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Comparative Salivary Proteome of Hepatitis B- and C-Infected Patients

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Abstract

Hepatitis B and C virus (HBV and HCV) infections are an important cause of cirrhosis and hepatocellular carcinoma. The natural history has a prominent latent phase, and infected patients may remain undiagnosed; this situation may lead to the continuing spread of these infections in the community. Compelling reasons exist for using saliva as a diagnostic fluid because it meets the demands of being an inexpensive, noninvasive and easy-to-use diagnostic method. Indeed, comparative analysis of the salivary proteome using mass spectrometry is a promising new strategy for identifying biomarkers. Our goal is to apply an Orbitrap-based quantitative approach to explore the salivary proteome profile in HBV- and HCV-infected patients. In the present study, whole saliva was obtained from 20 healthy, (control) 20 HBV-infected and 20 HCV-infected subjects. Two distinct pools containing saliva from 10 subjects of each group were obtained. The samples were ultracentrifuged and fractionated, and all fractions were hydrolyzed (trypsin) and injected into an LTQ-VELOS ORBITRAP. The identification and analyses of peptides were performed using Proteome Discoverer1.3 and ScaffoldQ + v.3.3.1. From a total of 362 distinct proteins identified, 344 proteins were identified in the HBV, 326 in the HCV and 303 in the control groups. Some blood proteins, such as flavin reductase (which converts biliverdin to bilirubin), were detected only in the HCV group. The data showed a reduced presence of complement C3, ceruloplasmin, alpha(1)-acid glycoprotein and alpha(2)-acid glycoprotein in the hepatitis-infected patients. Peptides of serotransferrin and haptoglobin were less detected in the HCV group. This study provides an integrated perspective of the salivary proteome, which should be further explored in future studies targeting specific disease markers for HBV and HCV infection.

Introduction

Hepatitis B and C are both potentially life-threatening liver infections caused by the hepatitis B and C viruses (HBV and HCV, respectively) and are important global health problems. These conditions can lead to chronic liver disease and put people at high risk of death from cirrhosis of the liver and liver cancer [1,2]. More than 240 million people have chronic (long-term) liver infections, and approximately 600,000 people die every year due to the acute or chronic consequences of hepatitis B and C. Different from hepatitis B, no vaccine against HCV is available [3].

The natural history of hepatitis B and C virus infections has a prominent latent phase, during which the patient is infected but does not manifest the disease. Infected patients may remain undiagnosed due to an unwillingness of the patient to provide a blood sample for testing. In such situations, HBV and HCV may continue to spread in the community. Therefore, it is of paramount importance to any health care system to detect latently infected individuals in an effort to prevent spreading the infection. This problem can be circumvented by the use of alternative specimens such as saliva for the detection of HCV and HBV infections [4].

Saliva is the secretion of the salivary glands that ensures stability in the oral cavity environment. The oral fluid is composed of saliva itself, gingival crevicular fluids contained in the dentogingival sulcus, mucosal transudate, cell detritus, bacteria and food remains. The basis of saliva is interstitial fluid from blood capillaries that enters via the salivary gland ducts where it is modified from an isotonic to a hypotonic fluid [5].

Saliva has the potential to replace screening using serum/plasma in community-based seroprevalence studies. In general, whole saliva is most frequently studied because its collection is easy, noninvasive and rapid to obtain without the need for specialized equipment. Human saliva harbors proteins of clinical relevance, and approximately 30% of blood proteins are also present in saliva [6,7]. The discovery of salivary biomarkers and the ongoing development of diagnostic technologies have addressed its diagnostic value for clinical applications [8]. Indeed, human saliva proteomics have proven to be a novel approach in the search for protein biomarkers for the detection of diseases. As a diagnostic specimen in the clinic, saliva has many advantages in terms of collection, storage, shipping and voluminous sampling: all of these processes can be carried out very economically compared with serum or urine. Saliva is also easier to handle during diagnostic procedures than blood because it does not clot, thus reducing the number of manipulations required. For the patient, the noninvasive collection approach could dramatically reduce anxiety and discomfort and increase willingness to undergo health inspections [9].

However, a major barrier to using saliva as a diagnostic fluid has been the fact that many informative analytes are generally present in lower amounts in saliva than in serum. Nonetheless, saliva-based diagnostics may offer a robust alternative for clinicians in the near future when making clinical decisions and predicting

post-treatment outcomes [10]. The performance of commercially available immunoassays has been evaluated for anti-HCV antibody screening in saliva. The OraQuick HCV Rapid Antibody Test was reported to have the best performance using these samples but was not better than serum samples, which was previously observed by Chau et al. [11]. This finding may be explained by the low concentration of anti-HCV antibodies present in oral fluid compared with blood or serum, which has been observed using anti-HCV rapid tests and immunoassays with oral fluid samples [12].

The recent availability of mass-spectrometry techniques has improved research on the salivary proteome and has produced qualitative and quantitative information on the protein composition of saliva. As proteomic technologies continue to mature, salivary proteomics can enhance the sensitivity and specificity of human disease detection and have great potential for biomarker research and clinical applications. Salivary proteomic studies are progressively resulting in a growing number of clinical applications for monitoring local and systemic human diseases or conditions such as cardiovascular disease [13], hyperglycemia and diabetes [14] and breast [15] and oral cancers [16]. Nonetheless, there are no results for hepatitis virus infection. Therefore, in this study, human saliva samples were collected from HBV- and HCV-infected patients and matched healthy control subjects. Salivary proteins were identified and compared in two pooled samples from each group by LTQ-VELOS ORBITRAP.

Materials and Methods

2.1. Patient selection

This study was conducted according to the principles expressed in the Declaration of Helsinki. To participate in this study, individuals signed informed consent to a research protocol that had been reviewed and approved by the Federal University of Rio de Janeiro Ethics Committee. All study subjects were a part of a public hepatitis program (Linhares, Espírito Santo – Brazil), and the controls were systemically healthy. Subjects were excluded from the study if they were nursing or pregnant, diabetic or drank alcohol. Each pooled group (HBV- and HCV-infected patients and controls) consisted of 10 individuals (5 female and 5 male). Two pooled samples of each group were collected (biological replicate).

2.2 Sample collection and processing

Prior to saliva collection, the volunteers were asked to rest for 15 min, sitting in an upright position, and were asked not to speak until the saliva collection was complete. Unstimulated whole-saliva samples were immediately placed on ice and then centrifuged at 14,000 × g for 15 min at 4℃ to remove insoluble materials, cell debris and food remnants. The supernatant of each sample was collected, and 1 mM PMSF (Sigma, St. Louis, MO, USA) was added to inhibit proteases. The

protein concentration was determined according to the Bradford protein assay. The supernatants were frozen at −70℃ until analysis.

2.3 Enzymatic digestion for LTQ-Orbritrap experiments and whole-saliva peptide ultrafiltrate preparations

The extraction of proteins was performed according to Gonçalves et al. [17]. An aliquot of pooled whole saliva (1 mg/mL) from each group was ultrafiltered through two types of Microcon concentrators (YM-10K and YM-3K, Millipore, Billerica, MA, USA) to separate the whole saliva into three fractions (fraction 1, salivary peptides <3 kDa; fraction 2, enriched for proteins and peptides between 3 and 10 kDa; fraction 3, enriched for proteins >10 kDa) (Figure 1). From 1 mL of whole saliva, approximately 500 μL of fraction 1, 100 μL of fraction 2 and 100 μL of fraction 3 were obtained. All fractions were treated with 10 mM DTT for 30 min and then with 50 mM iodoacetamide for 30 min in the dark. Trypsin (Promega, Madison, WI, USA) (150, w/w) was then added

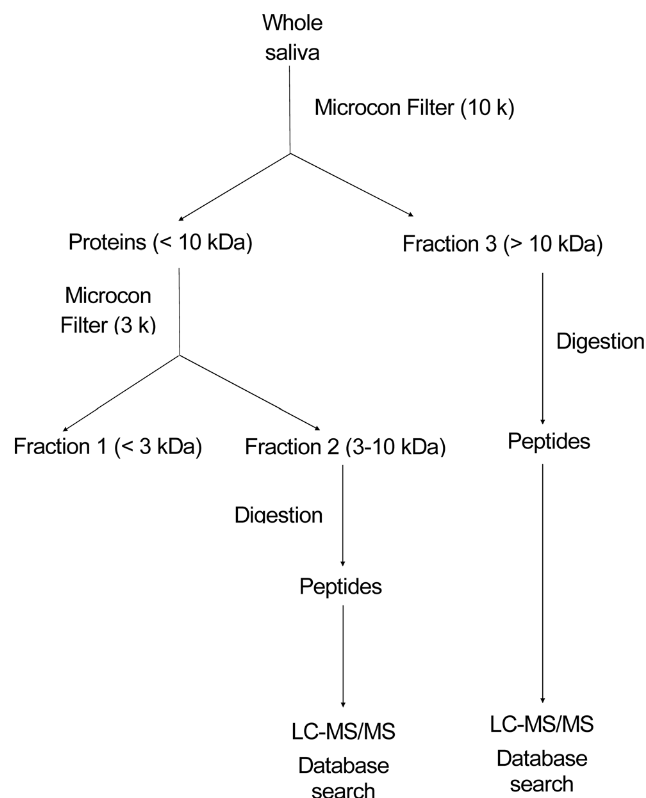

Figure 1. Outline of the procedures for the identification of whole-saliva proteins by prefractionation and shotgun proteomics. Two Microcon ultracentrifuge filters, YM-10K and YM-3K, were used to prefractionate whole saliva into three fractions. Fraction 2 contained proteins within 3–10 kDa, and fraction 3 contained proteins above 10 kDa. The proteins were digested into peptides with trypsin for subsequent protein identification by LCMS/MS and database searching. All steps were performed in duplicate with two biological samples.

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to the sample for overnight tryptic digestion at 37℃. All samples were concentrated in a Speed-Vac centrifuge to produce a 20-μL final volume. Before the experiment, the samples were vacuum-dried and reconstituted with 0.1% formic acid.

2.4 Nanoflow liquid chromatography coupled with LTQ Velos Orbitrap

An aliquot containing 4.5 μL of each pool of saliva sample was loaded onto an LTQ Velos Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) connected to a nanoflow LC (nLC-MS/MS) by an EASY-nLC system (Proxeon Biosystems, West Palm Beach, FL, USA) through a Proxeon nanoelectrospray ion source. The peptides were separated by a 2%–90% acetonitrile (ACN) gradient in 0.1% formic acid using an analytical column PicoFrit Column (20 cm × ID75 mm, 5 mm particle size, New Objective, Woburn, MA) at a flow of 300 nL/min over 45 min. The nanoelectrospray voltage was set to 1.7 kV, and the source temperature was 275℃. All instrument methods for the LTQ-Orbitrap Velos were set up in the data-dependent acquisition mode. Full-scan MS spectra (m/z 300–1,600) were acquired by the Orbitrap analyzer after accumulation to a target value of 1e6. The resolution of the Orbitrap was set to r=60,000, and the 20 most intense peptide ions with charge states ≥2 were sequentially isolated to a target value of 5,000 and fragmented in the linear ion trap by low-energy collision-induced dissociation (CID) as the dissociation or fragmentation method, with a normalized collision energy of 35%. The signal threshold for triggering an MS/MS event was set to 500 counts. Dynamic exclusion was enabled with an exclusion size list of 500, an exclusion duration of 60 s and a repeat count of 1. An activation q=0.25 and an activation time of 10 ms were used. The samples were analyzed in duplicate (technical replicate).

2.5 Database searching

Peak lists (msf) were generated from the raw data files using Proteome Discoverer version 1.3 (Thermo Fisher Scientific) with the Sequest search engine and searched against Human International Protein Database (200,740 sequences and 86,640,852 residues) downloaded from UniProt (http://www.uniprot.org) in September 2012. The following parameters were used: carbamidomethylation (+57.021 Da) as a fixed modification; oxidation of methionine (+15.995 Da); phosphorylation of serine, threonine and tyrosine (+79.966 Da) as variable modifications; one trypsin missed cleavage and a tolerance of 10 ppm for precursor and 1 Da for fragment ions. For protein quantification, the data files were analyzed in Scaffold Q+ (version3.3.1, Proteome Software, Inc., Portland, OR, USA); the quantitative value (normalized spectral counts) was obtained with the protein thresholds established at a minimum 90.0% probability and at least 1 peptide with a threshold set to a minimum 60.0% probability and filtered using

XCorr cutoffs (+1>1.8, +2>2.2, +3>2.5 and +4>3.5) to have less than 1% FDR. Only peptides with a minimum of five amino acid residues showing a significant threshold ($p<0.05$) in the Sequest-based score were considered as a product of peptide cleavage. The peptide was considered to be unique when it differed by at least 1 amino acid residue; covalently modified peptides, including N- or C-terminal elongation (i.e., missed cleavages), were counted as unique, and different charge states of the same peptide and modifications were not counted as unique. For the label-free quantization of endogenous peptides, the spectral count and number of unique peptides were assessed. The fold-change between proteins in the HBV, HCV and control groups was also calculated, and the resulting spectrum count values were used to analyze the distribution of identified proteins across the samples.

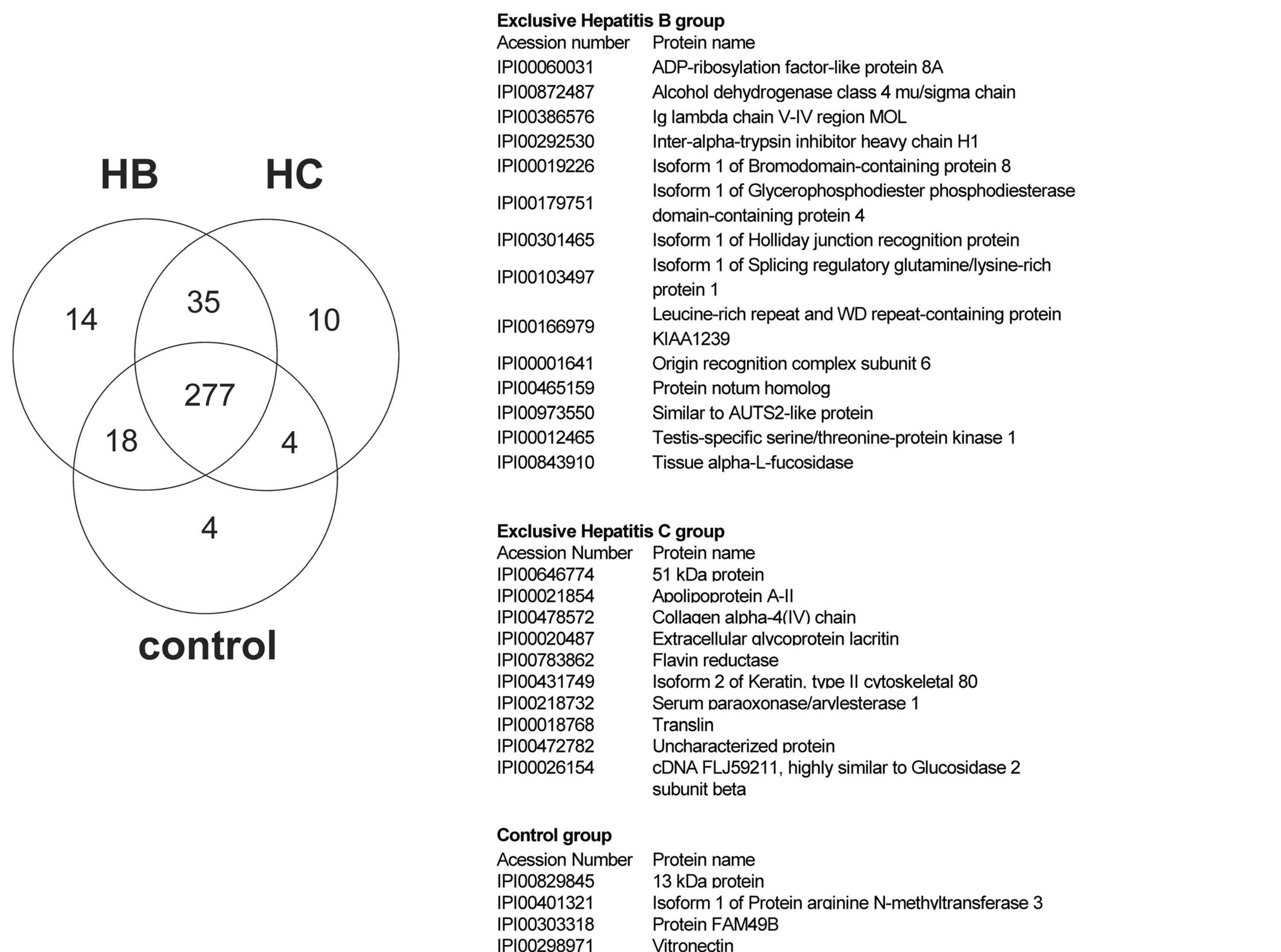
Results and Discussion

3.1. Human saliva proteomic profiles of hepatitis B- and C-infected patients

The salivary proteomes of hepatitis B (HB)- and hepatitis C (HC)-infected patients were analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS). Proteins were identified by the Sequest algorithm against the IPI human protein database and validated using Scaffold software by peptide hits of one or greater. We employed a prefractionation strategy to reduce high-abundance proteins from the samples prior to LC-MS/MS and to improve the representation of low-abundance proteins. Peptide mixtures generated from each sample (digested ultrafiltered pool of saliva – fractions >10 kDa and 3–10 kDa) were analyzed in duplicate, and the cumulative results were derived from the data combination of all 12 samples (two fractions of each salivary pool of the three groups – HBV, HCV and control – and two different pools of each group).

Pooling samples is a common way to reduce the cost of experiments as well as to provide equivalent power to experiments [18]. Because the purpose of our study is to identify robust biomarkers related to the presence HBV and HCV infections, the differences among the groups are more interesting than the differences between the patients within each patient group. We pooled the samples to smooth the intrinsic individual differences and enhance the common characteristic traits only related to the disease. It is also true that pooling samples may eliminate the number of biological replicates.

A consortium of three research groups has catalogued the proteins in human saliva collected as ductal secretions, with 1166 identifications: 914 in parotid and 917 in submandibular/sublingual saliva. The different analysis strategies showed that a high proportion of proteins found in plasma and/or tears are also present in saliva, along with a unique component [19]. Considering the convenience of our gel-free analysis, our study identified 362 proteins, and their distributions are displayed in the Venn diagram in Figure 2. All the human proteins identified in this study are detailed in Table S1. Approximately 273,636 MS/MS peptide spectra



Exclusive Hepatitis B group

Acession number	Protein name
IPI00060031	ADP-ribosylation factor-like protein 8A
IPI00872487	Alcohol dehydrogenase class 4 mu/sigma chain
IPI00386576	Ig lambda chain V-IV region MOL
IPI00292530	Inter-alpha-trypsin inhibitor heavy chain H1
IPI00019226	Isoform 1 of Bromodomain-containing protein 8
IPI00179751	Isoform 1 of Glycerophosphodiester phosphodiesterase domain-containing protein 4
IPI00301465	Isoform 1 of Holliday junction recognition protein
IPI00103497	Isoform 1 of Splicing regulatory glutamine/lysine-rich protein 1
IPI00166979	Leucine-rich repeat and WD repeat-containing protein KIAA1239
IPI00001641	Origin recognition complex subunit 6
IPI00465159	Protein notum homolog
IPI00973550	Similar to AUTS2-like protein
IPI00012465	Testis-specific serine/threonine-protein kinase 1
IPI00843910	Tissue alpha-L-fucosidase

Exclusive Hepatitis C group

Acession Number	Protein name
IPI00646774	51 kDa protein
IPI00021854	Apolipoprotein A-II
IPI00478572	Collagen alpha-4(IV) chain
IPI00020487	Extracellular glycoprotein lacritin
IPI00783862	Flavin reductase
IPI00431749	Isoform 2 of Keratin, type II cytoskeletal 80
IPI00218732	Serum paraoxonase/arylesterase 1
IPI00018768	Translin
IPI00472782	Uncharacterized protein
IPI00026154	cDNA FLJ59211, highly similar to Glucosidase 2 subunit beta

Control group

Acession Number	Protein name
IPI00829845	13 kDa protein
IPI00401321	Isoform 1 of Protein arginine N-methyltransferase 3
IPI00303318	Protein FAM49B
IPI00298971	Vitronectin

Figure 2. Venn diagram of proteins identified by LC-MS/MS exclusive to patients infected with hepatitis viruses B and C (HBV and HCV) versus the control group and those found in all these groups. The tables list proteins exclusive to the HBV, HCV and control groups.

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for the control group, 226,542 for the HBV group and 222,332 MS/MS for the HCV group were collected (fractions 2 and 3). A total of 344 proteins in the HBV, 326 proteins in the HCV and 303 in the control groups were identified. Of the total proteins identified, 14 were exclusive to the HBV samples, whereas 10 were exclusive to HCV. The control group presented only 4 exclusive proteins. The tables in Figure 2 show the list of proteins exclusive to the HBV, HCV and control groups.

3.2. Unique proteins of hepatitis B- and C-infected patients

With regard to HCV infection, one of the specific proteins identified was flavin reductase (IPI IPI00783862), an oxidoreductase that catalyzes NADPH-dependent

reduction and in the liver, converts biliverdin to bilirubin. Interestingly, however, both HCV and HBV cause hepatitis, yet HCV appears to be particularly more potent at inducing oxidative stress, suggesting mechanisms that are unique to HCV. Patients with HCV may experience increased oxidative stress due to the activation of NADPH oxidase, increased production of mitochondrial reactive oxygen species (ROS)/reactive nitrogen species (RNS), decreased antioxidants, iron overload and increased cytokines [20,21]. Clinically, the serum of HCV patients may show increased ROS [22] and RNS, which may lead to the chronic inflammation observed in these patients [23].

3.3 Comparative protein analysis of hepatitis B- and C-infected patients

Table 1 lists some of the identified proteins that were selected based on the overall increase or decrease in expression between the groups. As expected, the majority of the detected peptides in all of the fractions correspond to alpha-amylase or serum albumin. The diseased groups showed a reduced number of MS/MS spectra for complement C3, alpha(1)-acid glycoprotein and alpha(2)-acid glycoprotein, haptoglobin, serotransferrin and ceruloplasmin. For simplicity, these differences found in our study are discussed below in sections.

Complement C3

An interesting finding was the reduced number of MS/MS spectra of complement C3 identified in the saliva of HCV patients (Table 1). The C3 complement protein plays a pivotal role in both the classical and alternative pathways of complement activation. Interactions between HCV and the host immune surveillance system may play an important role in viral persistence. Serum C3 levels have been shown to be depleted in HCV-infected cirrhotic patients [24]. C3 is also an acute-phase protein, the levels of expression of which are either positively or negatively regulated by cytokines during inflammation, chiefly through the regulation of the activities of their cognate genes [25]. Mazumdar et al. [26] suggested that the HCV NS5A protein primarily suppresses C3 complement expression by inhibiting the expression of the IL-1-induced transcription factor C/EBP in human hepatocytes.

Alpha(1)-acid glycoprotein and alpha(2)-acid glycoprotein (AAG)

These proteins are acute-phase proteins synthesized predominantly in the liver. Cytokines can cause the plasma AAG level to increase as part of the inflammatory response [27]. The plasma concentration of AAG has been suggested to be a potential marker for cirrhosis and HCC [28], and decreased levels of α(1)-acid glycoprotein are found in patients with chronic hepatitis C [29]. Our study showed a reduced number of MS/MS spectra of these proteins in both groups of hepatitis-infected patients (Table 1).

Haptoglobin

This protein is one of the acute-phase proteins secreted by the liver and binds to hemoglobin, playing an important role in the response to inflammation and

Table 1. Spectrum counts of 12 representative proteins present in the saliva of HBV- and HCV-infected patients and identified using the LTQ-VELOS orbitrap.

Accession number	Protein name	Biological function	Spectrum counts		
			HBV-infected patients	HCV-infected patients	Control
IPI00300786	Alpha-amylase 1	Carbohydrate metabolism	2436	1608	1800
IPI00745872	Isoform of Serum albumin	Blood coagulation	942	512	1216
IPI00783987	Complement C3 (Fragment)	activation of the complement system	24	8	40
IPI00022429	Alpha-1-acid-glycoprotein	Acute phase and transport	8	0	24
IPI00020091	Alpha-1-acid-glycoprotein 2	Acute phase and transport	2	2	20
IPI00641737	Haptoglobin	Acute phase and immunity	24	2	36
IPI00465248	Isoform alpha-enolase of alpha-enolase	Glycolysis, Plasminogen activation Transcription, Transcription regulation	78	44	192
IPI00023011	Submaxillary gland androgen-regulated protein 3B	protection or detoxification	20	22	124
IPI00219018	Glyceraldehyde-3-phosphate dehydrogenase	glycolysis and nuclear functions	30	22	80
IPI00032293	Cystatin-C	inhibitor of cysteine proteinases	40	18	28
IPI00022463	Serotransferrin	Iron binding transport	122	76	188
IPI00017601	Cerulopasmin	Transport	8	8	24
IPI00022488	Hemopexin	Binds heme and transports it to the liver for breakdown and iron recovery	28	8	24
IPI00005979	Transthyretin	Transport	4	2	0

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malignancy. The overall N-glycans of the serum haptoglobin β chain have been found to be different in liver diseases. The Hp β chain contains four potential sites of N-glycosylation, and the site-specific characterization of N-glycans in glycoproteins has a potential clinical application [30]. Sarvary et al. [31] showed that haptoglobin α2 isoforms are differentially expressed in the serum from HCC-patients. Atta et al. [29] reported decreases in the serum levels of α(1)-acid glycoprotein, C3 complement and haptoglobin in HCV-infected patients, similar to our salivary analysis (Table 1). In the HCV group, for example, only two spectra of haptoglobin were detected.

Serotransferrin

The quantitative analysis in our study showed that levels of this protein were reduced in the hepatitis-infected patients (Table 1). An estimated 30–40% of patients with chronic hepatitis C have elevated serum iron, transferrin saturation and increased ferritin levels, and clinical data suggest that iron is a co-morbidity factor for disease progression following hepatitis-virus infection. Although iron is essential for a number of fundamental metabolic processes in cells and organisms, mammalian iron homeostasis is tightly regulated through the coordinated action of sensory and regulatory networks that modulate the expression of iron-related proteins at the transcriptional and/or posttranscriptional levels. Disturbances in

iron homeostasis have been implicated in infectious disease pathogenesis. Viruses, similar to other pathogens, can escape recognition by the immune system, but they need iron from their host to grow and spread [32]. Increased levels of ferritin and serum iron levels have been correlated with progressive hepatic parenchymal disease. A possible explanation for these elevations is that a necroinflammatory hepatic status can release iron and ferritin from damaged hepatocytes, a process also sustained by the concomitant high serum levels of serum alanine aminotransferase. Furthermore, iron accumulation in HBV and HCV infections [33] causes liver damage due to oxidative stress, which increases hepatocyte necrosis/apoptosis, hepatic stellate cell activation and fibrogenesis through the proliferation of actin and collagen [34,35]. However, iron markers do not differ significantly in patients with low or high HCV viremia [36]. Serum ferritin and transferrin levels appear to play an important role in determining the severity of liver disease related both to liver fibrosis and necroinflammatory activity but not the presence of infection.

Ceruloplasmin

Ceruloplasmin is an alpha 2-glycoprotein that is mainly synthesized in the liver and has been shown to play a role in acute-phase reactions, in which serum ceruloplasmin levels are upregulated during inflammation and/or tissue damage. Although the levels of this protein are downregulated in conditions such as severe hepatitis, fulminant hepatitis and decompensated cirrhosis [37-39], the clinical significance of ceruloplasmin has not yet been clearly defined [40]. In this study, we showed that peptides of ceruloplasmin were less detected in the HBV- and HCV-infected groups compared to the control group (Table 1).

Hemopexin, transthyretin, enolase-1, thrombospondim-1, cystatin C, glyceraldehydes-3-dehydrogenase (GADPH) and submaxillary gland androgen-regulated protein 3B

Proteomics has proven to be useful for elucidating the pathology of and discovering disease markers for hepatocellular carcinoma (HCC). Several studies have employed mass spectrometry techniques to compare sera from HCC patients and their possible correlation with HBV and HCV infections [41,42]. Some serum HCC biomarker candidates have been suggested, such as hemopexin [43], transthyretin, α-fetoprotein [44], GADPH [45], alpha-enolase and thrombospondim-1 [7,46] and cystatin C [47]. Except for α-fetoprotein and thrombospondim-1, our study also detected these proteins in saliva samples, but the quantitative alterations were not the same in the presence of hepatitis as detected in the presence of hepatic cancer (Table 1). Submaxillary gland androgen-regulated protein 3B, which has been detected as increased in oral squamous cell carcinoma, was reduced in the hepatitis-infected patients [16].

Conclusions

Due to its advantages of an easy, safe, cost-effective and non-invasive diagnostic approach, saliva shows a high potential for diagnostic hepatitis virus infections.

Considering the fact that a high proportion of proteins found in plasma and/or tears are also present in saliva, human saliva proteomics has proven to be a novel approach in the search for protein biomarkers for the non-invasive detection of human diseases. This is the first study to describe salivary protein alterations associated with hepatitis B and C. These findings may provide novel insight into salivary protein differences between HBV- and HCV-infected patients and help to identify candidate biomarkers that may lead to more efficient hepatitis diagnosis in an epidemiological setting.

Supporting Information

Table S1. Complete list of proteins identified in the proteome analysis of control, HBV and HCV-infected patients.
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Author Contributions

Conceived and designed the experiments: LDRG MRS. Performed the experiments: LDRG IBC RRD AFPL MRS. Analyzed the data: LDRG RRD MRS. Contributed reagents/materials/analysis tools: LDRG RRD AFPL MRS. Wrote the paper: LDRG MRS.

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