# Chapter 5
# The Lymph Proteome, Peptidome, and Degradome

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**Abstract** During the last decade advanced proteomic analysis have allowed detailed characterization of the composition of the lymphatic fluid. A picture start to emerge indicating that far from being an ultra filtrate of the plasma the lymph carries a rich repertoire of proteins and peptides reflecting the tissue of origin and its physiological state. Indeed in few pathological conditions it has been shown that proteins transported by the lymph closely represent a molecular signature of the diseased organs. Altogether the "omic analysis" (proteomic, peptidomic, degradomic and lipidomic) prove to be a powerful tool to fingerprint variations in the lymph fluid collected from different parenchymal organs and to discover molecular biomarkers for the early detection of diseases.

## 5.1 Introduction

Physiological and pathological conditions in parenchymal organs are associated with protein expression profiles whose molecular signatures are often reflected in the fluids bathing tissue/organs. A major challenge of clinical chemistry is to use modern proteomic and peptidomic technologies to qualitatively and quantitatively map these protein profiles in order to provide molecular fingerprints of healthy or diseased conditions. Proteomic approaches can be indeed utilized to discover molecular biomarkers for the early detection of diseases, monitoring disease progression, and assessing the efficacy of therapeutic regimens (Meng and Veenstra 2007, 2011; Veenstra et al. 2005; Zimmerman et al. 2012; Qian et al. 2006). To date, many proteomic analyses of human body fluids, including plasma/serum, urine, cerebrospinal fluid, saliva, bronchoalveolar lavage, synovial fluid, nipple aspirate

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fluid, tear fluid, lymph, and amniotic fluid have been performed (Meng and Veenstra 2007; Veenstra et al. 2005; Qian et al. 2006). However, to date the most comprehensive proteomic analysis has been performed on easily collectable biofluids, mostly plasma/serum and urine. In contrast, the composition of rodent and human lymph has been elusive, until very recently, due to the technical difficulty in collecting samples from the lymphatic capillaries (Veenstra et al. 2005; Qian et al. 2006).

In this chapter we will present a comprehensive overview of the proteomic and peptidomic composition of lymph as determined by advanced mass spectroscopy technologies performed by several groups. Since many of the lymph protein result from plasma ultrafiltration, the lymph protein expression profile will also be compared with that of plasma.

## 5.2 Lymph Formation

The pre-nodal afferent lymph is the fluid which directly derives from the extracellular milieu from every parenchymal organ and contains products derived from the organ metabolism/catabolism (Meng and Veenstra 2007; Interewicz et al. 2004; Clement et al. 2010, 2011). The detailed physiological studies on lymph circulation highlights the collection of this biological fluid into open end lymphatic capillaries, which form a mesh-like network distributed throughout the body (Clement et al. 2011). The lymph then enters through the cortical area of the node and conveys a representative sampling of the interstitial fluid to the nodal antigen-presenting cells before exiting through the efferent lymphatic vessels, or the central vein located in the nodal sinus (Clement et al. 2010, 2011).

Original work performed by Olsewski's and Miller's laboratories (Interewicz et al. 2004; Olszewski et al. 2001; Nanjee et al. 2000) emphasizes that the actual composition of the lymph is dictated by the blood capillary filtration rate, permeability of the capillary walls, metabolic state of parenchymal cells, and draining ability of the lymphatic capillaries. All these factors are constantly changing, depending on the physiological or pathological condition of the tissue or organ from which the lymph is drained (Interewicz et al. 2004; Olszewski et al. 2001). Indeed, in contrast to blood which is sampled from a large vein in the general circulation, the lymph is collected from the subcutaneous capillaries. Thus, the proteomic and peptidomic analysis of the lymph would reflect more closely the metabolism from which the capillaries drain (Olszewski 1991). For example, it was postulated that the composition of the lymph collected in a foot capillary, would be slightly different in resting conditions or during exercise, or cold and warm temperatures, the position of the leg extremity with respect to the heart level, and pathological skin processes. Similarly, the luminal content of the gut would influence intestinal lymph composition; hyperpnea would increase the lung lymph flow and change the chemical content of tissue fluids in the interstitial compartment and thus the peptidomic/proteomic composition of the lymph (Olszewski 1991). The new development and optimization of much more sensitive mass spectrometry-coupled assays will aid the discovery and validation of lymph biomarkers under physiological and pathological conditions.

## 5.3 Lymph and Plasma Proteome

Over the years very comprehensive databases have been compiled for human and rodent plasma proteins under physiological and pathological conditions (Tannu and Hemby 2006; Shen et al. 2006; Echan et al. 2005; Ahn and Simpson 2007; Armandola 2003; Whiteaker et al. 2007; Anderson et al. 2004; Zhou et al. 2004). So far, much less information is available for the lymph, mostly due to the difficulty in collecting the samples. Until a decade ago, the dominating notion was that the proteomic profile of pre-nodal afferent lymph would mostly overlap with that of plasma, since the former was considered to be an ultrafiltrate of the latter, although with a protein concentration anticipated to be lower in lymph compared to plasma. However, this notion was challenged by a few comparative analyses of lymph and plasma, which reported the presence of specific proteins in one fluid but not the other (Leak et al. 2004).

The first studies on lymph proteomics were performed on ovine lymph, since the size of the lymphatic capillaries allowed easy cannulation and enough material was retrieved for the analysis (Interewicz et al. 2004; Olszewski et al. 2001; Leak et al. 2004). Leak et al. (2004) described the first analysis of the pre-nodal, afferent ovine lymph and compared it with the matched plasma from the same ovine source. Their results, obtained by SELDI-TOF-MS and 2D-PAGE, demonstrated for the first time some relative differences in protein expression profiles between normal sheep lymph and plasma. Major proteins found in lymph, but also at lower concentrations in plasma, included fibrinogen alpha- and beta-chains, immunoglobulin (IgG), serotransferrin precursor, lactoferrin, and apolipoprotein A-1. Two proteins that were identified as uniquely expressed in lymph were glial fibrillary acidic protein and neutrophil cytosol factor-1 (Leak et al. 2004). The presence of the protease inhibitor, a-antiproteinase, demonstrated that the lymph also contains enzymatic components, confirming earlier studies which showed that the protease activator (tPA) and its inhibitor (PAI-1) were secreted by the lymphatic endothelium and were also present in the lymph (Leak et al. 2004).

In another independent study, performed by Goldfinch et al. (2008) ovine gastric lymph has been used to monitor the proteomic changes occurring in tissue fluid in response to infection with the parasitic nematode, *Teladorsagia circumcincta*. In this research, lymph was collected temporally over an experimental infection period and subjected to 2DE and proteomic analysis using MALDI-TOF MS and MS/MS. One important feature of the sample preparation was the immunodepletion of the lymph samples from albumin and IgG using immunoaffinity columns (Echan et al. 2005). Goldfinch et al. (2008) were able to identify a number of proteins by MS which had not previously been identified. These included multiple charged forms of alpha-1 acid glycoprotein and alpha-1 beta glycoprotein, two distinct forms of serum amyloid A and apolipoprotein A-1, and actin. The main serum/plasma proteins already identified by other groups, using 2-DE methodology (Whiteaker et al. 2007; Anderson et al. 2004), were also found in the gastric ovine lymph, including fibrinogen beta and gamma chains, alpha-2-HS-glycoprotein,

haptoglobins, some classes of apolipoproteins (AI, AIV), transferrin, zinc-alpha-2 glycoprotein, C-type lectin, plasminogen, retinol-binding protein, and alpha-1 antitrypsin (Goldfinch et al. 2008). The studies of Goldfinch et al. outlined for the first time a strong correlation between an inflammatory condition and the protein expression profile of the lymph. More importantly, the analysis of the gastric lymph proteome highlighted the first potential biomarkers associated with one particular type of infection (*T. circumcincta*). These biomarkers, which included gelsolin, alpha-1 beta glycoprotein, and hemopexin, were further validated by Western blot assays and indicated changes in concentration according to the phases of infection (Goldfinch et al. 2008). Thus, Goldfinch et al. demonstrated for the first time, that a semiquantitative analysis of the changes in the lymph proteome during the course of a parasitic infection could represent a reliable method for monitoring the stages of the disease (Leak et al. 2004; Gretz et al. 1996; Goldfinch et al. 2008).

One step further in the analysis of the lymph proteome was the characterization of the mesenteric lymph during acute pancreatitis by Mittal et al. (2009). The main hypothesis that originated the analysis of the protein expression profile of the mesenteric lymph was that the protein fraction of this biological fluid could contain different, yet uncharacterized, toxic factors during different phases of this critical illnesses (Mittal et al. 2008, 2009). An analogous hypothesis drove the analysis of the proteomic composition of the plasma in many traumatic and pathophysiological conditions (Qian et al. 2006). Mesenteric lymph was collected from control rats and rats with taurocholate-induced acute pancreatitis and subjected to proteomic analysis using iTRAQ and liquid chromatography-tandem mass spectrometry. Two hundred and forty-five proteins including 35 hypothetical proteins were identified in mesenteric lymph. Eight of the 245 proteins had a significant increase in their relative abundance in acute pancreatitis-conditioned mesenteric lymph. These eight protein hits were then classified by their cellular location and molecular function using the Gene Ontology classification system. Seven of the proteins were pancreatic enzymes; four peptidases (carboxypeptidase B1, chymotrypsinogen B, carboxypeptidase A2, and cationic trypsinogen), one ester hydrolase (pancreatic lipase), one endoribonuclease (ribonuclease), and the pancreatic amylase 2 (Mittal et al. 2009), and one was a cytosolic protein.

From proteomics perspective, the characterization of acute pancreatitis-conditioned mesenteric lymph represents a successful approach involving the combined methodologies of immunodepletion (of albumin and IgG) and state-of-the-art iTRAQ coupled with nano-LC-tandem MS/MS, which helped to reveal an increased number of proteins (245) and to characterize in a quantitative manner fold changes in selective biomarkers (Mittal et al. 2009).

Additional work on proteomic profiling of mesenteric lymph was performed in rat models of hemorrhagic shock by the group of Kirk C Hansen and Ernest E. Moore, from the University of Colorado Health Science Center, Denver, CO (Masuno et al. 2006; Jordan et al. 2007; Peltz et al. 2009; Zurawel et al. 2010; Dzieciatkowska et al. 2011) and from the group of Sen-Yung Hsieh, from the Clinical Proteomic Center, Chang-Gung University, Taoyuan, Taiwan (Fang et al. 2010).

Recently, Moore's group has used the lymph samples from 11 patients to map the human mesenteric lymph after hemorrhagic shock (Dzieciatkowska et al. 2011). The pre and post-shock lymph was loaded at equal protein concentrations on 2D gels, followed by standard trypsin digestion and protein identification with tandem mass spectrometry (MS/MS). In one study the authors demonstrated that gelsolin, a plasma protein that functions to depolymerize actin filaments and mediate the inflammatory response by binding proinflammatory lipids such as lysophosphatidic acid, sphingosine-1-phosphate, and phosphoinositides, changed in concentration in the mesenteric lymph after hemorrhagic shock. Clinically, reduced gelsolin concentrations have been associated with increased mortality in critically ill, trauma, and burn patients (Masuno et al. 2006; Jordan et al. 2007). Additional extensive animal work using mesenteric lymph has helped establish a mechanistic link between gut ischemia/reperfusion (I/R) and distant organ injury. The advanced proteomics profiling of the mesenteric lymph posthemorragic shock (Peltz et al. 2009; Zurawel et al. 2010; Fang et al. 2010) characterized several proteins upregulated at least twofold in the post-shock as compared with the pre-shock lymph including proteins released from tissue injury, depletion of coagulation factors, and evidence of hemolysis (Peltz et al. 2009). The most important discovery was that the components of coagulation system were depleted, whereas products of hemolysis were increased. In addition, the proteomics analysis from the group of Ernest E. Moore showed that the haptoglobin was decreased, which was proposed to be related to an early post-shock hemolytic process. Unexpectedly, several protective protease inhibitors including alpha-2-macroglobulin, alpha-1-inhibitor 3, and inter-alpha-inhibitor H3 and H4, were found to be decreased. These findings suggested that the loss of protective protease inhibitors could be correlated with subsequent higher protease activity and the development of post-shock inflammation (Jordan et al. 2007; Peltz et al. 2009; Zurawel et al. 2010).

Other proteomic analysis of the mesenteric lymph followed from the studies of the Ernest E. Moore group, to further map changes in the proteomic profile of this biological fluid in posthemorrhagic shock (Dzieciatkowska et al. 2011). The Hansen's group collected mesenteric lymph from 11 patients with either lymphatic injuries, or during spine reconstruction surgery, or immediately before organ donation. The lymph was analyzed by label-free proteomic analysis resulting in the characterization of a total of 477 proteins identified from the 11 subject's lymph samples with greater than 99 % confidence. The proteomic methodology didn't involve 2D-DIGE analysis as in previous work, but a combination of immune-affinity depletion (of albumin and IgG) and 1DEF-SDS-PAGE coupled with nano-LC-tandem MS/MS. Several of the identified proteins were markers of hemolysis, oxidative stress, matrix degradation, and general tissue damage. Out of the top 200 proteins of the dataset, several were not found in the 889 Human Plasma Proteome Project (HPPP) dataset (Farrah et al. 2011; Omenn et al. 2005). This finding was consistent with work by Leak et al. that showed ovine lymph contains plasma proteins and additional metabolic products of connective tissue, cells and proteins released from lymphatic endothelium (Leak et al. 2004). It is important to mention that most of the identified proteins in rodent lymph were also identified in human

mesenteric lymph (Jordan et al. 2007; Peltz et al. 2009; Zurawel et al. 2010; Dzieciatkowska et al. 2011; Fang et al. 2010).

While the methods employed in many of the above reported studies allowed for the identification of several hundred lymph proteins, the compiled datasets are far from being definitive. Indeed, the limited sample availability and inherent sensitivity of the analytical platform precludes, at least so far, the identification of many bioactive components such as cytokines or cellular mediators that are usually expressed at very low levels.

Our group recently reported the proteomic analysis of the afferent human lymph as compared to the plasma using a combination of immune-affinity depletion methods (for albumin and IgG) coupled with 1D SDS-PAGE and 2D-DIGE together with nano-LC-LTQ-Orbitrap tandem MS/MS. The 253 positively identified proteins ($p<0.05$) indicated a common proteome, shared by the two biological fluids (144 out of 253 proteins), which was significantly enriched with complement activation and blood coagulation components, transporters, and protease inhibitors. In addition, the enriched proteome of human lymph (72 proteins) consisted of products derived from the extracellular matrix, apoptosis, and cellular catabolism. In contrast, the enriched proteome of human plasma (37 proteins) comprised soluble molecules of the coagulation system and cell–cell signaling factors. This was the first analysis of the human lymph that established, in a semiquantitative manner, that the physiological lymph proteome is comprised of a series of proteins that derive directly from the catabolism of parenchymal cells and the extracellular matrix. (Clement et al. 2013).

## 5.4 Plasma Peptidome and Degradome

Over the last decade, new studies have identified the presence of a low-molecular weight serum proteome composed of fragments derived from protein processing and cleavage. The presence of this "degradome" was thought to provide insight into tissue-specific metabolism and disease pathogenesis/progression (Hood et al. 2005; Geho et al. 2006; Antwi et al. 2009; Zheng et al. 2009; Zou et al. 2011). The analysis of this degradome/peptidome was initially limited by the protein depletion strategies employed during sample preparation for proteomic assays, which made it difficult to retrieve the low-molecular weight protein/peptides which are often bound to major lymph proteins (such as albumin, retinol-binding protein, or vitamin D-binding protein) and, until few years ago, by the low resolution of mass spectrometric analysis (Hood et al. 2005; Geho et al. 2006). The strategy to retrieve the low-molecular weight serum peptidome and cleaved proteome was eventually improved and involved peptide separation by ultrafiltration, enzymatic digestion, and liquid chromatography coupled to electrospray ion trap tandem mass spectrometry (LC-ESI-MS/MS). While this approach uncovered a remarkable richness of protein fragments observed in the low-molecular weight proteome, the incorporation of the tryptic digestion step eliminated specific information about the peptide

sequences that occur naturally in the plasma or serum (Geho et al. 2006; Zou et al. 2011).

In addition, the intrinsic person-to-person variability, which is the case of most human biological samples, complicated the discovery of new biomarkers. As a consequence, initial efforts to discover a human plasma/serum degradome/peptidome were shifted to experimental mouse models of human diseases because they represented a highly controllable experimental system (Hood et al. 2005).

One of the most comprehensive approaches applied to mapping of the low-molecular weight cleaved proteome and peptidome of mouse serum is highlighted by the work of Brian L. Hood and colleagues (2005). By using differential labeling for trypsin-mediated incorporation of the stable isotope of oxygen, $^{18}O$, they compared the low-molecular weight serum proteome of tumor-bearing mice with control mice. The digests were combined, fractionated by strong cation exchange chromatography, and analyzed by nanoflow-reversed phase liquid chromatography coupled online with tandem mass spectrometry. The analysis resulted in the identification of 6,003 proteins containing at least a single, fully tryptic peptide. The low-molecular weight degradome/peptidome identified in the tumor-bearing mice was partially annotated using the GO (gene ontology) classifications for cellular compartmentalization. The GO annotations indicated that 22 % of the peptide fragments were derived from the turnover of extracellular proteins. At the same time, the GO classification predicts that only 14 % of the entire healthy mouse proteome encodes for extracellular proteins. Thus, the comparative analysis between the plasma degradome/peptidome in tumor and control mice indicated that about 8 % of the newly identified extracellular peptidome derived from a differential processing of the extracellular proteome in the tumor vs. the healthy control mice. The analysis also revealed that sensitive, accurate, and robust mass spectrometry technologies, aiming to sequence the low-molecular weight degradome/peptidome, can be used as reliable tools for the characterization of differential processing pathways involved in the proteome's turnover under physiological or pathological conditions (Hood et al. 2005).

Over the past few years several more degradomes have been mapped including serum, plasma, lymph, synovial fluid, urine, and cerebrospinal fluid (Shen et al. 2010a, b, 2011, 2012; Koomen et al. 2005). The human degradome has been shown to contain more than 500 proteases responsible for protein degradation and control of protein quality and functions (Shen et al. 2011, 2012). To date, all studies agree that in pathological conditions, the mapped peptidome/degradome of different biological fluids is enriched from the one found in healthy, physiological conditions (Shen et al. 2010b, 2011, 2012). Indeed, among the sequenced peptides, fragments derived from the most abundant proteins found in human plasma and serum have been shown to be processed differently by proteases highly active in different diseases, such as cancer, rheumatoid arthritis (RA), and osteoporosis (Shen et al. 2010b, 2011, 2012). As such, the associated degradome identified in pathological states, including tumor progression, invasion, and metastasis, has been proposed as a potential source of biomarkers for clinical diagnosis purposes (Shen et al. 2010b, 2011, 2012).

Another inclusive analysis of the plasma peptidome is from the group of Richard Smith, from the Pacific Northwest National Laboratory (Shen et al. 2010a). Their combined chromatographic methods and high resolution Fourier transform (FT) tandem MS/MS technologies highlighted the power of the chosen fractionation methods, as compared to the LC/MS/MS methodology, in mapping a high number of substrates of the degradome. The fractionation of human blood proteins using a combination of affinity and strong exchange chromatographic methods (AC)/(SEC) enabled depletion of the abundant proteins and enrichment of the small- to medium-sized blood plasma degradome/peptidome components. Additionally, the chromatographic fractionation strategies were used in conjunction with de novo sequencing for the identification of posttranslational modifications and mutations using high resolution liquid chromatography (HRLC)-FT-MS/MS. More than 200 peptides generated from 29 protein substrates were confidently identified from the plasma of a single healthy person (Shen et al. 2010a). The identified peptides had MWs in the range of 0.5–14 kDa and identifications were achieved with extremely low (near zero) false discovery rates through searching the IPI human protein database (Shen et al. 2010a). Alpha-1,2-mannosidase IA, alpha-1B-glycoprotein, alpha-2-antiplasmin, alpha-2-HS-glycoprotein, angiotensinogen, antithrombin III variant, apolipoprotein A-IV, beta-2 microglobulin, C1-esterase inhibitor, ceruloplasmin, complement C3, complement C4, complement C9, complement factor D, fibrinogen, gelsolin, inter-alpha-trypsin inhibitor H4, kininogen-(HMW), kininogen-(LMW), leucine-rich alpha-2-glycoprotein, multimerin-1, pigment epithelium-derived factor, plasma serine protease inhibitor, thymosin beta-4, and transthyretin were among the most abundant proteomic substrates. Some of the sequenced peptides were shown to be extensively modified posttranslationally, including acetylation, acetylhexosamine, amidation, cysteinylation, didehydro, oxidation, and pyro-glu, as the major posttranslational modifications (Shen et al. 2010a).

Combining different MS dissociation methods, including collision-induced dissociation (CID), high energy collision dissociation (HCD), and electron transfer dissociation (ETD) together with Fourier transform MS/MS, the group of Shen et al. identified >1,000 unique peptides with a low-peptide-level false discovery rate (FDRs) (e.g., 0.6 %).

The peptides were derived from the degradation of protein substrates of pooled plasma samples collected from early stage breast cancer patients (Shen et al. 2012). This analysis provided an improved basis for investigating disease-related peptidome components and further highlighted the advantages of the global analysis of the peptidome to identify specific protein substrate targets, characterize cleavage specificities of processed proteins substrates, and for diagnostic purposes (Shen et al. 2010a, b, 2011, 2012).

A major concern with the peptidome/degradome analysis is the limit of tryptic digests in revealing the cleavage sites of naturally processed peptides. This problem was overcome by using different fractionation procedures, which enabled the purification of naturally processed peptides with MW <5,000 Da. One characterization of naturally cleaved serum and plasma peptidome is highlighted by the LC

MALDI-Tof/Tof (MS/MS) studies of low-molecular weight proteome of heparinized plasma performed by John M. Koomen et al. in the research group of Ryuji Kobayashi (Koomen et al. 2005). In this analysis a combination of 96-well plate fractionation of plasma and LCMALDI-MS/MS were used for profiling and directly sequencing abundant peptides in the low-molecular weight plasma proteome. Approximately 250 unique peptides were detected, corresponding to approximately 20 proteins. The majority of the peptides were identified as fragments from fibrinogen, complement components, carrier proteins, and antiproteases. The cleavage patterns were consistent with those of known plasma proteases, including thrombin, plasmin, and complement proteins, followed by aminopeptidase and carboxypeptidase activity (Koomen et al. 2005). Altogether, the remarkable work of Koomen et al. outlines some mechanistic insights into the processing pathways generating the plasma degradome by molecular analysis of the cleavage sites of the peptidome (Koomen et al. 2005).

Another significant contribution to the discovery of new peptide markers in the low-molecular weight fraction of the human proteome was generated by Yusuke Kawashima et al. (2010). The research team from the Center for Disease Proteomics at the Kitasato University School of Science, Japan, developed a differential solubilization (DS) method to extract low-molecular weight proteins/peptides in serum. A combination of peptide extraction with denaturating agents (urea, thiourea) followed by ice-cold acetone precipitation, differential centrifugation, and trifluoroacetic acid (TFA 0.1 %) extraction of the final proteome/peptides allowed the development of a new extraction procedure characterized by good reproducibility and yield as compared to typical peptide-extraction methods such as organic solvent precipitation and ultrafiltration. Using the DS method combined with reverse-phase HPLC fractionation followed by MALDI-TOF-MS, they characterized more than 1,500 peptides from 1 μL of serum samples, including low-abundance peptides in the subnanomolar range and many peptides bound to carrier proteins such as albumin.

The results of Yusuke Kawashima et al. provided the evidence that serum peptide analyses based on the DS method could greatly contribute to the discovery of novel low-abundance biomarkers (Kawashima et al. 2010). The major protein hits identified were the family of apolipoproteins (AII, AIV, CI, CII, CIII, CIV, E precursors, haptoglobins, serum amyloid A-4 precursor, hemoglobin subunit alpha, beta, delta, clusterin precursor, transthyretin, alpha-1-antitrypsin precursor, leucine-rich alpha-2-glycoprotein precursor, pigment epithelium-derived factor precursor, antithrombin-III precursor, N-acetylmuramoyl-L-alanine amidase precursor, alpha-1B-glycoprotein precursor, serum albumin precursor, serotransferrin precursor, serum albumin precursor, Ig kappa chain C region, and Ig lambda chain C regions (Kawashima et al. 2010).

An independent, notable contribution for the characterization of the plasma peptidome was reported by Rustam Ziganshin et al. (2011), who developed a new method for desorption of low-molecular weight peptides from abundant blood proteins. The diluted blood serum was heated to 98 °C for 15 min in order to dissociate the low-molecular weight peptides from the most abundant blood proteins.

Blood plasma/serum fractionation was performed on magnetic beads with a functionalized surface followed by heating of the resultant fractions. This methodology significantly increased the number of peptides detected by MALDI–TOF MS and enhanced the general reproducibility of mass spectrometry profiles determined by LC–MS/MS using an Agilent 6520 Accurate-Mass Q-TOF (Ziganshin et al. 2011). The processed peptides covered the major classes of protein antigens from the plasma such as apolipoproteins (A-I, A-II, A-IV, B-100, C-I, C-II, C-III, E, clusterin (apo J)), coagulation factors (XII, XIIIa), complement system (C3, C4, factor B), fibrinogens (alpha chain), fibronectin, gelsolin, hemopexin, histidine-rich glycoprotein, inter-alpha-trypsin inhibitor heavy chain H4, kininogen-1, pigment epithelium-derived factor, plasma protease C1 inhibitor, plasma retinol-binding protein, plasminogen, prothrombin, serotransferrin, transthyretin, vitronectin, cytoplasmic actin, antithrombin-III, and alpha-HS glycoprotein (Ziganshin et al. 2011).

As a whole, the reported analysis on the low-molecular weight plasma peptidome/degradome indicated, as expected, that the major classes of plasma proteins could always be detected by the mass spectrometric analysis, independently of the method used for the peptidome extraction. Nevertheless, an additional peptidome/degradome derived from the low abundant intracellular and extracellular proteomes could be identified accurately only by using multiple chromatographic fractionation methods coupled with high resolution FT-MS/MS technologies (Shen et al. 2010a, b, 2011, 2012; Koomen et al. 2005; Kawashima et al. 2010; Ziganshin et al. 2011).

## 5.5 Lymph Peptidome and Degradome

Throughout the last decade (2002–2012), many research studies have been performed on human, bovine, rat, and goat lymph collected at different sites, thus providing the first glimpse into the proteomic composition of the lymphatic fluid. From early on a major question asked from the data analysis was how qualitatively and quantitatively different is the plasma/serum peptidome from the lymph one? (Shen et al. 2010a, b, 2011, 2012) Also, would the peptidome/degradome overlap with the proteins found in the lymph or would it reflect the tissue where the lymph originated? Moreover, would the cleavage site indicate a similar set of enzymes involved in the processing for the lymph and plasma peptidome or indicate a different set of proteases? Even though many of the questions are still unresolved, in the next sections of this chapter we will highlight the major advances in the qualitative and quantitative characterization of the lymph peptidome.

The first mapping of the peptidome transported by the human lymph was recently described by our laboratory at Albert Einstein College of Medicine (Clement et al. 2010, 2011). The primary hypothesis that initiated the research on the human lymph peptidome was that the lymph could potentially carry a wider antigenic repertoire than the plasma and could in turn be a richer source of tissue-specific antigens. Additionally, differently from plasma, the lymph could carry an enriched processed proteome/peptidome since it directly collects from the extracellular milieu where

products of tissue catabolism, tissue remodeling, cellular apoptosis, and extracellular matrix processing are collected before being transported to the draining lymph nodes (Clement et al. 2010, 2011).

Due to the difficulty in obtaining sufficient material, the peptidomic analysis was originally performed on pooled sample of human lymph from 18 healthy donors. Peptides were purified by ultrafiltration through 5,000 Da molecular weight filters or by reverse phase HPLC or gel filtration and then sequenced using both nanospray LC-MS/MS on a LTQ linear ion trap mass spectrometer (LTQ) and an Orbitrap/Velos (Clement et al. 2010 and unpublished data). More than 300 sequences were identified as self-peptides derived from both intracellular and extracellular proteins, mapping the variety of catabolic products transported by the human lymph (Clement et al. 2010). As expected, a large fraction of the peptidome was comprised of processed proteins derived from extracellular matrix proteins, cell adhesion molecules, and plasma membrane/receptors or from enzymes involved in the processing of the substrates which gave rise to the reported peptidome (metalloproteases/peptidases) (Clement et al. 2010).

Interestingly, many of the partially processed intracellular proteins were derived from the cytosolic fraction, nucleus (transcription factors and regulator of gene expression), mitochondria, golgi, and endoplasmic reticulum, most probably from parenchymal cells undergoing apoptosis. Indeed apoptotic cells have been found in the human lymph (Olszewski 1991). Pathway analysis of the mapped peptidome/degradome confirmed that the most abundant networks related to the biological turnover of the extracellular matrix, followed by cellular metabolism.

When the peptidome profile was compared to previously published MHC class II eluted peptidome, it appeared that the lymph peptide repertoire was partially skewed toward peptides derived from extracellular matrix proteins, cadherins, and cleavable or soluble receptors (43 %) (Clement et al. 2010). Likely, this was related to the source of the lymph, which was collected from the foot, and reflected the protein composition of the tissues (skin, adipose tissue, connective, and muscle) (Clement et al. 2010). In the future it would be interesting to collect lymph draining different organs and compare the degradome/peptidome for tissue-specific antigens.

A quantitative analysis of the peptides established that at least some of them were present in the circulating lymph in nanomolar concentration, proving that an immunologically relevant amount of partially processed proteins and peptides are carried by the human lymph (Clement et al. 2010, 2011). A preliminary analysis using the DAVID-WS program, which includes the characterization of the possible antigen processing machinery responsible for peptide cleavage, indicated that 8 % from the lymph peptidome is processed by metalloproteases and other peptidases.

This analysis further suggests that, unlike the proteins processed by local or nodal APC (antigen-presenting cells), which mostly produce epitopes constrained by endosomal activity, the identified self-antigens in the lymph could be derived from a wider variety of processing pathways, including caspases involved in cellular apoptosis, ADAMs and other metalloproteinases involved in surface receptor editing, cytokine processing, and/or matrix remodeling (Clement et al. 2010).

In the future it would be important to map the degradome machinery involved in the generation of the lymph peptidome. This type of analysis would involve mapping of the protease cleavage sites for the previously reported peptidome (Clement et al. 2010), using a similar approach to the one reported for the plasma peptidome by Yunfeg Shen et al. (2010a, b, 2011, 2012) or by Kobayashi's group (Koomen et al. 2005).

## 5.6 Conclusions

Over the course of the past 10 years, enormous progress has been made in the analysis of lymph fluid. In particular, advances in proteomic techniques have enabled a more comprehensive characterization of the lymph proteomic, peptidomic, and degradomic content than has heretofore been possible. However, much remains to be done. For example, it will be important to collect capillary lymph from different tissues in order to have an inclusive analysis of the tissue-specific self antigens that are transported to the draining lymph nodes. Additionally, many low abundant proteins and tissue-specific self antigens still remain to be mapped. Furthermore, with respect to the characterization of the lymph degradome, an analysis of the processing pathways that generate the previously reported peptidomes will also be an important endeavor, since it will specify the role of the over 500 mapped proteases in the human lymph and plasma that are involved in processing. Finally, since the lymph fluid carries the proteomic signature of the tissue that it drains, the immunological role of this tissue-specific proteome in maintenance of peripheral tolerance will also be an important question to answer.

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