High-dose vitamin B1 therapy prevents the development of experimental fatty liver driven by overnutrition

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Abstract

Fatty liver is an abnormal metabolic condition of excess intrahepatic fat. This condition, referred to as hepatic steatosis, is tightly associated with chronic liver disease and systemic metabolic morbidity. The most prevalent form in humans, i.e., nonalcoholic fatty liver disease, generally develops due to overnutrition and sedentary lifestyle and has yet no approved drug therapy. We earlier developed a relevant large-animal model in which overnourished sheep raised on a high-calorie carbohydrate-rich diet develop hyperglycemia, hyperinsulinemia, insulin resistance, and hepatic steatosis. Here, we tested the hypothesis that treatment with thiamine (vitamin B1) can counter the development of hepatic steatosis driven by overnutrition.

Remarkably, the thiamine-treated animals presented with completely normal levels of intrahepatic fat, despite consuming the same amount of the liver-fattening diet. The thiamine treatment also lowered the hyperglycemia and increased the liver's glycogen content, but it did not improve insulin sensitivity, suggesting that steatosis can be addressed independently of targeting insulin resistance. Thiamine increased the catalytic capacity for hepatic oxidation of carbohydrates and fats. However, at the gene-expression level, more pronounced effects were observed on lipid-droplet formation and lipidation of VLDL, suggesting that thiamine may affect lipids metabolism not only through its known classical coenzyme roles.

This discovery of the potent anti-steatotic effect of thiamine may prove clinically useful in managing fatty liver-related disorders.
Introduction

Fatty liver (FL) represents an abnormal metabolic condition of excess intrahepatic fat (> 5.5% w/w liver fat fraction), which is also referred to as hepatic steatosis. FL and its association with chronic liver disease (steatohepatitis, cirrhosis, and cancer) had long been known to result from excessive alcohol consumption or viral hepatitis (Asselah et al., 2006; Louvet and Mathurin, 2015). However, in recent decades the prevalence of nonalcoholic FL disease (NAFLD), which is largely attributed to excess caloric intake and sedentary lifestyle, has become more significant and is currently estimated to affect ~25% of the global population (Younossi et al., 2018). To better reflect the tight relationship of NAFLD with systemic metabolic dysfunction, such as insulin resistance and dyslipidemia, and its continuum of liver abnormalities, its coexistence with other liver diseases or in-parallel to alcohol consumption, an alternative name was recently proposed - ‘metabolic-dysfunction associated FL disease’ (MAFLD) (Eslam et al., 2020a; Eslam et al., 2020b). We have adopted this more inclusive term in the following.

Despite the increasing prevalence of MAFLD, and the accompanying rise in end-stage liver disease requiring liver transplantation (Goldberg et al., 2017; Wong et al., 2015) and in hepatocellular carcinoma (Mittal et al., 2016), there is as yet no approved drug therapy for the disorder. Although numerous therapeutic strategies are being developed and tested for targeting the steatotic, oxidative, inflammatory, fibrotic, and metabolic aspects of MAFLD, the responses observed in clinical trials have so far been inadequate (Friedman et al., 2018). This may reflect the heterogeneous and multifaceted nature of the disease, and highlights the unmet need for more robust preclinical models and for the development of combination therapies (Santhekadur et al., 2018). Likewise, therapeutic agents with the potential to counteract multiple aspects of this complex disorder may be of higher clinical value.

We recently reported a large-animal nutritional model for FL, characterized by excessive carbohydrate intake, hyperglycemia and hyperinsulinemia, which collectively drove liver steatosis in sheep (Kalyesubula et al., 2020). In a search for a pharmacological intervention with the potential to counteract both the systemic and liver-related metabolic aspects of this model, we decided to investigate thiamine, also known as vitamin B1, as a plausible beneficial agent, due to its fundamental role in energy metabolism. The biologically active form of thiamine is produced by its intracellular phosphorylation to thiamine pyrophosphate (TPP) (Voskoboyev and Ostrovsky, 1982), an essential coenzyme in the catalytic decarboxylation of α-keto acids and in transketolase reactions in all forms of life (Manzetti et al., 2014).
Of particular relevance to overnutrition and related metabolic disorders is the role of TPP in mitochondrial catabolism as a coenzyme for pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (α-KGDH). In the decarboxylation of pyruvate to acetyl coenzyme A (Acetyl-CoA) by PDH, TPP enables the terminal oxidation of carbohydrate-carbon sources in the tricarboxylic acid (TCA) cycle. Whereas this role places thiamine bioavailability at a pivotal checkpoint between aerobic and anaerobic glucose metabolism, the cofactor role of TPP as a cofactor for α-KGDH, which is involved in the regulation of the turnover rate of the TCA-cycle (Huang et al., 2003), emphasizes its importance in the catabolism of both carbohydrates and fats.

Regular ingestion of western-type diets, particularly rich in simple carbohydrates, can deplete physiological levels of thiamine (Elmadfa et al., 2001; Lonsdale, 2006; Maguire et al., 2018). Indeed, thiamine deficiency is associated with obesity and diabetes (Eshak and Arafa, 2018; Thornalley and Ali, 2007), and thiamine supplementation decreased hyperglycemia, diabetic complications, and oxidative stress (Al-Attas et al., 2014; Alaei Shahmiri et al., 2013; Arvidsson et al., 2003; Babaie-Jadidi et al., 2004; Gorlova et al., 2019; Hammes et al., 2003; Karachalias et al., 2010; Rabbani et al., 2009; Tunc-Ozdemir et al., 2009). In the metabolic context, it has been shown that overproduction of thiamine in Arabidopsis plants increases the oxidation of carbohydrates in the TCA-cycle (Bocobza et al., 2013). Taken together, these observations suggest that thiamine concentrations above the normal physiological levels may enhance carbohydrates combustion, presumably though not necessarily exclusively, by maximizing the oxidative activity of TPP-dependent enzymes. Whether such thiamine-dependent action can slow the rate of hepatic fat accumulation by reducing carbohydrate availability for de novo lipogenesis (DNL), and/or by directly enhancing fatty acid (FA) oxidation in the TCA-cycle remains unexplored.

The strong association between hepatic steatosis and hyperglycemia in the sheep model (Kalyesubula et al., 2020), together with the evidence that has accumulated with respect to the relevant metabolic benefits of thiamine supplementation, motivated us to investigate the potential of high-dose thiamine treatment to counteract the overnutrition-driven steatosis that develops in this model.
Results

To test the hypothesis that high-dose thiamine treatment can reduce hepatic steatosis, we conducted an experiment in which randomly assigned weaned lambs (N=36) were raised for a total of 135 days on three different regimes (Experiment flowchart): (i) On a low-calorie (LC) diet known to induce lean livers; (ii) on a high-calorie (HC) diet known to stimulate FL (Kalyesubula et al., 2020); (iii) thiamine-treated animals on the HC diet (THC).

Subcutaneous injections of thiamine (300 mg/animal) were initiated on day 50, to allow for some liver fat buildup. Metabolic and physical parameters were monitored in vivo weekly throughout the entire experiment, and the cumulative liver phenotypes were evaluated ex vivo on livers harvested postmortem. Differences between HC and LC animals were ascribed to effects of the diet, whereas differences between THC and HC animals (only from day 50 on) to the thiamine treatment effects.

To identify molecular signals associated with the observed phenotypes, we employed differential expression analyses of selected genes involved in carbohydrate and lipid catabolism, synthesis, transport and storage, since improper balance between these metabolic pathways may lead to abnormal accumulation of intrahepatic fat (Kawano and Cohen, 2013).

**High-calorie diet increased weight gain and adiposity**

As expected (Kalyesubula et al., 2020), animals raised on the HC diet gained substantially more weight than those consuming the LC diet ($P < .0001$; Table 1, Fig. S1). This increase in body weight was accompanied by significantly greater adiposity, as indicated by a higher body mass index (BMI; $P = 0.003$) and a higher body condition score (BCS; $P < .0001$), determined on a scale of 1 to 5, ranging from very thin to very fat (Kenyon et al., 2014) (Table 2). On average, the THC animals consumed a similar or slightly higher amount of the HC diet than the HC animals (Table S1 and Fig. S2a), but they tended to weigh less ($P = 0.09$; Table 1, Table S2).

Consistent with the adiposity induced by the HC diet, the HC animals presented with higher fasting plasma leptin levels than those receiving the LC diet ($P = 0.02$; Table 2; Fig. S3a). The circulating concentrations of triglycerides (TG) were also statistically higher in the HC relative
to the LC animals ($P = 0.02$; Table 1, Fig. S3b). However, no effects of thiamine on either plasma leptin or TG were detected.

**Thiamine raised the liver abundance of TPP, as measured by $\alpha$-KGDH activity**

As expected, the thiamine-treated animals displayed higher circulating thiamine concentrations compared to the untreated HC group ($P = 0.05$; Fig. 1a). This indicates that the subcutaneous injections of thiamine hydrochloride reached the bloodstream efficiently, but does not prove that tissue levels increased concomitantly. The latter needs to be directly evaluated by the determination of intracellular TPP levels.

Thiamine deficiency is generally diagnosed by measuring the response of erythrocytes transketolase activity to exogenous TPP (Mastrogiacomo et al., 1993). To examine the effect of the HC diet and of the thiamine treatment on the abundance of TPP in the liver, we employed the same approach to study the response to exogenous TPP of $\alpha$-KGDH activity in liver lysates. We found that $\alpha$-KGDH activity increased more substantially in HC than in THC animals in response to exogenous 50 $\mu$M TPP ($P = 0.01$; Table 2). Whereas the response to TPP was not significantly different between the THC and LC animals ($P = 0.3$), the response in HC animals also trended higher than in LC animals ($P = 0.08$). Taken together, these enzymatic data indicate that the HC diet induced low endogenous levels of TPP, and the thiamine treatment reversed this effect (Fig. 1b).

**Thiamine reduced blood glucose and increased liver glycogen levels**

As previously observed (Kalyesubula et al., 2020), the HC diet induced fed-state hyperglycemia as compared with the LC diet ($P < .0001$; Fig. 2a, Table 1). The difference in blood glucose between the HC and LC animals during fasting was not as substantial but still statistically different ($P = 0.01$; Table 2). Similarly, steady fed-state hyperinsulinemia was induced by the HC diet ($P < .0001$; Fig. 2b). The THC animals had reduced blood glucose concentrations compared to the thiamine-untreated HC animals ($P = 0.02$; Fig. 2a, Table 1). Despite its reducing effect on blood glucose levels, thiamine had no detectable effect on the diet-induced hyperinsulinemia (Fig. 2b). Since the average dietary intake was similar, or even slightly higher, in the THC compared to the HC group (Fig. S2), these suggest enhanced carbohydrate catabolism in the thiamine-treated animals. Consistently, we found that THC animals had mRNA higher
levels ($P = 0.002$; Fig. 1g) of the significant glycolytic factor, glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

In line with the high plasma insulin levels observed in both the HC and THC animals, the concentrations of circulating non-esterified fatty acids (NEFA) were low in these groups compared with the LC group ($P < .0001$; Fig. 2c, Table 1), indicative of reduced adipose lipolysis, presumably caused by the high plasma insulin. As previously observed (Kalyesubula et al., 2020), both the whole-body and adipose-tissue insulin resistance were increased with by HC diet, as evaluated respectively by homeostatic model assessment of insulin resistance (HOMA-IR; $P < .0001$) and ADIPO-IR ($P < .0001$). However, they were unaffected by the thiamine treatment (Fig. 2d, 2e; Table 2).

Interestingly, although blood glucose levels were lowered by thiamine, hepatic glycogen levels increased (Fig. 2f). Since thiamine did not affect plasma insulin, which, together with glucose as a substrate for glycogen, is known to up-regulated hepatic glycogen levels (Samuel and Shulman, 2016), the increase in hepatic glycogen by thiamine may indicate an improved hepatic sensitivity to insulin. At the gene expression level, no differences were detected between the THC and HC animals in genes related to insulin resistance (Fig. 2d,e) or to glycogen formation (Fig. 2g), suggesting that posttranslational effects that are known to regulate hepatic glycogen metabolism (Roach et al., 2012) may be involved.

**Thiamine prevented diet-induced hepatic steatosis**

The average hepatic-fat content determined for the HC sheep (8.1%; Fig. 3a) was virtually identical to that observed previously (Kalyesubula et al., 2020). The thiamine treatment, however, had a strong effect on the hepatic-fat content, since the average in the THC animals was substantially lower (4.8%; $P < .0001$, Fig. 3a). Notably, the THC fat content was below the typical 5.5% threshold for fatty liver (Szczepaniak et al., 2005), and statistically similar to the levels in the LC sheep (3.9%; $P = 0.164$). Hepatocellular steatosis, either as macrovesicular or microvesicular, which was correlated with advanced histology of MAFLD (Tandra et al., 2011), was significantly increased in the HC compared to both the LC and THC groups (Fig. 3b,c). Interestingly, the remarkable reduction in intrahepatic fat caused by thiamine was not associated with a decrease in whole-body insulin resistance (Fig. 2d). Whether the decrease in hepatic fat by thiamine involves a reduction in hepatic insulin resistance remains unknown. This possibility
is, however, consistent with the observed increase of the content of hepatic glycogen as a surrogate for insulin sensitivity (Petersen et al., 1998).

Consistent with the previous observation (Kalyesubula et al., 2020), the HC diet increased the hepatic index (liver weight/BW) compared to the LC diet, which is indicative of unproportional growth of the liver (hepatomegaly). Thiamine, however, had no apparent macroscopic effect on it (Fig. 3d, Table 2), but microscopically the hepatocytes were larger in the THC compared to both the LC and HC livers (Fig. 3e). Although the mechanisms remain to be investigated, it is possible that the increase in glycogen content by thiamine contributed to these observations, since hepatomegaly has been associated with increases in either hepatic fat or hepatic glycogen content (Reid, 2001; Sherigar et al., 2018).

The high-calorie diet decreased hepatic expression of genes involved in mitochondrial catabolism

Mammalian regulation of energy expenditure is primarily mediated by sensing of the cellular levels of adenosine monophosphate (AMP) and NAD⁺, respectively, by AMP-activated protein kinase (AMPK) and sirtuin 1 (SIRT1). In the setting of low-caloric intake, the resulting increased levels of AMP and NAD⁺ allosterically activate AMPK and SIRT1, respectively. The activation of these evolutionary-conserved sensors turns on signaling cascades for stimulation of catabolic and inhibition of anabolic processes. Peroxisome proliferator-activated receptor coactivator-1alpha (PGC-1α), a master regulator of mitochondrial biogenesis, is directly targeted by SIRT1 and AMPK, so as to stimulate mitochondrial oxidative processes in response to energy requirements and nutrients availability (Cantó and Auwerx, 2009).

In the current study, SIRT1 expression was similar in the three treatment groups. However, the overnourished sheep raised on the HC diet exhibited decreased expression of the genes coding for AMPK (PRKAA2), PGC-1α (PPARGC1A), and for peroxisome proliferator-activated receptor alpha, PPAR-α, (PPARA) (P = 0.05; Fig. 4a). Reduced expression of this molecular network, is expected to promote DNL and to inhibit FA oxidation (Smith et al., 2016), which is consistent with the increased hepatic steatosis observed in the HC animals both in the present study and in the previous one (Kalyesubula et al., 2020). Thiamine had no affect on the expression levels of these three genes.

Alterations in the expression of hepatic genes caused by thiamine favoured inhibition of fat storage
Under conditions of energy abundance, as caused by the HC diet, newly derived TG, resulting from hepatic synthesis and from liver uptake of circulating lipoproteins, can be either secreted into the bloodstream in very-low-density lipoprotein (VLDL) particles, or incorporated into cytosolic lipid droplets (LD) for storage as intrahepatic fat (Alves-Bezerra and Cohen, 2018). Expansion or reduction of the fat content within LD is dynamically regulated by LD-associated proteins (Gluchowski et al., 2017), and by the availability of cytosolic TG, which is partly controlled by the lipidation of VLDL particles by microsomal triglyceride transfer protein (MTP) encoded by the \textit{MTTP} gene. Hepatic steatosis has been associated with genetic defects in both MTP and ApoB100, the hepatic VLDL lipoprotein (Berriot-Varoqueaux et al., 2000; Tanoli et al., 2004). In the current study, thiamine increased the abundance of the \textit{MTTP} transcripts compared with the untreated HC group ($P = 0.001$; Fig. 4b). No effects were detected on \textit{APOB}.

Perilipins are the predominant hepatocellular LD proteins (Alves-Bezerra and Cohen, 2018). These surface-associated LD proteins stabilize the structure of LD and control substrate availability for certain LD-associated enzymes (Kimmel and Sztalryd, 2016). Perilipin 2, which positively correlates with hepatic steatosis (Chang et al., 2006; Fukushima et al., 2005; Motomura et al., 2006; Najt et al., 2016), is one of the most well characterized LD proteins associated with fatty liver disease (Okumura, 2011). Here, the HC treatment significantly increased the abundance of Perilipin 2 transcripts compared to the LC treatment ($P < 0.0001$; Fig. 4b), and the thiamine treatment lowered their levels substantially ($P = 0.002$; Fig. 4b). These findings are in accordance with earlier studies showing that both mRNA and protein levels of Perilipin 2 increased with hepatic TG accumulation (Motomura et al., 2006; Pawella et al., 2014; Straub et al., 2008), while inactivation of the perilipin 2 gene lowered hepatic steatosis (Chang et al., 2006; Greenberg et al., 2011).

The low-calorie diet increased the expression of genes involved in liver uptake of fatty acids

A variety of proteins associated with hepatic steatosis have been implicated in the liver uptake of circulating NEFA, including FA translocase/CD36, caveolin, and FA transport protein (FATP) complexes that possess very-long-chain acyl-CoA synthase activity (Alves-Bezerra and Cohen, 2018). Consistent with the elevated plasma NEFA concentrations in the LC group (Fig.
2c), the abundance mRNAs for FATP6 (*SLC27A6*) (*P* = 0.0008) and FATP5 (*SLC27A5*) (*P* = 0.03) was higher in these animals (Fig. 4c). Since PPARα positively regulates transcription of NEFA transporters (Pawlak et al., 2015), these data are consistent with the observed higher expression of PPARα (Fig. 4a). No effect of thiamine was observed on the expression of this energy-sensing axis (Fig. 4a), on genes involved in lipogenesis (Fig. 4d), or on the NEFA transporters (Fig. 4d).

The low hepatic-fat content in the LC lambs (Fig. 3a) suggests, therefore, that the NEFA influx was utilized primarily for energy production, which is consistent with their increased expression of genes promoting FA oxidation (Fig. 4a).

*Hepatic fat accumulation was associated with altered gene expression of proinflammatory cytokines and antioxidants; some were reversed by thiamine*

As a well documented factor in the pathogenesis of obesity, metabolic syndrome, and insulin resistance, inflammation also plays a role in the progression of MAFLD (Ibrahim et al., 2018). In addition to the common histological signs of steatohepatitis, inflammation in MAFLD is manifested by increased circulating proinflammatory cytokines and leukocytes that can infiltrate the liver to fuel local and systemic inflammatory processes (Tilg et al., 2020). In our study, the HC-fed sheep exhibited increased the abundance of CCL2 and CXCL8 mRNAs (*P* = 0.05; Fig. 5a) in circulating leukocytes as compared to the LC-fed animals. The thiamine treatment reversed the dietary effect on the expression of CXCL8 (*P* = 0.05; Fig. 5a). No effects of the treatments on the expression of *IL1B*, *TNF*, and *IFNG* were detected.

In the liver, thiamine lowered the expression of *TNF* (*P* = 0.005; Fig. 5b). Whereas no significant alterations were detected in the mRNA levels of of *NFKB1*, *IL1B*, *CCL2*, and *IL8*, the HC diet increased expression of *PTX3* compared with the LC diet (*P* = 0.01; Fig. 5b).

Overnutrition and hepatic steatosis are associated with an increase in reactive oxygen species and oxidative stress, which may lower endogenous enzymes that act as antioxidants (Spahis et al., 2017; Videla et al., 2004). Consistently, the expression of catalase was lower as a result of the HC diet (*P* = 0.005; Fig. 5c). Whereas thiamine did not affect the expression of catalase, it did increase the expression levels of superoxide dismutase 2 (*SOD2*) (*P* = 0.02) (Fig. 5c). There were no detected treatment effects on the expression of glutathione peroxidase 1 (*GPX1*).

**Discussion**
Hepatic steatosis is the hallmark of the MAFLD global epidemic. In this study, we set out to investigate the potential of high-dose thiamine therapy to ameliorate overnutrition-induced hepatic steatosis in a sheep model. The major metabolic phenotypes of this model, i.e., hyperglycemia, hyperinsulinemia, insulin resistance, and hepatic steatosis, were virtually identical to those previously observed (Kalyesubula et al., 2020).

Thiamine reduced the hepatic-fat content dramatically, decreased blood glucose levels, and increased the hepatic glycogen content. It is likely that the weekly dose of thiamine employed here, 900-1500 mg per animal, is realistic to investigate in other species of similar body weight, ~70 Kg (Table S2), as humans. Yet, clearly additional investigations are required to determine the minimal effective dose, duration and frequency of therapy.

Interestingly, the robust effect of thiamine on hepatic steatosis was not accompanied by an effect on whole-body or adipose insulin resistance (Fig. 2d,e), providing evidence that hepatic steatosis may be targeted directly and independently. Whether hepatic insulin resistance is involved in the development of steatosis or was is modulated by thiamine remains to be investigated. Yet, the increase in hepatic glycogen content by thiamine (Fig. 2f) may suggest that it reduced the hepatic insulin resistance.

To investigate the involvement of potential metabolic pathways in mediating the observed dietary and thiamine-treatment phenotypes, we performed ex vivo studies on postmortem liver extracts. While these have the capacity sensitively detect subtle molecular differences between treatment, it is important to realize that they reflect a final cumulative picture that highlights lasting and sustained rather than the temporary effects.

The change in the liver activity of α-KGDH, a key enzyme in setting the TCA-cycle turnover rate, in response to exogenous TPP (Fig. 1b), suggests that animals raised on the HC diet had relatively low levels of hepatic TPP. Consequently, the higher hepatic TPP in the thiamine-treated animals may have potentially boosted mitochondrial catabolism of pyruvate and acetyl-CoA by increasing both the catalytic activity of PDH and the oxidative capacity of the TCA-cycle. This is consistent with the observed lower blood glucose levels and reduced hepatic fat observed in the thiamine-treated animals. Interestingly, at the gene-expression level, the thiamine treatment may have also increased the glycolytic flux, since the mRNA abundance of GAPDH also increased.
In response to cellular energy levels, the AMPK/PCG-1α/PPARα axis orchestrates gene expression to trigger both FA β-oxidation and inhibition of lipogenesis. Whereas the functionality of this energy-sensing axis is mediated by posttranslational phosphorylation signals, transcriptional adaptations of the involved genes seem physiologically important and relevant to overnutrition, since lower expression levels are associated with increased dietary consumption, insulin resistance, and metabolic dysfunction (Cantó and Auwerx, 2009; Herzig and Shaw, 2018). Accordingly, strategies to combat insulin resistance, metabolic syndrome, and hepatic steatosis have been proposed based on stimulation of this axis (Gariani et al., 2016; Ruderman et al., 2013; Smith et al., 2016).

In our current study, the overnourished animals raised on the HC diet also exhibited decreased expression of AMPK/PGC-1α/PPAR-α (Fig. 4a), which is consistent with their increased insulin resistance. Moreover, since reduced AMPK signaling is expected to decrease FA oxidation, and to increase lipogenesis, these molecular observations are also in line with the steatotic phenotype of the HC animals.

Although thiamine decreased the hepatic-fat content robustly (Fig. 3), we found no direct evidence for increased sensitivity to insulin, or for an increase in the gene expression of the AMPK energy-sensing axis (Fig. 4a). This suggests that the mechanism of steatosis reduction or its inhibition by thiamine may not involve insulin sensitization and/or transcriptional regulation of AMPK signalling. This is consistent with the observation that transcript levels of lipogenic genes were not significantly altered by thiamine. (Fig. 4d). The possibility that thiamine is involved in posttranslational regulation of AMPK signaling remains to be investigated.

Surprisingly, no effects of thiamine were detected on the expression of genes involved in lipogenesis and mitochondrial oxidation. However, thiamine-treated animals presented with altered expression of genes involved in LD stability and VLDL lipidation. In particular, transcripts levels of perilipin 2, which is essential for the structural integrity of LD, and was shown to be positively correlated with hepatic steatosis (Alves-Bezerra and Cohen, 2018; McIntosh et al., 2012; Motomura et al., 2006; Najt et al., 2016), were substantially decreased. Notably, this effect of thiamine reversed the strong dietary effect of the HC diet in increasing perilipin 2 transcripts, thus reflecting the observed pattern of steatosis in the LC, HC, and THC animals. The simultaneous effect of thiamine in increasing mRNA levels of MTP, which is essential for hepatic VLDL lipidation, might have further contributed to the depletion of TG from cytosolic LD, and their export to extrahepatic tissues. In line with this, the liver-specific
loss of perilipin 2, which alleviated hepatic steatosis, was also associated with an increase in MTP (Najt et al., 2016). This coupling, of low levels of perilipin 2 with high levels of MTP, may serve as a customary module for efficient depletion of intrahepatic fat.

Progressive liver disease has not been established for the current sheep model, which more likely resembles NAFL, or MAFL, to use the new terminology. Therefore, it is unlikely that inflammation played a major role in the development of steatosis in this model, or in its reversal by thiamine. Nevertheless, since both inflammation and oxidative stress have been extensively documented in obesity and MAFLD (Ibrahim et al., 2018; Tilg et al., 2020), we investigated their association with overnutrition and the thiamine intervention by comparing transcripts levels of common proinflammatory cytokines and antioxidants.

The overnourished steatotic sheep displayed increases in the expression of \textit{CCL2} and \textit{CXCL8} in circulating leukocytes and of \textit{PTX3} in the liver. The thiamine treatment had an effect in opposite direction, \textit{i.e.}, it reduced this presumably higher inflammatory state of the HC animals, as lower expressions of \textit{CXCL8} in leukocytes, and of \textit{TNF} in the liver, were observed in the thiamine-treated animals. Interestingly, treatment of mice with thalidomide, an anti-TNF-α drug, exhibited improvements in hepatic alterations caused by a high-fat diet (Pinto et al., 2010). With regard to oxidative stress, the HC diet lowered the expression of catalase. Thiamine did not affect catalase expression, but it did increase the expression of \textit{SOD2}, which could potentially increase antioxidative capacity.

Conclusions

In this study, we have demonstrated the potential of pharmacological thiamine therapy to address hepatic steatosis resulting from overnutrition. Differential gene expression analyses indicate that the reduction of steatosis by thiamine may involve destabilization of LD and increased VLDL lipidation, rather than insulin sensitization. It would, therefore, be valuable to investigate the clinical benefits of thiamine therapy in management of hepatic steatosis, and of combination therapies using both thiamine and insulin sensitizers for management of FL disorders involving insulin resistance, such as MAFLD.

Materials and Methods
Animals and experimental design

All animal studies were approved by the Volcani Center Animal Care Committee (Permit 790/18IL), and performed at the Volcani sheep experimental farm, Rishon LeZion, Israel. The \textit{in vivo} investigation was started in August and ended in December 2018, corresponding approximately to the end-of-summer through mid-winter in Israel. Animals were maintained in open-shed pens that protected from direct sunlight and rain, with adequate ventilation and daylight illumination. Following weaning at \~45 days after birth, all the male lambs in the same crop (a cohort of ca. 250 born around June), of the Afec-Assaf breed (Gootwine et al., 2008), were fed a high-calorie (HC) concentrate-based diet (Table S1). Two weeks later, thirty-six lambs (2.0 ± .04 months old, 25.6 ± 0.87 kg in body weight) were randomly assigned to three treatment groups (n=12 each): (i) HC diet (control), (ii) HC diet + thiamine (THC) and (iii) low-calorie (LC) diet (Table S1) as a reference group of lean livers (see Experiment Flowchart). The animals were provided \textit{ad libitum} with their respective group rations, with free access to fresh drinking water. The group feeds and left-overs were weighed weekly. The group means of daily energy intake were 5.1, 5.29, and 3.5 MCal of metabolizable energy for the HC, THC, and LC, respectively (Table S1). These dietary treatments were provided for the entire experimental duration (135 days), whereas the pharmacological treatments were given only during the last 85 days.

The THC animals were given subcutaneous injections of thiamine hydrochloride (Duchefa Biocemie, Haarlem, The Netherlands) at 300 mg/animal dissolved in 2 mL of filter-sterilized 0.9% saline solution were administered 3 times weekly from day 50 onwards and 5 times weekly from day 93 onwards, to compensate for the increase in the lambs` body weights. The LC and HC lambs were administered with equal volumes of 0.9% saline to control for the potential stress effects of the subcutaneous injection.
Blood, plasma and liver sampling

Blood was drawn weekly from the jugular vein, and glucose concentrations were measured using a FreeStyle Optium glucometer (Abbot Diabetes Care Ltd., Oxfordshire, UK) (Pichler et al., 2014). Twice monthly, plasma was isolated from 5 mL freshly drawn blood as described (Kalyesubula et al., 2020). On day 128, the animals were fasted for 24 hours with free access to water to evaluate blood and plasma fasting parameters. Before fasting, the BCS (Kenyon et al., 2014) was determined, and heart girth, withers height, and body length were measured. Fasting plasma samples were stored at -20°C until biochemical analyses. Individual weights were measured weekly.

After day 135, the animals were slaughtered at a local abattoir, and their livers were immediately harvested and weighed. Samples of about 10 g from the left lobe of each liver were placed in cryo-tubes, flash-frozen in liquid nitrogen and later stored in -80°C until taken for molecular analyses. Another ~200 g of the left lobe of each liver was stored in a zip-lock bag at -20°C until used for analyses. Liver glycogen content was quantitated as described (Kalyesubula et al., 2020).

Fat content analysis

Hepatic fat content analysis was performed via the improved-Folch method (Mopuri et al. in press). Briefly, triplicates of ~1 g of liver tissue were sampled from the frozen left lobe. The exact wet weight of each piece was determined after thawing and dehydrating the excess moisture on a Whatman filter paper for 10 min at 25°C. Each sample was mechanically homogenized in 25 mL chloroform-methanol (2:1) solution, followed by sonication on ice, overnight agitation at 25°C, and 10 min centrifugation at 3000xg. For removal of polar and semi-polar lipids, 4 mL of 0.9% NaCl were added to the supernatant, and the mixture was vortexed, then centrifuged at 2500xg for 10 min. The upper phase was discarded, and the residual interface was further rinsed twice with 4 mL of 50% methanol. The lower chloroform phase containing the fat (triglycerides and cholesterol esters) was collected and evaporated in a rotary evaporator under vacuum. The residual fatty phase was oven-dried at 45°C for 2.5 hours to remove residual moisture. The fat weight was determined, and the hepatic-fat content was computed as the percentage of the wet liver weight.
**Histological Analysis**

Sample preparation fixation and staining were performed as described (Kalyesubula et al., 2020). Analyses of hematoxylin-eosin (H&E) and periodic acid-Schiff (PAS) stained sections were examined blindly by a veterinary pathologist. The assessment was performed by counting hepatocytes showing either macrovesicular or microvesicular steatosis at ×200 magnification in 3 randomly selected fields. Scores of 0, 1, 2, or 3 were assigned to samples presenting < 5%, 5–33%, > 33–66% or > 66% steatotic hepatocytes, respectively. Data are presented on a frequency plot (Fig. 3c), and differences between treatments were explored by univariate χ² analysis.

Assessment of the size of hepatocytes were made by counting their number under a fixed microscopic field at ×200 magnification, which is inversely related to their size. Each sample was observed at three homogeneous zones of liver tissue consisting of hepatocytes and no vessels, and the triplicate-average count was used for the statistical analysis by ANOVA and contrast t-tests.

**Biochemical analysis of Plasma**

Plasma triglycerides were measured using the Cobas C 111 analyzer (Roche Diagnostics, Rotkruez, Switzerland). Plasma NEFA was determined using a NEFA kit (Wako Chemicals, GmbH, Neuss, Germany), and plasma insulin was determined using a radioimmunoassay kit (Coat-A-Count insulin; Diagnostic Products, Los Angeles, CA). Plasma thiamine concentrations were analyzed by a Vitamin B1 ELISA kit (Aviva Systems Biology; San Diego, CA). Plasma leptin activity was determined by a cell-based bioassay in HEK-293T cells expressing exogenous full-length *leptin receptor* cDNA with the firefly luciferase as an intracellular reporter gene, as described (Seroussi et al., 2016).

**Determination of mRNA by quantitative PCR**

For isolation of RNA from leukocytes, blood was collected from sheep on day 126 via venipuncture into EDTA-coated tubes and kept on ice. RNA extraction was carried out using the Norgen leukocyte RNA purification kit (Norgen Biotek Corp., Ontario, Canada). DNAase treatment was performed using Promega RQ1 RNAase-Free DNase (Promega, Madison, WI,
USA). cDNA was synthesized from 500ng total RNA using a Revert Aid RT-PCR Kit (Thermo Fisher Scientific, USA).

For isolation of RNA from liver tissue, the Norgen animal tissue RNA purification kit (Norgen Biotek Corp., Ontario, Canada) was used following the manufacturer's instructions. cDNA was synthesized from 1 µg total RNA using a Revert Aid RT-PCR Kit (Thermo Fisher Scientific, USA), using the Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific, USA) following the manufacturer's instructions. RT-qPCR analysis was carried out using 5x HOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne, Tartu, Estonia). The reaction mixture contained: 4 µl of cDNA, 0.3 ul of each primer designed using NCBI Primer Blast (Table s3), 4 µl of 5x HOT FIREPol EvaGreen qPCR Supermix, completed with ultra-pure water (Biological Industries, Kibbutz Beit Ha’emmek, Israel) to a final volume of 20 µl. RT-qPCR was carried out using a Rotor gene Q instrment (Qiagen, Hilden, Germany) under the following conditions: 95°C for 12 min, 40 cycles of 95°C for 15 s, 60°C for 20 s and 72°C for 20 s.

Relative gene expression was computed using the ΔΔCT method (Livak and Schmittgen, 2001) with the mean of the HC group as the normalizer. For leukocytes, the geometric mean of two reference genes (GAPDH and YWHAZ; Table s3) was employed, while for liver, the geometric mean of three reference genes (YWHAZ, PPIA, and RPL19; Table s3) was employed.

Statistical Analysis

Data for continuous dependent variables (glucose, insulin, triglycerides, NEFA concentration and weight; Table 1) were analyzed by repeated-measures ANOVA (for variables measured repeatedly over time) with the linear mixed model approach in JMP (Version 14.0.0, SAS Institute Inc., Cary, NC, 2016). The model included Treatment (LC vs. HC – for dietary effects) or (THC vs. HC – for the thiamine effects) as a between-subject fixed factor, Time (from treatment initiation) as a nominal within-subject fixed factor, Treatment by Time interaction, and Individual Animal as a random factor nested within Treatment. For the effects of thiamine, only data obtained starting with the initiation of the thiamine treatment (day 50 onwards), were taken into consideration. The distributions of model residuals were visually confirmed for normality. Post-hoc pairwise comparisons between treatments at specific time points were done using Student's t-test, and the significance was Bonferroni-Holm corrected for multiple comparisons.
Differences between treatments for other response variables lacking the time dimension (as in Table 2), were determined by One-way ANOVA. Selected \textit{a priori} comparisons to investigate the effects of diet (HC vs. LC) and of thiamine (HC vs. THC) were carried out using contrast \textit{t}-tests. Two-tailed \(P\)-values are reported throughout. Data are presented as means \pm standard errors (SE), unless otherwise stated. A significance threshold level of \(\alpha = 0.05\) was employed. Exact \(P\) values are reported throughout, values lower than 0.0001 are presented as \(P < .0001\).
Acknowledgments

We thank Hilary Voet (Hebrew University) for statistical advice, Elisha Gootwine (Volcani Center, ARO) for valuable discussions, and Israel Silman (Weizmann Institute) for critical reading and editing of this paper.

Competing interests

An institutional patent application has been filed by Hay Dvir and Samual Bocobza, which is concerned with the potential use of thiamine for the management of fatty liver-related disorders. All other authors declare no competing interests.

Funding

N/A

Data availability

N/A

Author Contributions statement

H.D., Research conceptualization, methodology, validation, formal analysis, investigation, resources, data curation, writing original draft preparation, writing - review and editing, visualization, supervision, project administration, funding acquisition; M.K., formal analysis, investigation, data curation, writing – review and editing; R.M., formal analysis, investigation, data curation, writing – review and editing; S.Y., leptin assay; N.E., histopathological analysis; J.A., histopathological analysis; S.B., conceptual discussions; U.M., experimental advice.


Kalyesubula, M., Mopuri, R., Rosov, A., Alon, T., Edery, N., Moallem, U. and Dvir, H.


**Smith, B. K., Marcinko, K., Desjardins, E. M., Lally, J. S., Ford, R. J. and Steinberg, G.**


Figures

Figure 1. Plasma thiamine levels and liver activity of α-KGDH in response to exogenous thiamine pyrophosphate (TPP) in the low-calorie (LC), high-calorie (HC), and thiamine-treated HC (THC) sheep. (A) Plasma thiamine concentrations before thiamine treatment (day 43) and toward the end of the experiment on days 129 and 130 (Fasting). (B) A plot of the additive liver α-KGDH activity in response to exogenous addition of 50 μm TPP. Repeated measures ANOVA detects an effect of treatment ($P = 0.03$). * Denotes $P < .05$ by contrast t-tests. Negative values for the THC group imply that the endogenous levels of TPP were high and close to the maximal potential effect on α-KGDH activity, such that the exogenous TPP introduced an inhibitory effect.
Figure 2. Systemic and tissue metabolic responses to the low-calorie (LC), high-calorie (HC) and thiamine-treated HC (THC) groups. (A) Blood glucose. Data analysis for HC vs. LC by repeated measures ANOVA revealed an effect of the dietary Treatment ($P < .0001$), Time ($P < .0001$) and Treatment x Time interaction ($P < .0001$). Data analysis for HC vs. THC revealed an effect of the thiamine Treatment ($P = 0.02$), time ($P < .0001$) and a Treatment x Time interaction ($P = 0.0002$). *Denotes $P < .05$ for the difference between HC and THC. (B) Plasma insulin. Data analysis for HC vs. LC revealed an effect of the dietary treatment ($P < .0001$), time ($P = 0.006$) and treatment x time interaction ($P = 0.03$). Data analysis for HC vs. THC revealed no effect of treatment. (C) Non-esterified fatty acid (NEFA). Data analysis for HC vs. LC revealed an effect of the dietary treatment ($P < .0001$), time ($P = 0.006$) and treatment x time interaction ($P < .0001$). Data analysis for HC vs. THC revealed no Treatment effect. (D) Whole-body insulin resistance as measured by HOMA-IR. One-way ANOVA detects an effect of treatment ($P < .0001$). (E) Adipose tissue insulin resistance as measured by ADIPO-
IR. One-way ANOVA detects an effect of treatment ($P < .0001$). (F) Hepatic glycogen content. Data analysis by one-way ANOVA detected an effect of Treatment ($P = 0.001$). (G) Expression of GAPDH and genes involved in glycogen metabolism. One-way ANOVA detected an effect of thiamine on GAPDH ($P = 0.01$) and of the HC diet on glycogen synthase 2 ($P = 0.02$). * Denotes $P < .05$ by contrast t-tests.

Abbreviations:
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), glycogen phosphorylase, liver form (PYGL), Glycogen synthase kinase-3 beta (GSK3B).
Figure 3. Liver response to the low-calorie (LC), high-calorie (HC), and thiamine-treated HC (THC). (A) Hepatic fat content. The HC treatment promoted substantial hepatic fat accumulation that was ameliorated by thiamine treatment (One-way ANOVA, $P < .0001$). Similarly, contrast t-tests between HC and LC, as well as between HC and THC were both highly significant ($P < .0001$). (B) Representative livers and corresponding tissue sections that were analyzed by histopathology with H&E staining taken at a x200 magnification. Macroversicular steatosis was observed in the HC lambs, but microvesicular steatosis was more consistently present, mainly around lobular zone 3, and occasionally in zone 1. The arrow indicates macrovesicular steatosis where the hepatocyte nucleus is displaced to the side by a large lipid droplet. (C) Mosaic frequency plot of the hepatocellular steatosis (scored for the presence of macrovesicular or microvesicular steatosis) graded on a scale of 0 to 3. Differences between the treatments were significant for both the Pearson and the likelihood ratio $\chi$ squares ($P < .0001$). (D) The hepatic index values were computed as the percentage of liver weight per animal body weight. One-way ANOVA detects an effect of treatment ($P = 0.0002$). (E) Hepatocyte count under a fixed microscopic field at x200 magnification. A high count reflects smaller hepatocytes and vice versa. * Denotes $P < .05$ by contrast t-tests.
Figure 4. Comparative expression in liver of genes involved in: (a) fatty acid oxidation; (b) lipid droplet metabolism and VLDL secretion; (c) fatty acid uptake; and (d) lipogenesis, in the low-calorie (LC), high-calorie (HC) and thiamine-treated HC (THC) sheep. Effect of treatment by one-way ANOVA: SIRT1 ($P = 0.17$), PRKAA2 ($P = 0.002$), PPARGC1A ($P = 0.03$), PPARA ($P = 0.009$), AGPAT1 ($P = 0.1$), AGPAT2 ($P = 0.04$), SREBF1 ($P = 0.22$), PPARG ($P < .05$), FASN ($P = 0.02$), FOXO1 ($P = 0.56$), Perilipin 2 ($P < .0001$), MTTP ($P = 0.03$), APOB ($P = 0.76$), CD36 ($P = 0.46$), SLC27A6 ($P = 0.003$), and SLC27A5 ($P = 0.04$). * Denotes $P < .05$ by contrast t-tests.

Abbreviations: SIRT1, NAD-dependent protein deacetylase sirtuin-1; PPAR, peroxisome proliferator-activated receptor alpha; PRKAA2, 5’-AMP-activated protein kinase catalytic subunit alpha-2; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; AGPAT1, 1-acyl-sn-glycerol-3-phosphate acyltransferase alpha; AGPAT2, 1-acyl-sn-glycerol-3-phosphate acyltransferase beta; SREBF1, sterol regulatory element-binding protein 1; PPARG, peroxisome proliferator-activated receptor gamma; FASN, fatty acid synthase; FOXO1, forkhead box protein O1; MTTP, microsomal triglyceride transfer protein; APOB, apolipoprotein B-100; CD36, platelet glycoprotein 4; SLC27A6, long-chain fatty acid transport protein 6; SLC27A5, bile acyl-CoA synthetase.
Figure 5. Comparative expression of genes encoding for proinflammatory factors measured in leukocytes (A), in the liver (B), and for antioxidant enzymes in the liver (C). One-way ANOVA detected an effect of treatment for leukocyte CCL2 ($P = 0.04$) and CXCL8 ($P = 0.04$); and hepatic TNF ($P = 0.01$), PTX3 ($P = 0.02$), Catalase ($P = 0.008$), and SOD2 ($P = 0.02$). * Denotes $P < .05$ by contrast t-tests.

Abbreviations:

CCL2, C-C motif chemokine 2; CXCL8, interleukin-8; TNF, tumor necrosis factor; PTX3, pentraxin-related protein; SOD2, superoxide dismutase 2 (mitochondrial).
Fig. 6. Experimental flowchart.
Table 1. Effects of the thiamine and the dietary treatments on parameters measured progressively throughout the duration of the experiment for the low calorie (LC), high calorie (HC), and thiamine treated-high calorie (THC) sheep.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Groups</th>
<th>P-Values</th>
<th>Groups</th>
<th>P-Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LC</td>
<td>SEM</td>
<td>Treatment</td>
<td>Time</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>35.7</td>
<td>52.4</td>
<td>0.7</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>NEFA, µEq L⁻¹</td>
<td>257.5</td>
<td>148.2</td>
<td>11.3</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Glucose, mg dl⁻¹</td>
<td>60.4</td>
<td>77.3</td>
<td>0.5</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Insulin, uIU mL⁻¹</td>
<td>25.4</td>
<td>175.6</td>
<td>6.7</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Plasma TG, mg dl⁻¹</td>
<td>18.8</td>
<td>23.3</td>
<td>0.6</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Values are expressed as least squares means. SEM: Standard error of the mean. TG: Triglycerides, NEFA: non-esterified fatty acids, Independent analyses were made for the dietary effect and for the thiamine treatment effect. For the dietary effect, analyses included values for the entire experimental duration (135 days). For the thiamine effect, only data from the initiation of thiamine treatment until the end of the experiment were considered, viz., last 85 days. Pretreatment values were used as a covariate. The fasting values, corresponding to day 130 of the experiment, were excluded from these analyses.
Table 2. Final physical and biochemical parameters for the low calorie (LC), high calorie (HC), and thiamine-treated high calorie (THC) sheep.

<table>
<thead>
<tr>
<th></th>
<th>LC</th>
<th>HC</th>
<th>THC</th>
<th>SEM</th>
<th>ANOVA</th>
<th>Dietary effects(^1)</th>
<th>Thiamine effects(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver weight (g)</td>
<td>709.2</td>
<td>1232.4</td>
<td>1302.0</td>
<td>58.3</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.34</td>
</tr>
<tr>
<td>Hepatic index</td>
<td>1.48</td>
<td>1.74</td>
<td>1.86</td>
<td>0.04</td>
<td>0.0002</td>
<td>0.002</td>
<td>0.14</td>
</tr>
<tr>
<td>BCS</td>
<td>2.35</td>
<td>3.18</td>
<td>3.19</td>
<td>0.08</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.88</td>
</tr>
<tr>
<td>BMI (BW BL (^{-2}))</td>
<td>65.4</td>
<td>75.1</td>
<td>82.1</td>
<td>1.68</td>
<td>&lt;.0001</td>
<td>0.003</td>
<td>0.03</td>
</tr>
<tr>
<td>Fasting Insulin, µIU mL(^{-1})</td>
<td>11.5</td>
<td>44.1</td>
<td>51.4</td>
<td>4.1</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.32</td>
</tr>
<tr>
<td>Fasting Glucose, mg dL(^{-1})</td>
<td>51.5</td>
<td>59.2</td>
<td>59.7</td>
<td>1.3</td>
<td>0.01</td>
<td>0.01</td>
<td>0.86</td>
</tr>
<tr>
<td>Fasting NEFA, µEq L(^{-1})</td>
<td>943.7</td>
<td>863.4</td>
<td>982.0</td>
<td>33.5</td>
<td>0.35</td>
<td>0.33</td>
<td>0.16</td>
</tr>
<tr>
<td>HOMA IR</td>
<td>2.03</td>
<td>8.27</td>
<td>9.70</td>
<td>0.83</td>
<td>&lt;.0001</td>
<td>0.0003</td>
<td>0.36</td>
</tr>
<tr>
<td>ADIPO IR</td>
<td>75.6</td>
<td>260.0</td>
<td>355.5</td>
<td>29.0</td>
<td>&lt;.0001</td>
<td>0.002</td>
<td>0.08</td>
</tr>
<tr>
<td>Fasting leptin, Fold induction</td>
<td>1.21</td>
<td>2.08</td>
<td>2.21</td>
<td>0.17</td>
<td>0.02</td>
<td>0.02</td>
<td>0.71</td>
</tr>
<tr>
<td>Hepatic (\alpha)-KGDH activity*</td>
<td>1.14</td>
<td>8.1</td>
<td>-2.54</td>
<td>0.77</td>
<td>0.03</td>
<td>0.08</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Values are expressed as least squares means

BW: body weight, Hepatic Index: (liver weight)*100/BW, BCS: body condition score, BMI: Body Mass Index, BL: body length, SEM: Standard error of mean

\(\alpha\)-KGDH: \(\alpha\)-ketoglutarate dehydrogenase

All the variables were analyzed by one-way ANOVA except for \(\alpha\)-KGDH for which two-way ANOVA was employed.

\(^1\)Dietary effects were obtained from the contrast \(t\)-test between HC and LC animals.

\(^2\)Effects of thiamine were obtained from the contrast \(t\)-test between THC and HC animals.

\*The difference in activity between with and without exogenous 50 \(\mu\)M TPP, expressed in mOD\(_{450}\).
### Table S1. Composition of feeds and intake for the low-calorie (LC), high-calorie (HC), and thiamine-treated HC (THC) sheep, expressed as dry matter.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Oat Hay</th>
<th>Concentrate</th>
<th>Ground Corn</th>
<th>Soybean meal</th>
<th>Total Intake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (%)</td>
<td>11.0</td>
<td>14.9</td>
<td>16.3</td>
<td>13.6</td>
<td></td>
</tr>
<tr>
<td>Crude protein (%)</td>
<td>10.0</td>
<td>18.4</td>
<td>9.4</td>
<td>53.0</td>
<td></td>
</tr>
<tr>
<td>Crude Fat (%)</td>
<td>3</td>
<td>3.5</td>
<td>4.1</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>NDF (%)</td>
<td>56.0</td>
<td>22.6</td>
<td>9.2</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>ADF (%)</td>
<td>31.6</td>
<td>9.9</td>
<td>2.6</td>
<td>5.9</td>
<td></td>
</tr>
<tr>
<td>ME, MCal/kg</td>
<td>2.2</td>
<td>3.2</td>
<td>3.9</td>
<td>2.8</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>LC average intake (Kg) ± SD.</th>
<th>LC average intake (ME, MCal/kg)</th>
<th>HC average intake (Kg) ± SD.</th>
<th>HC average intake (ME, MCal/Kg)</th>
<th>THC average intake (Kg) ± SD.</th>
<th>THC average intake (ME, MCal/Kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (%)</td>
<td>0.96±0.35</td>
<td>1.87±0.67</td>
<td>0.09±0.02</td>
<td>0.17±0.03</td>
<td>0.09±0.01</td>
<td>0.18±0.03</td>
</tr>
<tr>
<td>Crude protein (%)</td>
<td>-</td>
<td>0.28±0.08</td>
<td>1.79±0.28</td>
<td>5.00±0.79</td>
<td>1.83±0.29</td>
<td>5.11±0.80</td>
</tr>
<tr>
<td>Crude Fat (%)</td>
<td>0.27±0.08</td>
<td>0.95±0.27</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NDF (%)</td>
<td>3.5</td>
<td>0.68±0.19</td>
<td>5.16±0.78</td>
<td>5.11±0.80</td>
<td>5.29±0.79</td>
<td></td>
</tr>
<tr>
<td>ADF (%)</td>
<td>1.52±0.48</td>
<td>3.50±1.07</td>
<td>1.88±0.28</td>
<td>5.16±0.78</td>
<td>1.93±0.28</td>
<td>5.29±0.79</td>
</tr>
<tr>
<td>ME, MCal/kg</td>
<td>1.87±0.67</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
### Table S2. Physical body and blood plasma parameters for the low calorie (LC), high calorie (HC), and thiamine treated-high calorie (THC) sheep.

<table>
<thead>
<tr>
<th></th>
<th>LC</th>
<th>HC</th>
<th>THC</th>
<th>SEM</th>
<th>ANOVA P-values</th>
<th>Dietary effects&lt;sup&gt;1&lt;/sup&gt; P-values</th>
<th>Thiamine effects&lt;sup&gt;2&lt;/sup&gt; P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial BW (kg)</td>
<td>25.8</td>
<td>25.2</td>
<td>25.8</td>
<td>0.9</td>
<td>0.94</td>
<td>0.76</td>
<td>0.77</td>
</tr>
<tr>
<td>Final BW (kg)</td>
<td>47.5</td>
<td>71.3</td>
<td>70.4</td>
<td>2.1</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.70</td>
</tr>
<tr>
<td>Body length (m)</td>
<td>0.85</td>
<td>0.98</td>
<td>0.93</td>
<td>0.01</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.02</td>
</tr>
<tr>
<td>Wither’s height (m)</td>
<td>0.69</td>
<td>0.74</td>
<td>0.74</td>
<td>0.006</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.7</td>
</tr>
<tr>
<td>Heart girth (cm)</td>
<td>89.0</td>
<td>103.3</td>
<td>102.3</td>
<td>1.4</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.61</td>
</tr>
<tr>
<td>Fasting Body weight, kg</td>
<td>44.5</td>
<td>72.5</td>
<td>68.5</td>
<td>2.3</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.11</td>
</tr>
<tr>
<td>Fasting Weight loss, kg</td>
<td>-1.08</td>
<td>-0.18</td>
<td>-0.78</td>
<td>0.14</td>
<td>0.03</td>
<td>0.009</td>
<td>0.08</td>
</tr>
<tr>
<td>Fasting Plasma TG, mg/dL</td>
<td>20.1</td>
<td>20.5</td>
<td>16.8</td>
<td>1.0</td>
<td>0.28</td>
<td>0.85</td>
<td>0.15</td>
</tr>
</tbody>
</table>


<sup>1</sup>Dietary effects were obtained from contrast t-test between HC and LC animals.

<sup>2</sup>Thiamine effects were obtained from contrast t-test between THC and HC animals.
### Table S3. Primer sequences used for real-time quantitative PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession</th>
<th>Primer</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leukocytes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| GAPDH         | NM_001190390.1    | Forward: AAGTTCCACGGGACAGTCAA  
Reverse: ATGTTGGCAGGATCTCGGCTC | 92   |
| YWHAZ         | XM_027972757.1    | Forward: AGACCGGAAGTGCTGAGAAA  
Reverse: CGTTGGGGATCAAGACCTTT | 123  |
| TNF           | NM_001024860.1    | Forward: CAACTTGTAGCACAACATCGC  
Reverse: GAGTGAAGAAGGCGATGCTT | 129  |
| CCL2          | XM_004012471.2    | Forward: TCGCTCAGCCAGATGCAATT  
Reverse: GACACTTGTGCTGATTCGACT | 112  |
| CXCL8 (IL8)   | NM_001009401.2    | Forward: AAGCTTGGCTGCTCTCCTG  
Reverse: GTGGAAGAGTGGTGAATGTT | 127  |
| IFNG          | X52640            | Forward: GGAGACTTTCAAAGGCTGA  
Reverse: GGTTAGATTGTTGGCCAGG | 110  |
| IL1B          | NM_001009465.2    | Forward: GTGCTGGATAGCCCATGTC  
Reverse: CAGAACACCACTTCTCGGCT | 74   |
| **Liver**     |                   |        |             |
| AGPAT1        | NM_001009746.1    | Forward: TTGGTCGTGCTAGCTACTCC  
Reverse: CTCATGATCAGCAAGAGGGTC | 141  |
| AGPAT2        | XM_015094112.2    | Forward: CCGTGGAACATGACATACCT  
Reverse: CGTGGAACATGACATACCT | 138  |
| SREBF1        | XM_027974781.1    | Forward: GAGCTTCGTGGTTTCCAGA  
Reverse: GTGAGGAATACCTCGCAGCAT | 86   |
| PPARG         | NM_001100921.1    | Forward: GACGCACAGGCAAACTACGGT  
Reverse: GGGCTGATGCTGTTGAACGAT | 88   |
| FOXO1         | XM_027973596.1    | Forward: ATCGGAGTTTTCCAAAGTGC  
Reverse: AATAGCCCTGAGCTGCTGCC | 100  |
| FASN          | AF479289.1        | Forward: CCCAGCTCAACGAAAACAC  
Reverse: GACAGGCTGACACCCCTTCC | 110  |
| SIRT1         | XM_01503477.2     | Forward: TTGGGTACAGAGATGACCTT  
Reverse: GATGGGCAAGTTCATGCTATACT | 94   |
| PPARA         | XM_012445989.2    | Forward: GTCCTCCATAGGAGCTTGTATG  
Reverse: GATCTCCGTTGGCCGGAATCT | 110  |
| PRKAA2        | NM_001112816.1    | Forward: GTCATCAGGCAAGCTGTA  
Reverse: AGGGTGCCAAAGAGGACG | 87   |
| PPARGC1A      | XM_004907938.4    | Forward: AATGAAGATGCTGCTGCTGCTG  
Reverse: CTGCTCTGATCCAGATCGTCT | 109  |
| Perilipin 2   | NM_00104932.1     | Forward: ACAGCTGAGTGGTGGAGCAAC  
Reverse: CTGATGTAAGCAGGAGGAC | 82   |
| Catalase      | XM_004016396.4    | Forward: GCAGTACCCCTCTGGACTG  
Reverse: CACTGAGGGCAAAACCTTGA | 105  |
| SOD2          | NM_001280703.1    | Forward: CAAATGGAGCAGGAGGACGCT  
Reverse: TAAAGCATGCTTCCACAGCCT | 111  |
| GPX1          | XM_004018462.4    | Forward: CACCACATTGAGCTGCAGGATTGTG  
Reverse: CGTCTCTGGCGTTTCTTCTGA | 102  |
| Glycogen synthase 2 | XM_004006794.4 | Forward: CAACTCAGCGGCAGTCTTCATG  
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**Abbreviations:**

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta; TNF, tumor necrosis factor; CCL2, C-C motif chemokine ligand 2; CXCL8, C-X-C motif chemokine ligand 8; IFNG, interferon gamma; IL1B, interleukin 1 beta; AGPAT1, 1-acylglycerol-3-phosphate O-acyltransferase 1; AGPAT2, 1-acylglycerol-3-phosphate O-acyltransferase 2; SREBF1, sterol regulatory element binding transcription factor 1; PPARG, peroxisome proliferator activated receptor gamma; FOXO1, forkhead box O1; FASN, Fatty acid synthase; SIRT1, sirtuin 1; SIRT3, sirtuin 3; PPARA, peroxisome proliferator activated receptor alpha; PRKAA2, protein kinase AMP-activated catalytic subunit alpha 2; PPARGC1A, PPARG coactivator 1 alpha; SOD2, superoxide dismutase 2; GPX1, glutathione peroxidase 1; PYGL, glyogen phosphorylase L; GSK3B, glycogen synthase kinase 3 beta; NFkB1, nuclear factor kappa B subunit 1; PTX3, pentraxin 3; MTTP, microsomal triglyceride transfer protein; APOB, apolipoprotein B; CD36, CD36 molecule; SLC27A5, solute carrier family 27 member 5; SLC27A6, solute carrier family 27 member 6; PPIA, peptidylprolyl isomerase A; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta; RPL19, ribosomal protein L19.
Figure S1. Body weight in the low calorie (LC), high calorie (HC) and thiamine-treated HC (THC) growing lambs. Repeated measures ANOVA analysis for HC vs. LC revealed an effect of the dietary treatment ($P < .0001$), time ($P < .0001$) and treatment x time interaction ($P < .0001$). Comparing HC with THC revealed an effect of time ($P < .0001$) and a treatment x time interaction ($P < .0001$) and a trend for thiamine treatment ($P = 0.09$). * Denotes $P < .05$ between HC and THC after Bonferroni-Holm correction.
Figure S2. Daily group-average caloric intake in the low calorie (LC), high calorie (HC), and thiamine treated HC (THC) animals.
Figure S3. Plasma triglyceride and fasting leptin levels in the low calorie (LC), high calorie (HC) and thiamine-treated HC (THC) sheep. (A) Fasting plasma leptin concentration. One-way ANOVA detected an effect of the dietary treatment ($P = 0.02$). (B) Plasma triglycerides levels. Repeated-measures ANOVA analysis revealed an effect of the dietary treatment (HC vs. LC) ($P = 0.02$), time ($P = 0.001$), and treatment by time interaction ($P = 0.01$). * Denotes $P < 0.05$ by contrast t-tests.
Figure S4. Comparative hepatic expression of genes involved in insulin sensitivity in the low-calorie (LC), high-calorie (HC), and thiamine-treated HC (THC) sheep. One way ANOVA detected an effect of treatment for Insulin receptor ($P<0.04$) and $IGF1R$ ($P<0.002$). * Denotes $P < 0.05$ by contrast t-tests.

Abbreviations:

$IGF1R$, Insulin-like growth factor 1 receptor