Gene expression profiling provides insights into pathways of Oxaliplatin related Sinusoidal Obstruction Syndrome in humans

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Abreviations list: SOS, sinusoidal obstruction syndrome, CRLM, colorectal liver metastases, SECs, sinusoidal endothelial cells, Ox, Oxaliplatin.

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ABSTRACT

Sinusoidal obstruction syndrome (SOS; formerly veno-occlusive disease) is a well-established complication of hematopoietic stem cell transplantation, pyrrolizidine alkaloid intoxication, and widely used chemotherapeutic agents such as oxaliplatin. It is associated with substantial morbidity and mortality. Pathogenesis of SOS in humans is poorly understood. To explore its molecular mechanisms, we used Affymetrix U133 Plus 2.0 microarrays to investigate the gene expression profile of 11 human livers with oxaliplatin-related SOS and compared it to 12 matched controls. Hierarchical clustering analysis demonstrated that profiles from SOS and controls formed distinct clusters. To identify functional networks and gene ontologies, data were analyzed using Ingenuity Pathway Analysis Tool. 913 genes were differentially expressed in SOS, 613 being upregulated and 300 down-regulated. RT-PCR results showed excellent concordance with microarray data. Pathway analysis demonstrated major gene up-regulation in six pathways in SOS compared to controls: acute phase response (notably IL-6), coagulation system (Serpine1, THBD and VWF), hepatic fibrosis/hepatic stellate cell activation (COL3a1, COL3a2, PDGF-A, TIMP1, and MMP2) and oxidative stress. Angiogenic factors (VEGF-C) and hypoxic factors (HIF1A) were upregulated. The most significant increase was seen in CCL20 mRNA.

In conclusion, oxaliplatin related SOS can be readily distinguished according to morphological characteristics but also by a molecular signature. Global gene analysis provides new insights into mechanisms underlying chemotherapy-related hepatotoxicity in humans and potential targets relating to its diagnosis, prevention and treatment. Activation of VEGF and coagulation (vWF) pathways could partially explain at a molecular level the clinical observations that bevacizumab and aspirin have a preventive effect in SOS.
Introduction

Chemotherapy-related hepatic sinusoidal obstruction syndrome (SOS, previously named veno-occlusive disease or VOD) is one of the limiting factors in the otherwise highly effective use of Oxaliplatin (OX) for patients with colorectal liver metastases (1-3). Its pathogenesis, preoperative diagnosis and prevention are thus the subject of intense investigation. In fact, since our initial report (1), several groups have confirmed the existence of OX-related SOS in 25-36% of treated patients. This has led to concerns that OX-associated hepatotoxicity may decrease the chances of curative liver surgery by increasing morbidity through preoperative hemorrhage, post operative liver failure, delayed regeneration and portal hypertension (4-13). Rare fatal cases have been reported, inducing a group of experts to publish a cautionary note on the use of chemotherapy before surgical liver resection (14).

SOS is morphologically characterized by centrilobular sinusoidal congestive dilatation and perisinusoidal hemorrhage, occasionally associated with perisinusoidal fibrosis and/or centrilobular hepatic vein fibrotic obstruction, nodular regenerative hyperplasia (NRH) or peliosis (15-17).

A rat model based on gavage with monocrotaline (a pyrrolizidine alkaloid) has made an important contribution to understanding SOS pathogenesis, both at the morphological and biochemical levels. Thus this model had shown that the main injury occurs in the hepatic sinusoids and that centrilobular vein involvement is not essential, leading to the replacement of the term VOD (18). Toxic injury to sinusoidal endothelial cells (SECs) is the key initiating event, followed by alterations of the perisinusodial space. SECs initially round up and detach, allowing erythrocytes to penetrate the perisinusoidal space with further dissection of the sinusoidal lining. Sloughed SECs and Kupffer cells intermingled with erythrocytes subsequently
embolize downstream within the sinusoidal lumen towards the centrilobular vein (19). Microcirculation plugging causes sinusoidal obstruction, a reduction of blood flow in the sinusoids, increasing portal pressure (20), leading to liver metabolic dysfunction, and affecting the viability of parenchymal cells through hypoxia (15-17). In humans, morphological studies have confirmed that the sinusoids are the main site of injury.

The aim of the present study was to gain further insight into the pathogenesis of SOS in humans using a molecular approach (global gene analysis with Affymetrix microarrays) to identify key genes through alterations in their mRNA levels. The clinical impact and the availability of human surgical liver specimen led us to study SOS lesions in CRLM surgical resection treated preoperatively by OX-based chemotherapy. We compared livers with severe OX related SOS to livers treated with OX without SOS, and to histologically normal livers.

Materials and methods

Patients and liver specimen

From the files of the Department of Pathology of University Hospitals of Geneva, Switzerland and of Cochin Hospital of Paris, France, three groups of patients with CRLM were established, matched for sex and age. Group 1 (n=20) was composed of patients treated with preoperative OX-based chemotherapy with histologically confirmed severe SOS (figure 1), based on our previous studies (1,2). Group 2 (n=20) was composed of patients treated by OX-based chemotherapy but without SOS. Group 3 (n=20) was composed of cases treated by surgery alone, and without histological lesions. For group 1 and 2, the protocol used was exclusively OX in combination with 5-FU and leucovorin with an equivalent number of cycles and a
similar delay between the end of chemotherapy and surgery. No portal embolisation was performed before surgery. The use of clinical and pathological records for our research was in agreement with Swiss and French laws and ethical guidelines related to the protection of the patient.

**Microarray analysis**

All snap frozen liver specimens from the selected cases were obtained from the biobanks of Cochin and Geneva Hospitals and reviewed by frozen section to confirm the presence (for group 1) or the absence (for group 2 and 3) of SOS in the frozen samples. Total RNA was extracted from 25 μm sections of surgical specimen using TRIzol (Invitrogen, Carlsbad, USA). RNA quality was assessed using an Agilent 2100 Bioanalyser with an RNA 6000 Nano LabChip kit. There was no obvious difference in RNA quality between the specimens from patients treated at the 2 centers. A RNA integrity number (RIN) was calculated for each sample of RNA and only high-quality samples were used for microarray hybridization, corresponding to intact RNA (n=23 samples). We generated a hybridization mixture containing 15 μg of double-stranded biotinylated cDNA and hybridized it to GeneChip HG U133 Plus 2.0 (Affymetrix). The data was RMA normalized (21).

As a first quality control of the dataset we performed a 1-way ANOVA (tested factor: group) and we visualized the result by hierarchical clustering in the Partek Genomics Suite (Partek) using the Pearson correlation similarity measure and average linkage algorithm (figure 2). Following this analysis, samples from groups 2 and 3 were merged.

To assess the difference in gene-expression values between cases (group 1) and controls (groups 2 & 3), we performed a Welch t-test in Partek Genomics Suite. P-
values were corrected for multiple testing by use of the false-discovery rate (FDR) method of Benjamini and Hochberg (22). We applied a conservative significance threshold of 5% FDR associated with fold change value $\geq 1.5$.

The gene expression data can be found in ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae) Accession No.:E-MTAB-503

Network and gene ontology analyses

Pathway analysis of the genes, which were identified as differentially expressed by microarray experiment was undertaken using the Ingenuity Pathway Analysis software (http://www.ingenuity.com) as previously described (23). All p-values are calculated using the right-tailed Fisher exact test.

Real-time quantitative PCR analysis

To validate our microarray results and further clarify the difference in the expression of the selected genes, we carried out a real-time RT-PCR on additional samples. cDNA was synthesized from 1 ug of total RNA using random hexamers and Supercript III reverse transcriptase (Invitrogen) following supplier’s instructions. Amplicons were designed using the software Primer Express v 2.0 (Applied Biosystems) with default parameters. Amplicons sequences were aligned against the human genome by BLAST to ensure that they were specific for the gene being tested. Oligonucleotides were obtained from Invitrogen. The efficiency of each design was tested with serial dilutions of cDNA. Oligonucleotides, amplicons, sequences and efficiencies can be obtained upon request. PCR reactions ($10 \mu l$ volume) contained diluted cDNA, 2 x Power SYBR Green Master Mix (Applied Biosystems), 300 nM of forward and reverse primers. PCR were performed on a SDS 7900 HT instrument
Rubbia-Brandt et al. (Applied Biosystems) with the following parameters: 50°C for two minutes, 95°C for ten minutes, and 45 cycles of 95°C 15 secondes-60°C one minute. Each reaction was performed in three replicates on 384-well plate. Raw Cq values obtained with SDS 2.2 (Applied Biosystems) were imported in Excel and normalisation factor and fold changes were calculated using the GeNorm method (Vandesompele et. al., 2002). The results were presented as box plot analyses, using StatView Software (SAS Institute Inc.).

Immunostaining:
Specimens showing representative sinusoidal lesions and control hepatectomy cases from colorectal metastasis without lesions were selected for immunohistochemical studies. For immunostaining, additional serial, 4 μm thick liver sections were deparaffinized in xylol and rehydrated in descending ethanol series. The epitopes were recovered by heating slides 30 secondes at 125°C in a buffer containing 0.01M Tris, 1mM EDTA pH9. Mouse monoclonal α-smooth muscle (α-SM) actin was purchased from DakoCytomation (Zug, Switzerland) and used at 1:400 dilution, while anti-CCL20 goat antibodies was purchased from R&D Systems (AF360) and used at 10ug/ml in diluent from Dako (S2022). After blocking endogenous peroxydases with H2O2/methanol, primary antibodies were incubated 1 hour at RT. For αSM actin, microwave pretreatment was used. Sections were incubated for 1 h at room temperature with the diluted primary antibodies, which were then revealed by ENVISION (DakoCytomation). For anti CCL20 staining, rabbit anti-goat antibodies labeled with HRP (sc-2768, Santa-Cruz Biotechnology) were used as secondary antibodies at 40ug/ml final concentration and were incubated 30mn at RT. The staining reaction was performed using the DAB substrate chromogen system.
from Dako (K3468). A negative control was performed with unspecific rabbit IgG. Sections were weakly counterstained with Mayer’s hematoxylin and mounted in Eukitt (Kindler GmbH, Freiburg, Germany). Negative controls were prepared by omitting the first antibodies.

Results

Microarray analysis

To gain insights into the pathogenesis of SOS, we compared expression profiles of one group of 11 human liver tissues with OX-related SOS with 2 control groups of histologically normal livers, one having received preoperative OX- chemotherapy but with no hepatic toxic lesions (n=7), the other without preoperative chemotherapy (n=5).

A hierarchical clustering of the 3 groups on the basis of similarity in the expression pattern of genes selected by ANOVA yielded two major clusters that separated samples with histologically severe SOS (group1) from histologically normal livers (group 2 and 3) (figure 2a). This result underlines the fact that SOS status has a greater impact on the expression profile than the presence or absence of OX-based chemotherapy. Based on this observation, groups 2 and 3 were pooled together for further analysis.

These analyses produced a list of 913 genes that were differentially expressed between SOS and controls (FDR adjusted p-value ≤ 0.05), 613 statistically up-regulated (see Supporting information, Table S1) and 300 statistically down-regulated (see Supporting information, Table S2) by more than 1.5 fold in SOS relative to histological normal liver tissue and a FRD adjusted p-value ≤ 0.05 (figure 2b).
Ingenuity Pathway Analysis tool was used to examine functional associations between genes and revealed that several pathways implicated with high significance in human SOS.

**Biological pathway analysis**

IPA revealed significant up-regulation of expression in SOS compared to controls in genes involved in acute phase response pathway ($p=0.00002$) (see Supporting information, Table S3), notably affecting the IL-6 pathway (IL-6, IL6ST, LBP, STAT3). The relative over-expression of STAT3 and IL-6 mRNA was confirmed by quantitative RT-PCR experiments that compared the expression levels of samples from group 1 (OX-related SOS $n=20$) with those observed group 2 (treated by OX-based chemotherapy but without SOS, $n=20$) and group 3 (treated by surgery alone, and without histological lesions, $n=20$) ($p<0.001$) (figure 3).

IPA revealed other biological pathways implicated in SOS with the coagulation system reaching high significance ($p=0.0001$), notably with up-regulation of genes such as SERPINE1, THBD, F3, PLAU and VWF (see Supporting information, Table S4) and the oxidative stress pathways ($p=0.006$) with up-regulation of genes such as JUN, SOD2. RT-PCR confirmed over expression of SERPINE1, VWF and SOD2 (figure 4) and was concordant with the microarray data.

IPA analysis also showed activation of the hepatic fibrosis/hepatic stellate cell activation pathway ($p=0.0002$) (see Supporting information, Table S5). Most of the activated genes encoded for extracellular matrix proteins, associated molecules or pro-fibrogenic cytokines (COL15A1, COL1A2, COL1A1, COL4A1, TIMP1, MMP2, CCL2, IFNGR1, LBP, PDGF-A, THBS1, THBS2). RT-PCR confirmed TIMP1 and MMP-2 over-expression in group1, compared to group 2 and group 3 (figure 5). The
over-expression of a number of genes involved in hepatic fibrosis was confirmed by Masson’s Trichrome- staining on histological section of SOS where centrilobular perisinusoidal fibrosis was present (figure 1). Hepatic stellate cell activation was illustrated by diffuse alpha smooth muscle cells expression on immunohistochemistry (figure 5).

In SOS, several cytokine and chemokine mRNAs were upregulated (see supporting information table S6); the highest change fold (10.97x) was observed in CCL 20 gene expression. Real-time quantitative RT-PCR revealed considerable over-expression of CCL20 in SOS (n=20), relative to histologically normal liver (groups 2 and 3) (Figure 6a). Immunohistochemistry for CCL20 localized expression in portal and sinusoidal cells aggregates, morphologically identified as macrophages and kupffer cells respectively (figure 6b,c).

Several genes involved in angiogenesis and hypoxia were also significantly over expressed. HIF1A and VEGF-C were upregulated in SOS (see Supporting information, Table S1), while VEGF-A and B, ANGPT1 and 2, NOS3 showed no significant changes. RT-PCR was concordant with the microarray data, notably for the increased HIF1a mRNA in SOS and the significant increase in VEGF-C RNA in both groups 1 and 2 compared to group 3 (figure 7). VEGF-A mRNA was present in the 3 groups, with no significant increase in SOS (figure 7).

Several cytochrome p450 enzyme mRNAs were down-regulated (see Supporting information, Table S7). RT-PCR for CYP7A1 showed excellent concordance with the microarray data (data not shown).
Discussion

To our knowledge, this is the first study to investigate the molecular mechanism of OX-related hepatotoxicity in humans using Affymetrix microarrays to evaluate quantitative gene expression profiles. The investigation provides new insights into the biological and cellular mechanisms involved in chemotherapy related SOS in humans.

We identified 913 genes differentially expressed in OX-related-SOS compared to 2 control groups with histologically normal livers. Interestingly, the two latter groups, one composed of patients who had received preoperative OX for CRLM but who did not develop toxic liver injury and the other composed of patients who had been treated by surgery alone, without chemotherapy, did not segregate on hierarchical clustering. Thus SOS can readily be distinguished from normal liver not only according to its morphological characteristics but also to its molecular configuration.

To pinpoint important functional networks and ontologies, genes over and under expressed in SOS were analyzed using the Ingenuity Pathway Analysis tool. The canonical pathways that were primarily implicated were acute phase response signaling, coagulation system, hepatic fibrosis/hepatic stellate cell activation and oxidative stress. We validated on additional samples 14 of the main deregulated genes by RT-PCR, all showing excellent concordance with the microarray data. In addition, over expression of VEGF revealed the up-regulation of angiogenic factors in SOS and that of HIF1 was indicative of presence of hypoxia in this condition.

Up-regulation of acute phase response signaling genes in SOS concerned in particular the IL-6 pathway. While inflammation is one of the main initiators of the acute phase reaction, this is unlikely to be the case in SOS; given that no significant inflammation is observed in human SOS (1,2) and studies on the rat model have shown that PMNs and Kupffer cells are not critical mediators of monocrotaline
hepatotoxicity (24). Interestingly, the IL-6 pathway may also be activated in response to toxic damage, where it plays a critical role in hepatic regeneration and hepatoprotection, or to ischemic injury. Both of these effects appear to be mediated through the actions of STAT 3 that blocks apoptosis, reduces oxidative injury (25) and maintains capillary integrity (26). An equivalent role could occur in SOS where the oxidative stress pathway is activated and ischemia is present. Interestingly the STAT 1 and STAT 2 genes were also both upregulated in SOS. STAT1 contributes to liver inflammation and injury and to suppression of liver regeneration (25). Further studies on animal models are needed to understand better STAT function in SOS pathogenesis, both at level of apoptosis and of liver regeneration impairment.

Interestingly, the highest mRNA up-regulation in SOS was observed for the chemokine CCL20. Interactions of CCL20 and its receptor CCR6, expressed in colorectal cancer, may play a role in development of metastasis by promoting cancer cell proliferation and migration (27,28) and increased expression of CCL20 has been reported in livers of patients with CRLM (29). These studies, however, did not address the effect of preoperative chemotherapy on this system. In our study, hepatic CCL20 expression could not be explained exclusively by the presence of CRLM, its level being higher in patients with SOS than in patients with CRLM alone. Immunohistochemistry demonstrated CCL20 expression in macrophages but not in endothelial cells (30). In monocytes/macrophages, CCL20 is a hypoxia-inducible gene as illustrated by ischemic/hypoxic transcriptome studies (31,32) where it constitutes an important mechanism to promote recruitment of specific leukocyte subsets at pathological sites. Thus in SOS, hypoxia possibly triggers CCL20 expression. Up-regulation of genes involved in the acute phase reaction could result in changes in serum concentrations of specific plasma acute phase proteins. Inflammatory mediators produced in SOS such
as CCL20 could circulate in the blood. Further studies are needed to evaluate if such proteins or cytokines could be used as serum diagnostic markers of severe SOS.

Interestingly, CCL20 and IL-6 signaling molecules have been reported to be up-regulated in inflammatory hepatocellular adenoma (33), a tumor characterized by inflammation and sinusoidal dilatation. IL-6 pathway activation in inflammatory adenoma has been related to GP130 mutation. Similar levels of over expression of CCL20 and STAT 3 mRNA were observed in SOS compared to 6 inflammatory adenomas (data not shown).

IPA analysis showed activation of coagulation pathways, underlining their role in human SOS. The role of clotting abnormalities in the experimental SOS model (34) and in humans is matter of debate. Injury to SEC creates a pro-coagulant condition in the sinusoids and over-expression of MMPs could be associated with increased platelet adhesion as is observed following cold preservation of liver (35). However ultrastructural studies of livers from individuals with bush tea related SOS revealed no evidence of clotting abnormalities (36) and immunohistochemical studies of autopsy livers did not detect platelets, although fibrinogen and factor VIII were detected in the hepatic veins (37). Conversely, thrombocytopenia has recently been shown as being associated with severe OX-related SOS in humans (38). Because of the role of platelets in liver regeneration through a serotonin-mediated mechanism (35), alteration in platelet function could play a role in postoperative liver insufficiency that may occur following severe SOS lesions. Our results underline the role of coagulation in SOS and open the possibility of therapeutic approaches to prevention OX-associated liver injury, such as Aspirin that has been associated with reduced risk for sinusoidal lesions (13). Treatment with anti-coagulants such as Heparin and Defibrotide, Prostaglandin E1, and plasminogen activator inhibitor 1, have been examined for their ability to prevent
hematopoietic stem cell transplantation related SOS. They have to be yet evaluated in OX-related hepatotoxicity.

Several drugs such as OX and other platinum compounds lead to the production of reactive oxygen species and glutathione depletion in SEC, resulting in SEC injury in vitro (30). Oxidative stress also contributes to SEC injury through monocrotaline induced depletion of cellular glutathione and the augmentation of reactive oxygen species production in vitro and in vivo (39). Our findings show an activated reactive oxygen species pathway in human tissue.

In SOS, genes involved in hepatic fibrosis/HSC activation pathways were also upregulated. This correlates directly with the histological features of human SOS where a significant proportion of patients develop centrilobular perisinusoidal and vein occlusive fibrosis (1,2,4, 15, 40). In the animal model deposition of collagen in the sinusoids also occurs, albeit as late event (18), when activated, HSC acquire a myofibroblastic phenotype and are essential actors in hepatic fibrosis (41) as underlined by the diffuse perisinusoidal αSM actin expression (1).

The animal model has demonstrated that MMPs (extracellular matrix remodeling proteins) play an essential role in SOS, where they contribute to SECs detachment and sinusoidal lining denudation (42). Drugs such as Cisplatin may cause up-regulation of MMP activity in vitro (43). We demonstrate here that MMPs up-regulation also occurs in human hepatic tissue, underlining its role in Ox-SOS pathogenesis.

VEGF-C and HIF1a mRNA were significantly upregulated in OX-related SOS. Hepatic VEGF mRNA expression is significantly increased in the rat model in parallel with sinusoidal damage (44) and serum VEGF increase correlates with the development of SOS in patients after hematopoietic stem cell transplantation (45). VEGF plays a major role in maintaining SEC differentiation (46-48). The increased
expression of VEGF could therefore be a response to endothelial barrier disruption or to cellular hypoxia following SOS. In fact, with microcirculatory disturbances leading to insufficient energy supply, and reduced of hepatic tissue oxygenation (46). The increase HIF1α mRNA levels that we detected underlines the presence of hepatic hypoxia in SOS and could contribute to VEGF induction.

Bevacizumab is a monoclonal anti-VEGF antibody that is used in combination with OX to increase response rates in patients with stage IV colorectal cancer and thus improve progression free survival. Bevacizumab treatment has been shown to have a protective effect against OX-induced sinusoidal injury (2, 49, 50). Our molecular observation could partially explain this clinical observation. VEGF blockade by bevacizumab could lead to down-regulation of MMP expression by SEC (41.46), and thus attenuate sinusoidal lesions. VEGF-C mRNA was increased in SOS, while VEGF-A remained at the same level as in controls.

Finally, the few data that exist regarding the interaction of platinum compounds with the human liver microsomal cytochrome P450 system, come mainly from in vitro studies. Our results on human liver tissue documents the cytochromes that are altered in OX induced hepatotoxicity.

In conclusion, global gene analysis of OX-related SOS provides new insights into the mechanisms underlying SOS and opens new opportunities for diagnosis and therapeutic interventions.
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Legends

Figure 1: Low-power image of Masson’s Trichrome stain of the liver reveals areas of sinusoidal congestion involving centrilobular and mediolobular lobular areas. The small hepatic vein is occluded by fibrous tissue. Perisinusoidal centrilobular spaces are fibrotic.

Figure 2: (a) Hierarchical clustering (Pearson distance – average linkage) on the RMA normalized expression values using the genes that vary across all samples according their group (as selected by ANOVA). Samples showing similar expression profiles are clustered together. Following this analysis, samples from groups 2 and 3 were merged. (b) Heat map representation of genes differentially expressed between cases (group 1) and controls (groups 2 & 3) according a fdr p-value ≤ 0.05 and a fold change value ≤ 1.5.

Figure 3: Box-plot analysis of (a) STAT3 and (b) IL-6 expression by real-time quantitative RT–PCR. Ox-related SOS (n = 20) is compared to liver treated with OX without SOS (n = 20) and histological normal livers (n = 10). Student’s t-test was used to calculate p values (*p ≤ 0.05; **p ≤ 0.01). Means and standard errors are indicated.

Figure 4: Box-plot analysis of (a) VWF, (b) Serpine and (c) SOD2 expression by real-time quantitative RT–PCR. Ox-related SOS (n = 20) is compared to liver treated with OX without SOS (n = 20) and histological normal livers (n = 10). Student’s t-test was
used to calculate p values (*p ≤ 0.05; **p ≤ 0.01). Means and standard errors are indicated.

Figure 5: Box-plot analysis of (a) MMP-2 and (b) TIMP-1 expression by real-time quantitative RT–PCR. Ox-related SOS (n = 20) is compared to liver treated with OX without SOS (n = 20) and histological normal livers (n = 10). Student’s t-test was used to calculate p values (*p ≤ 0.05; **p ≤ 0.01). Means and standard errors are indicated. Expression of alpha-smooth muscle actin by immunohistochemistry (c) Representative view in normal liver shows strong cytoplasmic staining in portal vessel walls and few portal myofibrobasts. No staining is observed in the lobules. (d) Representative view of IHC reveals strong and diffuse staining in perisinusoidal spaces in the lobules in Ox-related SOS.

Figure 6: Box-plot analysis of (a) CCL-20 expression by real-time quantitative RT–PCR. Ox-related SOS (n = 20) is compared to liver treated with OX without SOS (n = 20) and histological normal livers (n = 10). Student’s t-test was used to calculate p values (*p ≤ 0.05; **p ≤ 0.01). Means and standard errors are indicated. Expression of CCL20 by immunohistochemistry. (b) Representative view of a portal tract shows cytoplasmic staining several portal macrophages. No staining is observed in endothelium. (c) Representative view of IHC reveals Küpffer cells in the lobules in Ox-related SOS.

Figure 7: Box-plot analysis of (a) VEGF-C, (b) VEGF-A and (c) HIF-1 expression by real-time quantitative RT–PCR. Ox-related SOS (n = 20) is compared to liver treated with OX without SOS (n = 20) and histological normal livers (n = 10). Student’s t-test
was used to calculate $p$ values ($p \leq 0.05$; $**p \leq 0.01$). Means and standard errors are indicated.
Group 1: OX+SOS
Group 2: OX
Group 3: no chemo

613 up-regulated genes in SOS compared to control livers
300 down-regulated genes in SOS compared to control livers
**Figure 5a**

**MMP-2**

- OX+SOS
  - *p<0.05
- OX
  - **p<0.01
- no chemo

**Figure 5b**

**TIMP-1**

- OX+SOS
  - *p<0.05
- OX
  - **p<0.001
- no chemo
Figures 6a, 6b, and 6c show the expression levels of CCL20 under different conditions.

- **Figure 6a**: Box plot showing relative gene expression levels under OX+SOS, OX, and no chemo conditions.
  - OX+SOS: ** p<0.01
  - OX: **
  - no chemo: **

- **Figure 6b**: Image of tissue sample stained with CCL20.

- **Figure 6c**: Image of another tissue sample with different staining, possibly highlighting another marker or condition.

The data suggests significant differences in gene expression levels among the conditions tested.
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