Metabolomics, peptidomics and glycoproteomics studies on human schistosomiasis mansoni

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1 GENERAL INTRODUCTION

1. HUMAN *SCHISTOSOMA* INFECTION

In 1851, Theodor Maximilian Bilharz, a German pathologist working in Egypt, was the first to identify a blood-dwelling fluke as the etiological agent causing a parasitic infection nowadays known as Bilharzia or schistosomiasis. However, the palaeoparasitology of human schistosomiasis demonstrated the long history of this parasite. Wei *et al.* identified *Schistosoma japonicum* in a mummified body of the Han Dynasty (1) and other investigations showed the presence of this parasite in Egyptian mummies (2-5). In addition, the introduction of new diagnostic techniques facilitated the identification of other historical cases (6;7) including different species of this parasite.

Taxonomy, morphology, distribution and prevalence

Schistosomes belong to the class of digenic trematodes, having a snail (intermediate) host and a vertebrate (definitive) host. They have a basic bilateral symmetry, oral and ventral suckers and a body covered by a syncytial tegument. The blind-ending digestive system consists of a mouth, an oesophagus and a bifurcated caeca. The area between the tegument and alimentary canal is filled with a loose network of mesoderm cells. The excretory system is based on flame cells (8;9). Adult worms range in size from 10 to 30 mm in length and 0.2 to 1 mm in diameter.

Unlike most other trematodes, the schistosomes are dioecious i.e., the sexes are separate. The two sexes display a strong degree of sexual dimorphism, and the male is considerably larger than the female. The male surrounds the female and encloses her within his *gynaecophoric canal* for the entire adult life. The average life span has been estimated to be between 3 and 10 years, but it has been reported that some worm pairs may live up to 40 years in the blood stream (10;11).

The three main schistosome species infecting humans are *Schistosoma mansoni, S. haematobium and S. japonicum. S. intercalatum, S. mekongi, S. malayensis* and S. *guineensis* (12) are less prevalent. The geographical distribution of schistosomiasis primarily comprises developing countries in tropical areas, where hygienic conditions are poor, and climate and ecological conditions are optimal for the intermediate host. *Schistosoma mansoni* is found in Africa and parts of South America, the Caribbean, and the Middle East, *S. haematobium* in Africa and the Middle East, *S. japonicum* in the Far East. Despite progress in schistosomiasis control, an estimated 600 million individuals are at risk of exposure and at least 165 million are infected in sub-Saharan Africa, and a further 30 million in Northern Africa, Asia and South America (13).

Even within an endemic area, the distribution of the infections can be very focal. Obviously, this is not only related to demographic and geographic characteristics but is certainly also determined by the presence and density of the snail intermediate host (14;15).

Life cycle

As illustrated in Figure 1, schistosomes have a complex life cycle involving asexual replication in the intermediate host and sexual reproduction within the vertebrate host. All human *Schistosoma* infections are the consequence of direct contact with fresh water that harbors free-living cercariae which are shed by the snail host. Cercariae are highly motile and infect humans by direct skin penetration. The cercaria attaches to the human skin and secretes proteolytic enzymes facilitating the entrance into cutaneous capillary vessels. Upon penetration, the cercaria sheds its tail and transforms into a schistosomulum. The schistosomulum migrates to the lungs (in 3-4 days), and after passing through the pulmonary capillaries, enters the systemic circulation. During the migration process, the parasite undergoes stages of transformation and maturation and finally ends up in venules at sites characteristic for the different species. *S. mansoni* and *S. japonicum* worms usually reside in the superior mesenteric veins and *S. haematobium* in the venous plexus of the urinary bladder but occasionally also in the rectal venules. There they mature into adult schistosomes in approximately a month.

After maturation the sexual reproduction of the parasite begins and the females start laying eggs. The eggs are deposited in the blood venules of the host. During their

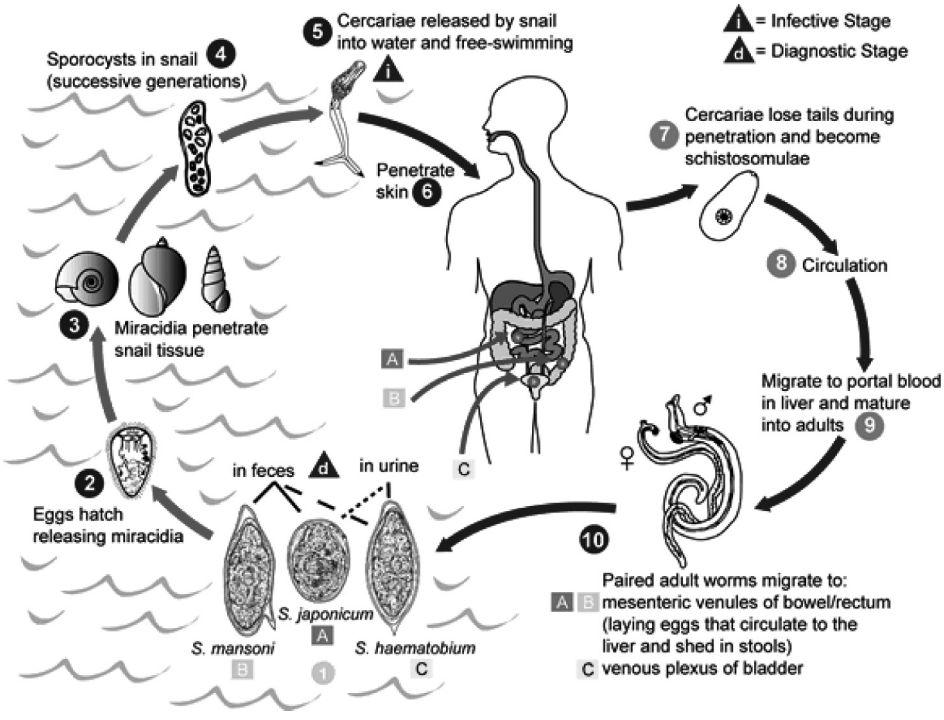

Figure 1. Life cycle of *Schistosoma*. (http://lamarck.unl.edu/zoo/lifecycles/Schistosoma.gif)

reproductive lives, a pair of *S. mansoni* adult worms may produce up to 300 eggs per day while a pair of *S. haematobium* worms produces about 160 eggs daily. Approximately half of these eggs pass through the wall of the intestines (*S. mansoni*) or urinary tract (*S. haematobium*) and are then excreted with the faeces or urine, respectively. Following contact with freshwater, the eggs hatch and the released miracidium is able to infect the intermediate snail host. After infection, the miracidium transforms into a primary (mother) sporocyst. Germ cells within the primary sporocyst will then start dividing to produce secondary (daughter) sporocysts. Subsequently, germ cells within the secondary sporocyst begin to divide again, producing cercariae. These leave the snail at a rate of thousands per day and the shedding of these cercariae can continue for months (13).

Pathology and morbidity

Cercarial dermatitis

In human, the first clinical manifestations of *Schistosoma*-infection are related to skin penetration by cercaria. Up to 40% of schistosomula released from cercariae die in the skin and can induce allergic hypersensitivity reactions (16). These skin manifestations mainly take place in individuals coming into contact with schistosomes for the first time. With prior sensitization, it can also result in a pruritic papular rash within the inner layers of the skin.

Acute schistosomiasis; Katayama syndrome

Primarily travelers to endemic areas can develop acute schistosomiasis, the so-called Katayama syndrome, two to ten weeks after infection. The severity of acute schistosomiasis varies with the intensity of the infection from general malaise to severe illness, as reviewed by Ross *et al.* (17). The most frequent clinical symptoms are: fever, headache, loss of appetite, abdominal pain, diarrhoea, myalgia, hepatomegaly, splenomegaly, urticaria and loss of weight (18;19). The above mentioned clinical symptoms may be associated with other types of infections as well, and therefore diagnosis may still be very difficult at this stage, especially due to the inconsistent excretion of eggs. A history of fresh water exposure in an endemic area and positive serology for schistosomal antibodies in combination with high eosinophilic counts are additional indications of acute schistosomiasis (20-22). In case of late diagnosis, patients suffering from acute schistosomiasis may present severe pathology such as alterations of the intestines, liver and sometimes of the spleen. These pathological changes are usually reversible after adequate treatment (23).

Chronic schistosomiasis

The term schistosomiasis generally refers to the chronic stage of the disease, which is the most common form and has the biggest impact on human populations. Chronic schistosomiasis can last for decades and is primarily related to egg-induced responses. As mentioned above, part of the eggs produced by the worms are not excreted, but are swept away by the blood and can become trapped in different organs. In chronically infected human individuals, long-term exposure to the potent imunogens released by the miracidia within the eggs produces a state of delayed hypersensitivity and each egg may be the initiator of granuloma formation.

This inflammatory reaction around the eggs trapped in the bladder wall in the case of *S. haematobium* can cause obstructive uropathy, i.e. hydroureter and hydronephrosis, which can be visualized by ultrasonography. In addition, *S. haematobium* infections are characterized by the presence of blood in the urine (haematuria). The eggs of *S. mansoni* that do not leave the body are trapped in the presinusoidal capillary venules of the liver, inducing a granulomatous response leading to fibrosis (24). Individuals with persistent or heavy *S. mansoni* infections may suffer from severe clinical manifestations such as hypertension, hepatomegaly, splenomegaly, ascites, haematemesis, oedema and pre-umbilical varices. Some individuals seem to be more vulnerable to the development of severe symptoms than others. This can partially be explained by the intensity and duration of the infection, but immunological and genetic factors of the host also play an important role.

Schistosome infections also have an indirect effect on nutrition, growth and physical fitness due to diarrhoea, decreased appetite or loss of nutrients. The unspecific and indirect morbidity such as pain is difficult to quantify and it is often difficult to identify schistosoma-related mortality. Consequently, a recent study suggests that the true public health burden of schistosomiasis is probably considerably larger than previously estimated (25).

Control and treatment

A variety of drugs such as antimonials, niridazole, lucanthone, hycanthone, cyclosporin A, levamisole and oltipraz have been abandoned as potential drugs against schistosomiasis due to undesirable side effects and/ or poor effect (26). Oxamniquine and metrifonate have been extensively used for the control of schistosomiasis (26;27), although oxaminique is effective against *S. mansoni* only *(26;28)*. Currently, praziquantel is the drug of choice for the treatment of schistosomiasis caused by any of the human schistosome species (WHO, 2002). It is administrated orally at a standard single dose of 40 mg/kg body weight. The drug is well-tolerated, side effects are mild and transient and it can be prescribed to all age groups. Praziquantel is extensively metabolized in the liver, yielding mainly monohydroxylated and dihydroxylated phase-I metabolites,

but also polyhydroxylated metabolites have been described (29-31). Praziquantel and its metabolites are mainly excreted in the urine within 24 hours after a single dose.

The drug has become less costly, and average treatment is now available for less than €0.3, resulting in an easier access of the drug in the developing countries. A single dose of praziquantel is generally sufficient to give cure rates between 60-90% and to significantly reduce the average number of excreted eggs. However, re-infection rates are high (32;33) due to continuous exposure to freshwater containing cercariae (34). Nevertheless, repeated treatment during childhood is suggested to reduce or at least delay the risk of development of severe urinary and hepatic morbidity (WHO, 2002). Moreover, a higher dose of praziquantel or a combination of drugs (praziquantel combined with artemether) may help to improve the treatment success rates (35).

However, widespread use of praziquantel has raised the concern for development of resistance (36;37), especially after the observation of very low cure rates in a highly endemic area in Northern Senegal (38;39). These observations initiated studies to examine the possible upcoming resistance against praziquantel (40;41) but both field and laboratory studies failed to show strong evidence for this. The low cure rates were attributed to high initial worm loads, intense transmission in the area and the presence of immature worms, not affected by praziquantel treatment. Nevertheless, the possibility that ultimately praziquantel resistance may emerge, requires alertness.

Since chemotherapy does not prevent re-infection, regular treatment in endemic areas is needed due to continuous exposure. Therefore, this therapy is only a feasible and effective strategy for the control of morbidity (14). The development of a vaccine that could offer at least partial immunity would be an important step forward in the control of schistosomiasis. Despite intensive efforts in vaccine research (42-44), at present no vaccine is available. Since many of the glycans present on the surface of each life cycle stage of the parasite are clearly distinctive from those of their host, these might be used as conjugates for vaccine candidates (45).

Diagnostic methods

Decisions regarding treatment, assessment of morbidity, evaluation of chemotherapy and control measures are based on the results from diagnostic tests. Therefore, the ideal diagnostic test has a high specificity, sensitivity and predictive value providing both qualitative and quantitative information. Currently, no diagnostic tests are available that fulfill all these criteria. The current tools for diagnosis of schitosomiasis fall into two categories: indirect and direct methods.

Indirect diagnostic methods

In hospital settings, cystoscopy and endoscopy are used to visualize bladder lesions and oesophageal varices (46;47). Laparoscopy and biopsy can reveal the macroscopic

and histological presentation of granulomatous inflammation or periportal fibrosis (48;49). Renal, urethral, and bladder pathology can be visualized by radiography. Additionally, the reagent strip for microhaematuria is a cheap, easy, and effective tool for the screening and rapid epidemiological assessment of urinary schistosomiasis (50). In hepatic schistosomiasis, contrast radiography can show portal-vein distension or gastro-oesophageal varices. CT, myelography, and MRI are useful for detailed imaging, especially for neuro-schistosomiasis (51) In addition, portable ultrasonographic equipment has allowed major advances in the study of schistosomiasis pathology (52;53). Therefore standard protocols have been developed to classify hepatic fibrosis and urinary-tract lesions. However, their use requires specific expertise and experience. As a result, the diagnosis may show a large variation within and between observers.

Direct parasitological methods

Diagnosis of schistosomiasis is usually based on the microscopic detection of parasite eggs in faeces (*S. mansoni*) or urine (*S. haematobium*), the shape of the *Schistosoma* eggs allowing species differentiation. The Kato-Katz thick smear (54) is the most extensively used method for examining the presence of *S. mansoni* eggs in faeces of infected individuals while urine filtration and centrifugation enables the diagnosis of urinary schistosomiasis. These microscopy-based methods are very specific, simple and cheap but due to the uneven distribution of *S. mansoni* eggs in solid excreta and the considerable day-to-day fluctuation, infections and especially light infections, are easily missed. Therefore, parasitological examination needs to be repeated several times, which is not always feasible in large-scale epidemiological and control studies. Moreover, the samples need to be processed within 24-48 hours and the processing itself is rather laborious. In short, this method is highly specific but lacks sufficient sensitivity.

Recently, conventional PCR methods for the detection of *Schistosoma* DNA in human samples have been developed (55;56). PCR-based methods have shown high sensitivity and specificity for the detection of parasitic DNA, yet their use in epidemiological studies has so far been limited. Compared to the Kato-Katz parasitological examination, it demands more sophisticated equipment and a more complex operational effort. Nevertheless, PCR appears to be more sensitive than the Kato-Katz technique. PCR based methods may be particularly suited for the diagnosis of the *Schistosoma* infection in cases where high sensitivity and specificity are required and a proper infrastructure is available. Recent developments in the simplification of DNA isolation procedures and the developments in PCR technology, especially real-time PCR, have positioned molecular diagnosis as a good alternative to microscopy-based diagnostic methods (57-60).

Alternatively, detection of antibodies is a highly sensitive and specific method to diagnose schistosomiasis. High antibody responses are generally seen upon first exposure, for example in travelers originating from areas where schistosomiasis is

non-endemic. However, in individuals with a life-long history of exposure, antibody responses are mostly moderate to low. In addition, antibody levels are not associated with the actual worm load and may remain unchanged after treatment. Consequently, serology mostly gives straightforward answers for patients tested within months after their first exposure, but data are difficult to interpret for those who have a history of previous infection.

Detection of parasite derived circulating antigens by immunological means is an effective strategy to distinguish active from past infections. A variety of schistosome specific molecules are released into the host circulation by the adult parasites' regular regurgitation of the gut content. Some of these molecules have been partially characterized, for example the two major polysaccharide antigens: a negatively charged circulating anodic antigen (CAA) and a positively charged circulating cathodic antigen (CCA) (61;62). Sensitive and specific immunoassays using monoclonal antibodies have been developed for the detection and quantification of these two antigens in serum and urine of patients (63-66). Although these methods work well in endemic areas with moderate and high prevalence of the disease, they are less sensitive in low infection areas (65;66). The recently developed 'dipstick' qualitative test for the detection of CCA (65;67) has been a major improvement. It has the advantage of being easy and rapid to perform and might be implemented in the field, although it additionally needs to be evaluated in areas of light infections. In addition to specific molecules released by the adult parasite, the eggs also secrete antigens (68;69). A part of these antigens can be detected by ELISA in the host circulation or urine using monoclonal antibodies (mAbs) that recognize specific glycan motifs (70;71).

2. BIOMARKER DISCOVERY

During the past decade the comprehensive analysis of an organism's biomolecules has been extensively used to investigate biomolecular changes associated with an organism's phenotype. Consequently, these approaches have become an essential element both in the explorative phase of clinical research ("biomarker discovery") as well as in more focused, in-depth analysis in biomedical research. The National Institutes of Health defined a biomarker as: "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention". Large scale biomarker discovery research includes the integrated application of various research areas, such as biochemistry, bio-analytical analysis (primarily mass spectrometry (MS) and Nuclear Magnetic Resonance (NMR) coupled to a variety of analytical separation techniques), clinical chemistry and bioinformatics.

Urine

Urine and blood are the preferred biofluids for biomarker discovery due to their ease of collection and extensive history of use in clinical analysis and diagnostics. Urine, in particular, can be collected non-invasively and in large quantities and comprises a plethora of inorganic salts and organic compounds, including proteins, peptides, hormones, and a wide range of metabolites.

Urine is an obvious "target" fluid used in many studies that are investigating renal and urological disorders. However, other diseases might also be reflected by changes in the urine composition and the fact that urine is an ultrafiltrate of plasma has led researchers to look into this body fluid for biomarkers of disease from more distant organs.

Robust sample work-up is a key element for conducting biomarker discovery studies and different biological samples need different sample preparation for further analysis. Although urine is an easily obtainable body fluid, the variability of its composition, such as salt, pH and other interfering compounds are making systematic analysis of urinary components such as proteins, peptides and metabolites a challenging task. The standardization of urine sample collection, storage and shipment are some of the challenges to be overcome when working with urine for biomarker discovery. Recently, a research paper by Thongboonkerd *et al.* has described step-by-step-practical points to perform urinary proteome analysis, providing detailed information for study design, sample collection, sample storage, sample preparation, proteomic analysis and data interpretation (72).

The collection and processing of urine samples have a profound influence on the urinary composition (73-82). The information obtained from 24-hours or midstream first morning urine might seem the most suitable for the analysis of urinary compounds. The 24h urine provides information of average urinary protein excretion over a day and offers the possibility to correct for diurnal variation but has a number of drawbacks such as logistical difficulties and potential bacterial growth during extended storage (83;84). The diurnal variation can also be avoided by the collection of the first morning urine. However, several studies have demonstrated that the collection of first-morning urine is less practical as this may be contaminated with proteins from bladder epithelial cells (85;86). The use of the midstream urine is recommended as an alternative to the first-stream urine, due to the lower epithelial and urethral "contribution". A midstream sample of the second morning urine has been found to be optimal and this has been used with success in several studies (87;88). Nevertheless, due to logistical issues, most clinical studies rely on random, non-timed urine collection.

The variable dilution of urine is considered to be a major drawback for systematic analysis of urine in biomarker discovery studies. However, in clinical practice sample normalization on creatinin appears to adequately compensate for variable dilution of specimens and to circumvent the need for a standardized time point collection.

Urinary peptidomics

"Peptidomics" has been introduced in analogy to "proteomics" and emerged as one of the new "omics" technologies (89;90). It deals with the analysis of small endogenous peptides in a biological sample (91-100). Peptidomics is one of the major areas within the field of clinical proteomics: the application of proteomics technologies to investigate peptide expression differences, especially in biofluids, with the aim to identify disease-related biomarkers. These potential biomarkers, identified in the discovery phase, may form the basis for early and accurate diagnosis of a disease.

Peptides are usually generated from a larger precursor by proteolytic cleavage events and typically have a molecular weight of up to about 10,000 Da. In contrast to the protein precursor, peptides exhibit better permeability between tissue compartments due to their relatively low molecular weight (101;102) and therefore the probability of detecting proteolytic fragments in biofluids is higher than the probability of detecting the protein precursor. Proteases can initiate, modulate and terminate many important cellular functions by highly specific substrate cleavages and there are many pathological processes that influence protease activities and protein expression. Therefore it is expected that *in vivo*-generated peptides have the potential to mirror pathological events (103). Consequently, the peptidome is not only associated with protein expression levels but may additionally reflect protease activities. Some peptides, such as neuropeptides, are actively involved in biological processes and have several unique characteristics that make them well suited as biomarkers (104). The peptidomics concept has been metaphorically described by Schultz-Knappe *et al.* as being the new direction in proteomic research to analyze the "terra incognita" of proteomics (the low molecular mass window) (105).

The urine of healthy individuals contains only a low amount of proteins. These proteins are originating from ultrafiltration of plasma through the renal glomeruli and from the urinary tract itself, especially the renal tubes, which reabsorb and degrade the filtered proteins (106;107). The efficiency of the removal of proteins during ultrafiltration is size dependent. For example, more than 99.9% of proteins larger than 60kDa are retained in the plasma space (108). Therefore the urine:plasma ratio of most proteins (the size of albumin or larger) is less than 0.001, while the ratios for small proteins and peptides are usually close to 1. As a result, in comparison to plasma, urine has an approximately 1000-fold relative enrichment of low molecular-weight proteins and peptides.

Initial studies examining the peptide content of urine have described fragments of a wide range of proteins, and have indicated that urine would be a potentially rich source of peptide markers (109-112). As a consequence, several studies have aimed at analyzing the complex mixture of urinary peptides. The methods used in urinary peptidomics are based on the detection of native peptides by mass spectrometry without prior trypsinization. Pre-MS fractionation or extraction of peptides is often used in order to improve the dynamic range.

Urinary metabonomics

Metabolomics and metabonomics have originated from two fields of biological science: metabolomics from plant science and metabonomics from animal biochemistry and medicine.

As defined by Nicholson, "metabonomics" describes the global biochemical profile of an organ or organism, while "metabolomics" describes the biochemical profile of an isolated cell (113). Using these definitions, metabonomics may be seen as the sum of all metabolomic changes and it deals with integrated, multicellular biological systems. All metabolites present in a biological cell, tissue, organ or organism, which are the intermediates and end-products of cellular processes, represent the metabolome. In practice, therefore, the term "metabolomics" is also often used when dealing with studies of the metabolome of multicellular systems. With respect to human studies metabolomics/metabonomics may be seen as a "quantitative measurement of the dynamic multiparametric metabolic response to pathophysiological stimuli or genetic modification" (114). Metabolomics is directly linked to the physiology of a given cell at a given moment, such in contrast to proteomics and transcriptomics. This may be one of the main reasons why the field of metabolomics/ metabonomics has developed rapidly, merging strategies to identify and quantify cellular metabolites using sophisticated analytical technologies with statistical methods for data interpretation and information extraction. Metabolomics and metabonomics use similar analytical approaches: a targeted metabolite analysis and an untargeted screening (global analysis, profiling, fingerprinting). Targeted analysis has been applied for many decades for the determination and quantification of specific groups of metabolites (amino acids, lipids, fatty acids, steroids, sugars, etc), based on previous knowledge of the biological system or a given metabolic pathway. In contrast to targeted analysis, metabolite profiling aims at the analysis of a wide range of compounds, resulting in a metabolic "signature" or mass profile of the sample of interest. These fingerprints are then compared in a large sample population to screen for differences such as phenotyping of genetically modified plants, determination of gene function, monitoring of a disease, treatment effects, responses to chemical and physical environmental stress, etc.

The human metabolome database (HMDB) contains about 1000 metabolites identified in urine. These metabolites are characterized by a large dynamic variation and different physical and chemical properties being the result of the mechanisms maintaining the homoeostasis of the individual (115;116). It is likely that different physiological stresses may be reflected in the urine metabolite fingerprint. Therefore, global metabolomics analysis of urine might benefit from multiple extraction procedures as well as different methods of analysis. The methods used in "global metabolomic" analysis are generally based on mass spectrometry (MS) and nuclear magnetic resonance (NMR) since these techniques can handle complex biological samples with a high sensitivity, selectivity and

throughput. Mass spectrometry is usually combined with chromatographic separation methods such as gas chromatography (GC), liquid chromatography (LC) or capillary electrophoresis (CE). In a recent paper Issaq *et al.* have discussed in detail the critical role of separation of metabolites prior to analysis (117).

Peptidomics and metabonomics technologies

Surface-enhanced laser desorption ionization mass spectrometry (SELDI-MS) and Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-ToF MS)

Clinical or epidemiological biomarker studies, where typically hundreds of samples are analyzed, require a high throughput approach. Because of this, SELDI-MS has often been used in these types of studies, emerging as one the preferred platforms for urinary peptide profiling (88;118;119). SELDI uses "ProteinChips" with different affinity surfaces such as hydrophobic, cation exchange, anion exchange, copper (IMAC) and silica coated chips. These can be used to reduce the sample complexity and combining different matrices facilitates the detection of a larger number of compounds (120). Two independent studies have presented specific urinary biomarkers associated with the rejection of renal allografts using the SELDI-MS platform (118;119). Shaub and colleagues could define three prominent peak clusters that discriminated between patients with biopsy-confirmed rejection episodes and patients without histological evidence of rejection. Clark and coworkers were able to detect five biomarker candidates with a high diagnostic prediction, allowing discrimination between renal transplant patients with no rejection and those with acute allograft rejection (sensitivity 83%, and specificity 100%). The fact that these two studies used different ProteinChip arrays resulted in differences in the biomarkers observed to be associated with the renal allograft rejection. Rogers and colleagues have investigated urine of renal cancer patients (121). They were able to differentiate between patients with renal cell carcinoma, patients with benign diseases of the urogenital tract and healthy controls. Additional SELDI-MS studies on urine have shown that renal, bladder and prostate cancers produce peptidomic changes in urine (88;121-126). Recently, Ye *et al.* reported polypeptide changes in the urine of ovarian cancer patients (127) while Ward and colleagues showed that changes in the urine peptidome may aid in the early detection of colorectal cancer (128). Despite the progress made in biomarker discovery using the SELDI-MS technology there are certain limitations. The fact that SELDI is based on the specific binding of a fraction of the peptides/ proteins to different matrices limits the number of compounds under investigation. Moreover, because of large inter-individual differences in urinary peptide/protein concentration and factors like pH and salt conditions, reproducibility and comparability of datasets is generally poor, especially when working with very low volumes of urine (few µl) (125;129;130). In

addition, SELDI-MS profiles are characterized by complex spectra, high dimensionality, and significant noise, which all together make the discovery of potential biomarker in clinical samples a challenging task.

MALDI-ToF/ToF MS combines high throughput biomarker discovery with the identification of the peptides of interest in one instrument and from the same set of samples. (131). However, the identification of native peptides is more challenging than identification of peptides generated after tryptic digestion, as applied in more common proteomics approaches. Consequently, many general search algorithms used for the identification of tryptic peptides may not be particularly suited for the analysis of native peptides. This is primarily related to the large search space, because no enzyme can be specified, but also fragmentation issues and the size of the peptide of interest may complicate straightforward identification. In addition, post translational modifications (PTMs) may be a serious obstacle to sequence and identify potential biomarkers. Manual inspection of fragmentation spectra and *de novo* sequencing may additionally be required for confident identification. Information about sequencing and identification of native peptides has recently been summarized by Mischak and colleagues (132) and Theodorescu and co-workers (133).

Recently, Merchant and colleagues identified peptides in the low molecular weight fraction of urine that correlate with early renal function using a combination of manual inspection of spectra and database searches using a common search algorithm. These peptides reflected changes in both tubular and glomerular protein expression that are associated with the formation of stress granules and may define a new cellular mechanism by which diabetic nephropathy is initiated or progresses (134).

Although the high-throughput capabilities of MALDI-ToF MS make this technology suitable for large-scale metabolomic studies, its application in this field has been rather limited. This is due to the fact that the conventional matrices produce interfering low-mass ions complicating the detection of metabolites. The development of new matrices such as ionless matrices has facilitated the detection of small molecules (0–1,000 Da). Shroff *et al.* demonstrated the applicability of MALDI-ToF MS for targeted metabolomics on plant, insect and blood samples (135) using these ionless matrices. Recently, in a non-targeted approach, Wang *et al.* could predict acute cellular renal allograft rejection by urinary metabolomics using MALDI-FT MS (136).

The use of MALDI-MS as an analytical tool in clinical research still needs optimization (137). Peptide and metabolite concentrations in biological samples cover a large dynamic range and therefore only part of the compounds in a sample is detected. In addition, overlapping signals and ion suppression introduce additional limitations. As a result, extensive off-line fractionation steps may be necessary to improve the number of compounds detected by MALDI-ToF MS. Obviously, the efficiency, recovery and reproducibility of these fractionation and purification steps are important issues.

Capillary electrophoresis coupled mass spectrometry (CE/MS)

Capillary electrophoresis (CE) is one of the most powerful analytical separation methods, being characterized by high-speed separation, high resolution, and high sensitivity for ionic compounds. CE is applicable for separation of both urinary peptides and metabolites. The analytes are separated based on their migration through a liquid-filled capillary column in an electric field.

A detailed review illustrating CE/MS as a powerful peptidomics tool in biomarker discovery and clinical diagnosis has been published recently (138). Neuhoff *et al.* have shown in a comparative study that CE/MS has a higher sample resolution and mass accuracy resulting in a larger number of biomarkers compared to SELDI-based approaches (139). By on-line coupling of CE to an ESI-TOF-MS, combining the high resolving power of CE with the mass accuracy of the MS, it has been shown that 6,000 polypeptides can be resolved within a 45–60 min time window (140). The enhanced capability of CE/MS to resolve thousands of polypeptides in a single processing step was subsequently confirmed in a number of clinical studies, aiming to discover disease-specific polypeptide marker profiles for various chronic kidney diseases. The identification of 20-50 urinary polypeptide markers has, among others, allowed diagnosis and discrimination of IgA nephropathy, focal-segmental glomerulosclerosis (FSGS), membranous glomerulonephritis (MGN), and minimal-change disease (139;141-143). Recently, Julian *et al.* reported on the identification and validation of biomarkers for urinary polypeptide biomarkers of renal disease in patients with IgA-associated glomerulonephritides (144). In another study, CE/MS resolved different polypeptide profiles in urines from different age groups (145).

A recent study by Zimmerli *et al.* has shown the applicability of CE/MS in diseases other than renal and urological disorders. They examined urine from patients who underwent coronary artery bypass grafting and from patients after acute myocardial infarction (146) to identify coronary artery disease (CAD)-specific biomarkers.

In the field of metabolomics, CE/MS has also repeatedly been used for the analysis of a subset of metabolites in human urine but there are only a limited number of studies that have used CE for separation of metabolites in global metabolomics approaches (reviewed by (147-149)). The applicability of CE/MS in the field of metabolomics was successfully shown by Garcia-Martinez *et al.* They have used CE/MS to study the influence of a *Dunaliella salina* extract on the urinary metabolic profile in control and diabetic rats and correlated this with the beneficial effect of the treatment (150). In another study, Ramautar and colleagues have described potential markers to differentiate complex regional pain syndrome (CRPS) patients and controls based on CE metabolic profiling of urine (151).

All these CE/MS based studies have shown the applicability of this technique based on the efficient separation of a wide variety of compounds in biological samples.

Additional advantages include low sample and solvent consumption and relatively short analysis times. One of the drawbacks of CE/MS is the fact that coupling of CE and MS is not so straightforward. Moreover, CE/MS suffers from poor concentration sensitivity and detection due to the low sample loading capacity (1-100 nL) and migration times may shift during the analysis of biological samples, especially in case of minimal sample pretreatment. Consequently, CE/MS is not yet a commonly applied approach for studies of epidemiological scale.

Liquid chromatography coupled mass spectrometry (LC/MS)

LC is regarded as the method of choice for the separation of both peptides and small molecule metabolites, providing a powerful fractionation method compatible with practically any mass spectrometer (152-154). Depending on physical and chemical proprieties of the compounds, these can be separated by using reverse phase (RP), normal phase, ion exchange, chiral, size exclusion, hydrophilic interaction chromatography (HILIC), and mixed modes. For the analysis of peptides, a combination of methods in a two-dimensional design is particularly suited for in-depth analysis of body fluids such as urine (155). For example, Cutillas and coworkers have compared urine from patients with Fanconi syndrome with urine of healthy individuals and have described differences in levels of proteins implicated in progressive renal injuries (109;156). Although 2D-LC MS is widely recognized as a versatile technique for in-depth analysis of biological fluids, the considerably long analysis and data processing time limits its use for biomarker identification in an initial discovery phase. The latter is related to the fact that multiple separation dimensions create some difficulties in comparative analysis due to the variability of the multidimensional separation.

The first analyses of metabolites using LC have dealt mostly with "targeted metabolomics". Two recent reviews have illustrated the potential, challenges and application of a LC/MS-based global metabolomics analysis (157;158). Fukui and colleagues have described one of the applications of an ultra-performance liquid chromatography-mass spectrometry (UPLC/MS) based metabonomic approach to identify a candidate metabolite associated with interstitial cystitis (IC) aiming at non-invasive diagnosis of the early stage of IC (159). In another UPLC/MS study urinary metabolites from liver cancer patients and healthy volunteers were studied using a metabonomic based approach, resulting in the identification of 21 metabolites as potential biomarkers (160). Issaq *et al.* have shown that metabolomics based on high performance liquid chromatography-mass spectrometry has the potential to become a non-invasive early detection test for bladder cancer (161).

Gas chromatography coupled mass spectrometry

GC/MS was a pioneering technique used for the profiling of urinary metabolites, in particular steroids, acids and drug metabolites (162). GS/MS is a promising approach,

since GC is an excellent separation technique but is obviously limited to small compounds that are thermally stable and volatile, or can be made chemically volatile by derivatization

To date, GC/MS for large-scale metabolite analysis is mainly applied in the field of plant metabolomics (163). Relatively few studies have used GC/MS for the metabolic profiling of urine. As an example, Lee *et at.* have shown a non targeted metabolic profiling method for the evaluation of the hepatotoxicity of valproic acid and demonstrated the proof-of-concept that the metabolomic approach with GC/MS has great potential for predicting valproic acid–induced hepatotoxicity and discovering novel biomarkers (164). In another study, GC/MS was used to investigate the urinary metabolic difference between hepatocellular carcinoma male patients and normal male subjects. The authors found 18 metabolites to be significantly different between the HCC and control groups (165). Lin and colleagues, using GC/MS, could identify potential markers of intestinal inflammation using a mouse model of Crohn's disease (166).

Nuclear magnetic resonance (NMR)

Nuclear Magnetic Resonance (NMR) is a powerful technology for metabolic analysis of biofluids and as such has significantly contributed to the field of metabolite profiling (113;167-171). Metabolite profiling involves the combination of high-resolution spectroscopy with multivariate statistical methods allowing the investigation of small differences in sample cohorts by detecting metabolites signatures (172-174). The use of NMR for this purpose has many advantages, such as a high information content of the resulting spectra, nondestructiveness, the relative stability of the resulting chemical shifts, the ease of quantification of multiple classes of metabolites and the lack of any need to pre-select the conditions employed for the analysis.

Although 1D NMR has laid the foundation of metabolomics studies, this methodology seriously limits the unambiguous identification of metabolites, due to the high complexity of the NMR spectra of biofluids. The availability of metabolite databases has improved the peak assignment process, but a close examination of these databases suggests that they are often inadequate for a complete and unambiguous assignment, particularly for low abundant compounds (115;116). Various approaches for metabolite identification have been discussed in several recent reviews (175;176). Despite these minor drawbacks the use of NMR spectroscopy for the analysis of metabolomics has become very popular (168;175;177).

In the clinical field, the use of the NMR-generated metabolic data sets in combination with multivariate statistical analysis has allowed sample classification, effective interpretation of alterations in biochemical pathways and demonstrated the potential for identifying candidate biomarkers. Several encouraging results have been obtained using urinary metabolite profiling. In a recent study, using NMR to quantitatively measure the profile of metabolites excreted in the urine of patients

with pneumonia caused by *Streptococcus pneumoniae,* Slupsky and colleagues have found that this profile was significantly different from the profiles for viral and other bacterial forms of pneumonia (178). In addition, Saude *et al.* have shown that urine NMR analysis is a promising, noninvasive technique for monitoring asthma in humans (179). Ishihara *et al.* performed a 1D NMR metabonomics-based study using an animal model and have shown that this approach might be useful for determining the mechanisms of toxicity, and as such may be implemented in drug safety evaluation and biomarker discovery (180).

Data processing and analysis

Data handling and analysis form a critical step in any biomarker discovery workflow. Signal processing is essential for successful extraction of adequate information. Signal processing of mass spectra is performed through a series of steps: smoothing, baseline correction, normalization, calibration (alignment) and peak labeling. In analogy to this, NMR spectra are processed using apodization, zero-filling, fourier transformation, phasing, baseline correction, referencing (alignment), binning, normalization and peak labelling. The purpose of this data pre-processing is to correct intensity of *m/z* values or NMR chemical shifts in order to reduce noise, reduce the amount of data points, and to enable comparison of the spectra. Normalization reduces systematic variations caused by variation in the concentration of the samples, degradation in the sample or variations in the detector sensitivity. To allow the comparison of compounds across samples, spectra have to be aligned. The application of an external calibration using peptides of known molecular mass that are analyzed alongside the samples is a common approach in mass spectrometry. Referencing of an NMR spectrum is achieved using one reference peak (internal standard) whose chemical shift is accurately known.

The dimensions of a MS or NMR spectrum may be reduced by grouping intensity measurements at adjacent values into bins. Discrete wavelet transformation has also been used to reduce the dimensionality of a data set. Wavelet coefficients with a value lower than a given threshold are discarded, yielding a new feature set consisting of the wavelet coefficients.

Before starting the statistical analysis it is important to decide whether to use all recorded *m/z* values or chemical shifts as features or to use peaks identified by a peak detection algorithm. The use of peak detection algorithms may have the advantage of filtering out the noise but at the same time may also eliminate low-abundant features.

After the pre-processing, multivariate methods can be applied for data analysis. Commonly, unsupervised analysis is initially performed to get a visual representation of the signal strength of the data. There are many unsupervised methods such as principal component analysis (PCA) and hierarchical cluster analysis. PCA is most often the method of choice to investigate clustering tendency, detect outliers and visualize data structure. PCA searches for a small number of the mutually orthogonal linear

combinations of variables, that explain the gross of the variance within a data set (181). The resulting linear combinations may not reflect the underlying class structure and therefore the maximum variance directions do not necessarily guarantee maximum discrimination. Moreover, one of the drawbacks of unsupervised methods is that they do not exploit the information provided by class structures, completely ignoring the meta data. Therefore, PCA is often followed by a supervised analysis technique such as partial least square analysis (PLS) (182). In this step, the processed data are usually split into a training set and a test set. The training set is used to build a classification model and the test set is held aside for the evaluation of the trained classifiers. Many computational methods and statistical tests have been developed to support the recognition of intra-group similarities and inter-group differences.

PLS is a regression method that incorporates features transformation methods that construct new features as functions that express relationships between the initial features (183). PLS is adapted to a small number of samples with a high dimensional representation. It is therefore one of the most commonly used supervised methods relating a data matrix containing independent variables from samples, such as spectral intensity values, to a matrix containing dependent variables such as e.g. infection intensity. Partial least squares discriminant analysis (PLS-DA) is performed in order to improve the separation between groups. The recent advanced development of PLS-DA, the orthogonal projection on latent structure discriminant analysis (O-PLS-DA) may further improve the interpretation of "omics" data sets.

Other types of supervised analysis methods are based on feature selection methods that generate a subset of the original input features without transforming them. The feature selection is done by using univariate methods that assume mutual independence of the predictive variables and by using multivariate methods that assess the predictive power of features subsets rather than individual features. The selected features set can be used in any learning algorithm to build a predictive model. For example, support vector machine (SVM) provides a machine learning algorithm that can be applied for classification. Different parametric tests such as t-test and F-ratio, and nonparametric tests including the Kolmogorov-Smirnov test, Wilcoxon rank test (equivalent to the Mann-Whitney) and AUC (area under the ROC curve) test have been used for feature selection in "omics" biomarker discovery. The ROC curve describes the trade off between sensitivity and specificity of a model depending on its parameters. The sensitivity calculated for a model is defined as the proportion of people with a disease being predicted to have the disease, and the specificity of a model is defined as the proportion of people without the disease that are predicted not to have the disease.

The supervised methods mentioned before all use a training data set to improve predicting ability of a model. However, models that fit the training dataset very well may mispredict new data points. Such over-fitting of the training data most likely will yield a model that does not generalize well and, consequently, will not be useful.

Therefore, for each prediction model the generalization error to an independent data set is estimated by cross validation. *Cross-validation* is an important tool to avoid over-fitting models on training data, as over-fitting will give low accuracy on validation. Besides using cross-validation simply to estimate the generalization error of a given model, it can also be used to choose the best algorithm and parameters by minimalizing the estimated generalization error. In this case double cross-validation is used. This means that the generalization error is estimated using three groups: a training group and two testing groups. The training and one testing group are used to choose the model parameters by optimizing the generalization error. The optimized model and the second testing group are used to estimate the generalization error.

3. SCOPE OF THE THESIS

Global urinary peptidomics and proteomics approaches may provide new insights in the host parasite interaction and might improve the monitoring of *Schistosoma*-infection and morbidity.

Previously, CE has been used to analyze the metabolites present in urines from *S. mansoni*-infected and non-infected mice and a few potential biomarkers were identified, demonstrating the potential of this technique as a diagnostic tool for this parasitic infection (184). Systematic NMR metabolic fingerprinting has also been applied in studies on parasitic infections and the metabolic consequences of parasitic worm infections in rodent models have been clearly demonstrated (185-190). The metabolic analysis of urine from mice infected with *Schistosoma mansoni* provided a metabolic signature of the infection (189). Recently, four parasite-rodent models, namely *Plasmodium berghei*-mouse, *Trypanosoma brucei brucei*-mouse, *S. mansoni*-mouse and *Fasciola hepatica*-rat were used to investigate the metabolic signature which is associated with inflammation and to determine specific metabolites that correlate with parasite-induced changes in plasma cytokine levels (191).

To date, little is known about urinary metabolic or peptide biomarkers for *Schistosoma*-infections in humans. In comparison with animal studies, human studies are more challenging due to variable infection intensities and differences in the time course of the infection. Moreover, the inter- and intra-individual biological variability, different food patterns, co-morbidities with other infections, differences in age and gender are features that should be considered in the statistical analysis (192-194).

The conclusions drawn from peptidomics / metabolomics data become more powerful when study designs are implemented that rely on a large numbers of samples. For human studies it is important to analyze hundreds to thousands of samples, necessitating the application of high throughput methodologies, including an easy, robust and reproducible sample preparation technique. In **Chapter 2**, we describe a novel automated workflow for global urinary peptidomic analysis. We have tested the

applicability of this novel method on a small cohort of urines from *S. haematobium* infected individuals. In **Chapter 3**, we have applied the same method on a large number of urine samples collected in an area endemic for *S. mansoni* infection and we have incorporated novel data processing methods for data analysis. In **Chapter 4**, we have used these urine samples for a global NMR-based metabolomics analysis.

For more targeted biomarker discovery approaches related to schistosomiasis, glycoconjugates constitute a class of interesting compounds. A large part of the antibodies produced by infected subjects are directed against glycan epitopes of such schistosome glycoconjugates (195;196). All *Schistosoma* life cycle stages express a substantial amount of glycoconjugates that are developmentally regulated, and intensive research has been carried out aiming at the structural characterization of the carbohydrates structures (197-200). The role of schistosome-specific glycosylation and the different immune responses induced by distinct epitopes present on these glycoconjugates has been the focus of many recent studies (196;201-204). In **Chapter 5**, we have characterised the glycan structure of one of the major proteins secreted from *S. mansoni* eggs.

As stated above, glycan and glycoconjugate antigens such as the worm gut-associated CAA (circulating anodic antigen) and CCA (circulating cathodic antigen) are released in the circulation of the host and form the basis for one of the methods for the diagnosis of *Schistosoma* infection, using a sandwich immunoassay with anti-carbohydrate monoclonal antibodies (205;206). Schistosomes express many different glycans with a high degree of fucosylation and the (±Fucα1-2)Fucα1-2Fuc moiety seems unique for schistosomes. Recently, the schistosome-specific multifucosylated glycan epitope recognized by a carbohydrate-specific antibody that binds to egg glycoprotein antigens has been characterized (207). Interestingly, this antibody immunocaptured free oligosaccharides containing the same multi-fucosylated structural elements from urine of *Schistosoma*-infected individuals (208). In **Chapter 6**, we have performed a comprehensive mass spectrometric analysis of urine samples with the aim of identifying specific glycopeptides in urines of *S. mansoni*-infected individuals.

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