% of the total carnitine pool resides here (9). Additionally the volume of the muscle compartment largely exceeds that of other organs, explaining the high percentage of the carnitine pool residing in muscle tissue. Therefore it was surprising to find the fluxes to and from the muscle compartment to be very low, except for the net C2-carnitine flux. The latter may be considered as either supply of carnitine or alternatively supply of acetyl-CoA (chapter 4). In general, muscle showed no correlations of acylcarnitine profiles with other tissues or plasma, nor did it show pronounced production or uptake of acylcarnitines (except for C2-carnitine). This demonstrates that muscle must interact only moderately with other compartments when it comes to acylcarnitine metabolism per se.

**Intervening in the acylcarnitine pool**

As carnitine is essential for the oxidation of fatty acids, it has been used and studied extensively in conditions where endogenous carnitine levels are low due to inborn errors of metabolism, leading to pathological carnitine deficiencies (34, 35). Additionally, carnitine is often used as a supplement for potential beneficial effects on lipid oxidation and muscle performance (22, 36-40).

We showed that the administration of the precursor of carnitine, gamma-butyrobetaine, in lean and obese mice, increased levels of carnitine and almost all plasma acylcarnitine species, as well as the shorter chain species in liver (chapter 7). But we were unable to interfere in the acylcarnitine levels of muscle or white adipose tissue (WAT). Even in obese mice, where FAO rates were potentially higher, no effect on carnitine levels was observed. When administering a high amount of C2-carnitine as a bolus in lean mice, acylcarnitine levels in plasma and liver showed a strong increase. But apart from an increase in carnitine and C2-carnitine, again the profiles in muscle or WAT were hardly affected. This illustrates how the compartments of muscle and adipose tissue apparently do not (fully) rely on constant supply of carnitine from the plasma and the other compartments. As we did not substantially interfere with the energy demand in these mice, it might be that the lacking effect in muscle and WAT resulted from an unchanged need for carnitine. Possibly, more pronounced effects after C2-carnitine administration could be observed in settings where carnitine is depleted or where metabolism is otherwise stressed. It has been suggested earlier by Muoio et al that mitochondrial capacity is sufficient to handle sudden increases in substrate flux (41). We assume that carnitine is present in muscle tissue in great excess, to secure the capacity for adjustment to moderately increased FAO rates. Additionally carnitine might be excessively present as a potential buffer for acetyl-CoA, as CrAT can regulate PDH activity by converting acetyl-CoA to C2-carnitine and vice versa.

Another interesting aspect of the lacking effect of increased carnitine availability on muscle and WAT acylcarnitine profiles, is that our tracer studies showed that the acylcarnitine pool behaves as a single pool, which exceeds the plasma compartment up to 50-fold. This means that the pool must include the interstitium and possibly the cytosol of certain tissues as well, and that exchange of acylcarnitines between plasma and interstitium or cytosol must occur unrestrained. We hypothesize that the boundary of
the pool is the mitochondrial membrane where acylcarnitines are uncoupled, and the acyl-CoA enters beta-oxidation.

**Acylcarnitines in various metabolic conditions**

As most acylcarnitines are mainly FAO derived intermediates, they are often studied under fasted circumstances as fasting induces higher FAO rates. But in HFD-induced obesity and insulin resistance in mice, FAO rates are often increased at the expense of CHO. Acylcarnitine levels are also found to be elevated in obese humans (3). A possible causal role for acylcarnitines in the induction of insulin resistance has been proposed. Here acylcarnitines accumulate as a consequence of increased or incomplete FAO in obesity. This hypothesis is supported by the observation of several statistically significant associations between specific acylcarnitine species and markers of glucose intolerance and insulin resistance, further suggesting a causal role (1, 3, 42-44). However, when studying acylcarnitines in insulin sensitive and insulin resistant mouse models, we were unable to reproduce some of the results from available studies. Moreover, the results from these studies are not always consistent. Therefore we question the direct role of acylcarnitines as modifiers of insulin sensitivity.

In fasted HFD mice, plasma acylcarnitine levels were lower when compared to fasted chow mice (chapter 7). In liver, the shorter chain species were lower in HFD fed mice as well. However, in muscle and adipose tissue no difference was found between HFD and chow fed mice, in accordance with the absent effect of gamma-butyrobetaine administration. These effects could have been detectable when mice were not fasted. However, we also showed that in HFD mice, no differences in kinetics of both C2-carnitine and C16-carnitine were detected. In the chow-fed mice, there was a difference in C2-carnitine kinetics, meaning that this effect is concealed by the HFD intervention. Moreover, changes in acylcarnitine profiles in the different studies were not associated with insulin resistance. We were unable to influence insulin resistance, glucose tolerance or energy expenditure by administering gamma-butyrobetaine (chapter 7). And in addition, improvements in insulin sensitivity in obese humans on a weight loss intervention did not correlate with alterations in acylcarnitine levels (chapter 6). Here, changes in acylcarnitine levels did correlate with plasma FFA, suggesting that acylcarnitines may reflect WAT lipolysis during weight loss. We did not find any effect on production or elimination rates of C16-carnitine in diet- or fasting induced insulin resistant mice, even though this is one of the main species of interest in the discussion on acylcarnitines and insulin resistance (chapter 5). Again lowering the probability of acylcarnitines as causal factors in diet-induced insulin resistance.

An exception to the modest contribution of most acylcarnitines is the role of C2-carnitine in insulin resistance. Although we do not propose that C2-carnitine induces insulin resistance, it may have a crucial role in switching between lipid and carbohydrate oxidation. C2-carnitine can be produced by carnitine acetyltransferase (CrAT), an enzyme which converts acetyl-CoA into C2-carnitine and vice versa (22, 42, 45, 46). However the affinity of CrAT is higher for acetyl-CoA than for C2-carnitine, shifting the equilibrium towards C2-carnitine formation. As the presence of acetyl-CoA inhibits PDH activity,
CrAT can buffer the amount of acetyl-CoA into C2-carnitine, in order to allow CHO via PDH. It has been suggested that CrAT function is lower in obese and insulin resistant subjects, leading to impaired substrate switching and metabolic inflexibility (42). It was demonstrated in a CrAT knockout mouse model that when CrAT activity is diminished, the ability to switch to CHO is impaired and FAO is the main source for energy production. Several studies including that of Muoio et al. (22, 42, 45) suggest that CrAT activity is mainly found in skeletal muscle and that skeletal muscle is the main source for C2-carnitine. It was proposed that the high activity of CrAT causes a high efflux of acylcarnitines from muscle tissue. However the expression and activity of CrAT varies between different species. In rodents, CrAT expression is indeed highest in skeletal muscle, but humans mainly express CrAT in liver tissue (11). The pigs in our study on trans organ fluxes, which exhibit CrAT activity in both liver and muscle (11), had low fluxes of all acylcarnitines from muscle tissue. Therefore we question if muscle is indeed responsible for the largest fraction of the plasma acylcarnitine pool, as proposed by Noland et al. (22). In our study, much higher fluxes of C2-carnitine came from the liver. This could imply that the purpose of C2-carnitine formation in muscle and liver differs.

**Conclusion**

To summarize, our results do not directly implicate a causal role for acylcarnitines in insulin resistance. We even question if there is a relevant association between these metabolites and glucose tolerance, as levels of acylcarnitines varied under different metabolic circumstances and independent of the degree of insulin resistance. However, we do think that there is a relevant association between C2-carnitine and glucose tolerance via the interplay between lipid and glucose oxidation.

**References**


