UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA
FACULTY OF MEDICINE

PhD THESIS

Abstract

Evaluation of adipose tissue and liver inflammation in patients with alcoholic liver disease: focus on the pathogenic role of alcohol

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Introduction

Excessive alcohol consumption is the third largest risk factor for disease and disability worldwide, resulting in approximately 2.5 million deaths each year (4% of all deaths) (World Health Organization report 2011). The liver, as the main site of ethanol metabolism, can be deeply affected by alcohol abuse, resulting in alcoholic liver disease (ALD). Histological features of ALD include a wide spectrum of hepatic injuries ranging from simple steatosis (lipid accumulation in hepatocytes) to liver inflammation (alcoholic hepatitis), hepatocyte necrosis and fibrosis/cirrhosis. From the clinico-pathologic point of view, ALD can be considered in 3 overlapping phases with distinct pathological and clinical features: fatty liver (or steatosis) which is reversible and benign in its pure form, alcoholic hepatitis (AH) (liver inflammatory infiltrate, predominantly neutrophilic in nature) which can lead to acute hepatic failure in its severe form, and hepatic cirrhosis which is the final stage of ALD (Day, 2007).

The intensity of alcohol exposure is a major determinant of ALD pathogenesis. The risk increases in a dose dependent manner with increasing daily ethanol intake (Bellentani et al., 1997). In spite of this, the prevalence of hepatic cirrhosis is relatively low in heavy drinkers. Therefore, key factors other than the quantity of alcohol intake influence the onset and progression of ALD, and my PhD research will focus on the role of AT.

Before my arrival the team reported that excess weight for at least 10 years is an independent risk factor for pure steatosis, AH and cirrhosis in alcoholic patients.
Furthermore, the body mass index (BMI) is an independent risk factor for fibrosis in non-obese alcoholics (Raynard et al., 2002). It could be speculated that dysregulation of AT metabolism may account for the deleterious effects of excess weight on the generation and progression of ALD.

It is now recognized that adipose tissue (AT) is not merely a location for fat storage but a true endocrine and immune organ that can synthesize and secrete a large range of pro- and anti-inflammatory cytokines and chemokines, generally termed adipokines. The adipokines mediate communication within AT and with other organs. The dysregulation of adipokine secretion may exert deleterious effects on distant organs, such as liver and muscles. Obesity increases the risk of nonalcoholic steatohepatitis and dysregulation of AT metabolism can be a major contributor to obesity-related hepatic injury (Perlemuter et al., 2007). Chronic alcohol consumption is also associated with dysregulation of AT adipokine production. The epididymal adipose tissue of rats chronically fed an ethanol containing diet shows increased pro-inflammatory cytokine production (Lin et al., 1998). A recent research performed by our team before my arrival has revealed that a pro-inflammation state can be seen not only in the liver but also in the AT, in non-obese alcoholic patients (Naveau et al., 2010). However the causal role of alcohol in the pathogenesis of AT inflammation as well as the significance of this inflammation in ALD is unknown.

Key words: alcoholic liver disease, adipose tissue, cytokine, adipokine, inflammation
A. Background

The first chapter of the literature review, entitled “The immune mechanisms involved in alcohol-induced liver injury”, describes the main mechanisms of alcohol-induced liver inflammation. It is generally accepted that Kupffer cell activation is the key event for the initiation of liver inflammation related to chronic alcohol consumption. Therefore, the factors that contribute to Kupffer cell activation are described: endotoxin, reactive oxygen species, iron, histone acetylation (Voican et al., 2011). Activated Kupffer cells further release pro-inflammatory cytokines and chemokines that attract and activate immune cells, mostly neutrophils and lymphocytes.

The second chapter of the literature review, entitled “Adipose tissue and alcoholic liver disease”, describes the mechanisms involved in the pathogenesis of AT inflammation and its possible involvement in alcohol-induced liver injury.

The second chapter of the literature review, entitled “Specific therapeutic approaches for alcoholic liver disease”, describes available therapeutic approaches for ALD, and is focused primarily on the treatment of acute alcoholic hepatitis.
B. Personal contribution

I. The study of housekeeping genes in alcoholic liver disease

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is the most commonly used method for the quantification of mRNA expression among different samples. PCR signal relative quantification of a target mRNA requires normalization to obtain reliable data. Careful normalization to an endogenous reference is essential for correcting variations of amplification related to different amounts of input RNA and variation of experimental conditions (Pfaffl, 2001). In general, normalization involves accurately quantitated total RNA or the use of one housekeeping gene (HKG). Such genes should be present in all nucleated cell types and synthesis of HKG mRNA is considered to be stable among individuals, tissue types and physiological states (Ho-Pun-Cheung et al., 2009). However, numerous studies have already shown that many of the commonly used HKGs are regulated and vary under experimental conditions.

There is no published data available to assess which HKG shows the lowest variability and should be used for normalization in the liver of alcoholic patients. This point is crucial as the variability of HKG expression can lead to erroneous results and conclusions. In patients with ALD, HKG expression may vary according to the extent of steatosis, liver inflammation and fibrosis. I therefore aim to study in a group of alcoholic patients the
variability of four HKG commonly used for RT-qPCR normalization: 18S, β-actin, GAPDH and arginine/serine-rich splicing factor (SFRS4). The results of my first study will permit to identify the most suitable HKG to be used for gene expression normalization in the liver of ALD patients.

**Patients and methods**

I prospectively included 50 patients admitted to the Hepatogastroenterology Department of Antoine Béclère University Hospital, Clamart, France, due to alcoholism and ALD. Patients were eligible for inclusion if they had high serum aminotransferase levels, had drunk at least 50 g of alcohol per day over the previous year and had not stopped drinking before admission, had no detectable hepatitis B surface antigen or antibodies against hepatitis C virus in their serum, gave informed consent and had no contraindications (including blood coagulation defects or ascites) for intercostal liver and adipose tissue biopsies. The exclusion criteria were gastrointestinal bleeding, bacterial infection, hepatocellular or other carcinoma, acute pancreatitis, severe associated disease, the presence of anti-HIV antibodies, dyslipidemia and diabetes mellitus. A specific questionnaire was used to obtain information about alcohol consumption (Naveau et al., 2000). Informed consent was obtained from all participants.

All patients underwent ultrasound-guided liver biopsy within one week of admission for the evaluation of their ALD. One specimen of liver tissue was used for histological analysis. Another was immediately frozen in
liquid nitrogen and stored at -80 °C for subsequent RNA extraction. All biopsy samples were evaluated by a pathologist blind to the patient’s clinical and biological data and to the duration of alcoholism and daily alcohol intake. Internationally accepted morphological criteria were used for this semi-quantitative evaluation (group, 1981).

*RNA isolation.* Total RNA was extracted with the RNeasy Lipid Tissue kits (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RNA was quantified by the average of duplicate measuring of the absorbance at 260 nm (A260) in a spectrophotometer (Eppendorf Biophotometer). Purity of total RNA was determined by the A260/A280 and A260/A230 ratio, respectively.

*Amplification of total RNA.* Due to the reduced biopsy size, we isolated only a small quantity of total RNA. In order to obtain a higher quantity of RNA for gene expression analysis, 1µg of each patient total RNA sample was amplified with the Message Amp II aRNA kit (Ambion, Austin, TX, USA) following the manufacturer’s instructions. The antisens RNA that resulted from amplification protocol was used for further experiments.

*RT-qPCR.* In the first step, 5µg of each antisens RNA sample was reverse transcribed into cDNA. In the second step, I performed the RT- qPCR in a LightCycler 480 instrument (Roche) using the LC FastStart DNA Master SYBR Green I kit (Roche) and primers specific for the genes of interest: 18S, SFRS4, GAPDH and β-actin. For each gene, sample expression levels are presented per microgram of RNA.
Statistics. To compare the dispersion of values for each HKG, the absolute value of deviations from the median (AVDM) was calculated for each patient and the means were compared between the 4 HKGs. The Mann-Whitney test was used to analyze the differences between two groups and the Wilcoxon test for paired comparisons; the Kruskal-Wallis Z test was used for comparing more than two groups and the Friedman test for paired comparisons; p value was adjusted using Benjamini and Yekutieli method for the multiple comparisons. The Number Cruncher Statistical System 2007 software (NCSS, Kaysville UT) and R-2.12.1 software were used for statistical analysis.

Results and discussions

41 men and 9 women were included in the study. The mean period from admission to liver biopsy was 5.6 ± 0.5 days. Twenty-four patients had alcoholic hepatitis, 44 had moderate steatosis (6 to 32 % of hepatocytes) and 17 had cirrhosis.

The most stable genes were 18S and SFRS4. Conversely, the other HKGs showed clear differences in their expression level. Coefficient of dispersion was the smallest for 18S (1.4) and the highest for β-actin (23.9). If female samples were excluded from the analysis the coefficient of dispersion was still the smallest for 18S (18S: 0.95; actin: 16.2; SFR: 2.1; GADPH: 2.3).

The extent of steatosis varies between alcoholic patients. I therefore studied the modulation of liver HKG according to the presence or not of steatosis. GAPDH and β-actin mRNA was decreased in patients with
steatosis. 18S and SFRS4 were not significantly modified according to the presence of steatosis.

Alcoholic hepatitis may also be involved in the modulation of HKG expression. I therefore assessed HKG expression in patients with and without AH. 18S and SFRS4 mRNA were not significantly different between the two groups of patients. Oppositely, GAPDH and β-actin were increased in patients with AH.

I further compared the expression level of HKG between patients with low levels of fibrosis and patients with extensive fibrosis. β-actin, 18S and SFRS4 mRNAs were not significantly modified according to the fibrosis score. However, there was a significant increase of GAPDH mRNA expression in patients with a high fibrosis score.

Fibrosis is present to some degree in almost all cases of alcoholic hepatitis and most patients with AAH have cirrhosis. Given this very strong association, I investigated HKG expression between 4 groups of patients: patients with neither cirrhosis nor AAH; patient with cirrhosis and AAH; patients without cirrhosis but with AAH; patients with cirrhosis without AAH. Both β-actin and GAPDH mRNAs were significantly different between the 4 groups of patients. However, 18S and SFRS4 mRNA were not significantly modified according to the extent of liver lesions.

I clearly show in my study that the gene demonstrating the lowest level of variation according to pathological liver lesions was 18S, and to a lesser extent SFRS4. Both β-actin and GAPDH were highly variable according to liver lesions, and their use as a HKG for
normalization of a RT-qPCR would lead to misinterpretation of the results.

**Conclusions**

1. Although my sample size is a bit small to make large generalizations, I have shown that 18S and SFRS4 expression in liver of alcoholic patients are not influenced by steatosis, alcoholic hepatitis, significant fibrosis and cirrhosis.
2. 18S and SFRS4, but not β-actin or GAPDH are appropriate reference genes for RT-qPCR normalization in percutaneous liver biopsies of alcoholic patients. However, 18S was the housekeeping gene that showed the lowest variability.
3. Based on my results, I recommand 18S as a reference gene for RT-qPCR normalization in percutaneous liver biopsies of ALD patients.

**II. Evaluation of subcutaneous abdominal adipose tissue and liver inflammation in patients with alcoholic liver disease**

The severity of alcohol-induced liver injury greatly varies between cases and only a small proportion of heavy alcohol drinkers develop advanced liver disease (AH and cirrhosis). It is therefore considered that factors other than quantity of alcohol intake are involved in the pathogenesis of ALD and AT is one of them. In this context, my PhD researches focused on AT as a risk factor for ALD.
The first evidences that AT may have a deleterious effect on liver lesions in ALD patients came from the team 14 years ago (Naveau et al., 1997). Before my arrival the team has showed that being overweight for at least 10 years is an independent risk factor for steatosis, AH and fibrosis in alcoholic patients. Furthermore, it was also showed that BMI is an independent risk factor for fibrosis in non-obese alcoholics (Raynard et al., 2002). A recent research performed by our team before my arrival further indicates that subcutaneous adipose tissue shows an inflammatory pattern in ALD patients (Naveau et al., 2010). However, the role of alcohol in the induction of adipose tissue inflammation has never been studied in humans. Furthermore, the significance of AT inflammation in the context of ALD remains also unknown.

**Hypotheses and objectives**

I hypothesized that chronic alcohol consumption could alter the balance between the pro- and anti-inflammatory factors in the adipose tissue. The increased production of TNFα and leptin by the AT following chronic alcohol consumption (humans and animal models) and the participation of these factors to the hepatic inflammatory process and fibrogenesis suggest that alcohol-induced activation of adipocyte and non-adipocyte cells may account for the correlation between BMI and severity of ALD in humans.

The causal role of alcohol in the induction of AT inflammation has never been studied in alcoholic
patients. In this context, the first objective of this part of my research is to prove the involvement of alcohol in the pathogenesis of adipose tissue inflammation in ALD patients. I therefore study the expression of pro- and anti-inflammatory cytokines, adipokines and their receptors, and inflammatory cell markers in the subcutaneous adipose tissue and serum of patients with different stages of ALD, before and after 7 days of alcohol abstinence. In this way, each patient becomes its own control.

The second objective of my study is to identify new actors driving AT and liver inflammation during chronic alcohol consumption which could open new research directions in ALD. I therefore analyzed the production of key adipokines/cytokines/chemokines in the liver and adipose tissue as a function of histological features of liver injury.

Patients and methods

I prospectively included 47 patients with different stages of ALD admitted to the Hepatogastroenterology Department of Antoine Béclère University Hospital, Clamart, France. Patients were eligible for inclusion if they had high serum aminotransferase levels, had drunk at least 50 g of alcohol per day over the previous year and had not stopped drinking before admission, had no detectable hepatitis B surface antigen or antibodies against hepatitis C virus in their serum, gave informed consent and had no contraindications (including blood coagulation defects or ascites) for intercostal liver and adipose tissue
biopsies. The exclusion criteria were gastrointestinal bleeding, bacterial infection, hepatocellular or other carcinoma, acute pancreatitis, severe associated disease, the presence of anti-HIV antibodies, dyslipidemia and diabetes mellitus. A specific questionnaire was used to obtain information about alcohol consumption (Naveau et al., 2000). Informed consent was obtained from all participants.

At admission, we performed a liver and subcutaneous adipose tissue biopsy. Alcohol withdrawal was performed during hospital admission following the usual alcohol detoxification protocol of the department. After 7 days of alcohol abstinence we performed a second subcutaneous adipose tissue biopsy. One specimen of liver tissue of 15 mm in length was fixed in formol 10%, included in paraffine and then used for histological analysis. Another specimen of liver tissue (at least 3 mm in length) was immediately frozen in liquid nitrogen and stored at -80°C for subsequent analysis of gene expression. All liver biopsy samples were evaluated by a pathologist blind to the patient’s clinical and biological data and to the duration of alcoholism and daily alcohol intake. Internationally accepted morphological criteria were used for this semi-quantitative evaluation (group, 1981). Adipose tissue samples were immediately frozen in liquid nitrogen and stored at -80°C for subsequent gene expression analysis. A blood volume of 10 ml was collected from each patient at admission and after 7 days of alcohol abstinence for subsequent assessment of cytokine (adipokine) serum levels. The blood was centrifuged after clotting and the serum was stored at -80°C.
**Gene expression analysis.** Total RNA was extracted with the RNeasy Lipid Tissue kits (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The purified RNA was quantified by the average of duplicate measuring of the absorbance at 260 nm (A260) in a spectrophotometer (Eppendorf Biophotometer). RNA purity was determined by the A260/A280 and A260/A230 ratio, respectively. RNA integrity was assessed by capillary electrophoresis on an Agilent 2100 bioanalyzer (Agilent Technologies) using an RNA 6000 Nano LabChip kit (Agilent Technologies) according to the manufacturer’s instructions. We then synthesis the complementary DNA by reverse transcription of 0.35 µg of total RNA per sample using RT² First Strand kit (SABiosciences) following the manufacturer’s protocol. Custom-made RT² Profiler PCR arrays (CAPH09709, SABiosciences) were used to simultaneously quantify the relative expression levels of several cytokines (adipokines) (table 1) in the liver and AT (at admission and after 7 days of alcohol abstinence) of my alcoholic patients according to manufacturer’s instructions. 18S was used as an endogenous control (housekeeping gene) for gene expression normalization.
Table 1. The groups of genes assessed by RT²-qPCR arrays.

<table>
<thead>
<tr>
<th>Pro-inflammatory cytokines (Th1/M1)</th>
<th>Anti-inflammatory cytokines (Th2/M2)</th>
<th>Adipokines/receptors</th>
<th>Total macrophages</th>
<th>M2 macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL18, IL8, CXCL12, SEMA7A, CCL2, CCL5, OPN, IL1β, IFNγ, IL6, TNFα</td>
<td>IL10, IL1RA, TGFβ1</td>
<td>Leptin, LepR, Adiponectin, AdipoR1, AdipoR2, Apelin</td>
<td>CD68, CD14</td>
<td>CCL18, MRC1</td>
</tr>
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</table>

Serum cytokine (adipokine) levels. The serum levels of several cytokines/adipokines were determined on admission and after 7 days of alcohol withdrawal using Luminex technology (Millipore).

Statistics. Statistical significance of the differential gene expression between the subgroups with different degrees of liver injury was determined using the non-parametric Mann-Whitney test with the ΔCt of each group. Statistical significance of the differential gene expression in the AT before and after 7 days of alcohol abstinence was determined using paired-samples Wilcoxon signed rank test with the ΔCt of each group. Correlations were analyzed using the Spearman’s rank correlation test. A p value < 0.05 was considered as significant. Number Cruncher Statistical System 2007 software (NCSS, Kaysville UT) and R-2.12.1 software were used for statistical analysis.
Results and discussions

Forty seven consecutive patients (38 men, 9 women) were included in the study. No patient had an abnormal blood glucose level and/or an abnormal HbA1c level, suggesting the absence of insulin resistance in our patients. Most of our patients did not have advanced liver injury.

I first analyzed the effect of alcohol abstinence on the production level of pro- and anti-inflammatory cytokines by AT. The AT production of IL18, osteopontin and SEMA7A decreased at D7 (day 7 of alcohol abstinence) compared to D1 (day of inclusion), but the difference was not statistically significant. I further analyzed the expression level of total macrophage markers (CD68 and CD14) and M2 anti-inflammatory macrophage markers (CCL18 and MRC1). In the overall study group, the mRNA expression levels of CD68 and CD14 were not significantly influenced by alcohol abstinence (Fig. 1). However, the adipose tissue CCL18 gene expression level showed a statistically significant 1.52 fold-increase (p=0.026) after only 7 days of alcohol withdrawal (D7) compared to its expression at enrolment in the study (D1) (Fig. 1). Overall, my results suggest that 1 week of alcohol withdrawal do not influence AT macrophage infiltration in alcoholic patients, but abstinence from alcohol may alleviate AT inflammation by promoting macrophage switch toward an alternative, anti-inflammatory (M2) phenotype. It is very likely that 1 week of alcohol withdrawal was not enough to see a reduction in AT cytokine production.
Figure 1. Macrophage marker expression in the subcutaneous adipose tissue during alcohol withdrawal.

I further analyzed the effect of alcohol withdrawal on cytokine production and macrophage marker expression in the subcutaneous adipose tissue of our patients, as a function of ALD severity. I show that the response of adipose tissue inflammation during the first week of alcohol abstinence is significantly different in patients with severe ALD (fibrosis score ≥ 3 and/or AH grade ≥ 2) compared with those with mild ALD (fibrosis score < 3 and AH grade < 2) (Fig. 2).
Figure 2. Effect of alcohol withdrawal on subcutaneous adipose tissue inflammation according to the presence (dark-grey bars) or absence (light-grey bars) of severe liver injury.

The AT mRNA expression of Th1/M1 pro-inflammatory cytokines (IL18, osteopontin and SEMA7A) (Fig. 2A) and total macrophage marker CD68 decreased following one week of alcohol withdrawal (Fig. 2B) in patients with mild ALD lesions, while the ATM switched toward an anti-inflammatory phenotype (Fig. 2B). Oppositely, the production of Th1/M1 pro-inflammatory cytokines (Fig. 2A) and macrophage infiltration (Fig. 2B) increased in the SAT of patients with severe ALD lesions during the first week of alcohol withdrawal. However, a macrophage polarization toward a M2 phenotype could also be seen (Fig. 2B).

Overall, these results suggest that alcoholic patients with mild liver injury show a clear reduction of AT inflammation during the first week of alcohol abstinence. Oppositely, patients with severe liver damage upregulate their pro-inflammatory cytokine expression in the AT during the first week of alcohol abstinence, despite the switch of ATM towards an M2 anti-inflammatory phenotype, which may indicate a
more intense inflammatory process in the subcutaneous adipose tissue of this subgroup of patients.

The mRNA expression level of leptin, adiponectin, adipoR1 and adipoR2 significantly decreased at D7 compared to the level at D1 (Fig. 3). One week after admission (D7) to the alcohol abstinence protocol adiponectin serum levels were significantly lower than at D1 (Fig. 4). The serum levels of leptin had also a tendency to decrease following alcohol withdrawal, but these differences were not statistically significant.

![Figure 3. Leptin, adiponectin and adiponectin receptor (adipoR1 and adipoR2) mRNA expression level in the subcutaneous adipose tissue during alcohol withdrawal.](image)
Figure 4. Adiponectin serum levels during alcohol withdrawal.

The pro-inflammatory and pro-fibrotic properties of leptin have already been proven. The serum levels of leptin are increased in alcoholic patients (Nicolas et al., 2001). I show here that one week of alcohol withdrawal decreases leptin mRNA level in the SAT of ALD patients, demonstrating the causal role of alcohol in leptin upregulation. The high leptin production is probably involved in both SAT and liver inflammation in alcoholic patients. Leptin may be therefore partially responsible for the harmful association between AT and severity of liver damage. Adiponectin displays anti-inflammatory, anti-fibrotic and hepatoprotective properties against alcohol-induced liver injury (Xu et al., 2003). The pro-inflammatory cytokines inhibit adiponectin production. The concomitant induction of pro-inflammatory cytokines and adiponectin in AT in alcoholic patients is unexpected as they are mutually
antagonistic. Two hypotheses can be proposed to account for the upregulation of the adiponectine expression in alcoholic patients: (a) there is a compensatory production of adiponectin attempting to limit inflammatory process; and, (b) alcoholic liver becomes resistant and unresponsive to the hepatoprotective effects of adiponectin.

Osteopontin and CXCL12 are potent chemoattractant factors for immune cells. They have been previously incriminated in the pathogenesis of chronic liver diseases such as NASH or viral hepatitis. Their implication in ALD may be therefore speculated. Given the urgent need to develop pathophysiology-oriented therapies for ALD, I analyzed the expression level of OPN and CXCL12 genes in the liver and SAT of our patients, as a function of liver injury.

I demonstrate that gene expression of osteopontin was increased in patients with mild (F1), moderate (F2) and severe fibrosis (F3/F4) compared with patients with no fibrosis (F0) (Fig. 5). Hepatic osteopontin mRNA expression levels progressively increased with the severity of liver fibrosis. A similar pattern is observed also in the subcutaneous adipose tissue (Fig. 6).
Figure 5. Osteopontin gene expression in the liver, as a function of fibrosis score. #, p=0.013, compared with F0; $, p<0.01, compared to F0; &, p<0.001, compared to F0; §, p<0.001, compared to F1; £, p<0.05, compared to F2.

Figure 6. OPN gene expression in the subcutaneous adipose tissue, as a function of fibrosis score. #, p<0.01, compared with F0; $, p<0.05, compared to F0.
Serum concentration and hepatic expression of osteopontin was positively correlated with liver fibrosis and inflammation, as well as with liver fibrosis markers (TGFβ1, αSMA). Furthermore, osteopontin expression in the AT correlated with hepatic fibrosis but not with liver inflammation and steatosis. Overall, my results suggest that osteopontin may be involved in the pathogenesis of alcohol-induced liver injury in humans and circulating osteopontin level reflects its hepatic expression. On the other hand, osteopontin may also play a role in AT inflammation related to chronic alcohol consumption in humans. The specific blockade of osteopontin may be a potential therapeutic approach that needs further investigation.

CXC-motif chemokine ligand 12 (CXCL12), a chemokine constitutively expressed in many tissues (e.g. bone marrow, liver, lung), plays a key physiological role in hematopoietic stem cells homing to the bone marrow (Peled et al., 1999). CXCL12 is a potent chemoattractant factor for lymphocytes, macrophages and neutrophils (Balabanian et al., 2005). CXCL12 can also induce activation of hepatic stellate cells via CXCR4 pathway (Hong et al., 2009). However, the role of CXCL12 in ALD is unknown. I therefore assessed the hepatic and subcutaneous adipose tissue mRNA expression level of CXCL12 in my alcoholic patients as a function of liver injury. The hepatic production of CXCL12 was significantly higher in alcoholics with AH when compared with those without AH (Fig. 7). Furthermore, liver CXCL12 expression strongly correlated with hepatic inflammation and fibrosis, as well as with liver fibrosis markers (αSMA, TGFβ1).
CXCL12 mRNA expression level was also upregulated in the AT of alcoholic patients with AH compared with alcoholics without AH (Figure 8), and positively correlated with both hepatic fibrosis and inflammation.

Overall, the results of my study indicate that CXCL12 can be involved in the modulation of alcohol-induced inflammatory process in the liver and adipose tissue of ALD patients. However, these are only preliminary results opening the door for new studies that will be performed in our laboratory to investigate the therapeutic potential of CXCL12 in ALD.
Conclusions

In conclusion, the results of my study demonstrate that alcohol is directly involved in the initiation of AT inflammation in ALD patients. I therefore propose a model in which alcohol induces an M1 pro-inflammatory phenotype in adipose tissue macrophages with an increased production of pro- and anti-inflammatory cytokines, and alcohol withdrawal leads to an M2 phenotype orientation and downregulation of cytokine expression (Fig. 9). These findings may account for the harmful interaction between adipose tissue and alcohol-induced liver injury.

OPN and CXCL12 seem to be important actors in the pathogenesis of alcohol-induced liver injury and should be further investigated for their therapeutic potential in ALD.
Figure 9. Effects of alcohol on the adipose tissue.

III. Final conclusions

1. There was no previous work studying HKG in the liver of patients with various stages of ALD. I show here that hepatic β-actin and GAPDH are highly variable with respect to alcohol-induced liver injuries and their use for gene expression normalization in the liver of alcoholic patients would lead to erroneous results. Oppositely, I first show here that 18S and SFRS4 are the most stable HKG in liver samples from patients with ALD. These genes are therefore appropriate reference genes for normalization of RT-qPCR in the liver of ALD patients and should be used in future gene expression studies.
2. I show for the first time that alcohol withdrawal alleviates inflammatory process in the subcutaneous adipose tissue of ALD patients by promoting macrophage polarization toward an anti-inflammatory (M2) phenotype. These findings reinforce the previous results of the team that showed the presence of inflammation not only in the liver but also in the AT of alcoholic patients, and further demonstrates that alcohol is the cause of adipose tissue inflammation in human ALD. I therefore speculate that alcohol-induced changes in the AT may account for the harmful interaction between BMI and hepatic inflammation, fibrosis and cirrhosis in alcoholic patients.

3. Common mechanisms could be involved in the pathogenesis of liver and AT inflammation in alcoholic patients. Osteopontin and CXCL12 may be key mediators of these pathogenic mechanisms and should be investigated for their therapeutic potential in ALD, particularly for alcoholic hepatitis.

**IV. Perspectives**

1. The first perspective consists of the study of underlying mechanisms mediating alcohol-induced AT inflammation.

2. A second perspective would be the study of inflammatory cell infiltration in the subcutaneous adipose tissue of alcoholics and healthy non-alcoholic subjects by immunohistochemistry, flow cytometry and RT-qPCR. This will allow us to identify the cellular
origin of pro-inflammatory cytokine production in SAT of alcoholic patients.

3. The third perspective would be to confirm the results obtained in alcoholic patients by studying the expression of CXCL12 gene in the liver and adipose tissue of ethanol-fed mice. If our hypothesis is confirmed we will further assess the effect of CXCL12 specific blockade on the development of alcoholic liver disease in the same animal model. Our experiments could lead to the identification of new therapies for alcoholic liver disease, and particularly for acute alcoholic hepatitis.

References


Curriculum Vitae

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Publications in PhD research area

I. Articles published in ISI indexed journals in PhD research area


II. Oral and poster presentations at the international conferences in PhD research area

**Oral presentations**

Posters

1. Gual P; Patouraux S; Bonnafous S; Voican CS; Anty R; Saint-Paul MC; Rosenthal-Allieri MA; Agostini H; Njike M; Barri-Ova N; Naveau S; Calès P; le Marchand-Brustel Y; Perlemuter G; Tran A. L’augmentation de l’expression de l’ostéopontine dans le foie, le tissu adipeux et le sérum de patients alcooliques est corrélée à la fibrose hépatique. Abstract book. 69ème Journées Scientifiques de l’Association Française pour l'Etude du Foie, Paris-La Villette 2011.