Urinary Clara cell protein and alpha 1-microglobulin – biomarkers of changes in kidney and airway function

Lena Andersson

Institute of Medicine
at Sahlgrenska Academy
University of Gothenburg

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Lena Andersson
Occupational and Environmental Medicine,
School of Public Health and Community Medicine,
Institute of Medicine, University of Gothenburg, Gothenburg, Sweden

ABSTRACT

Validated biomarkers are useful tools for screening large populations. The benefit of screening may be improved risk assessment or early indications of adverse effects before detection or onset of symptoms. Screening is valuable in studies of environmental exposures and their effects on human health, for example after exposure to air pollution and toxic metals in our daily environment. The overall aim of this thesis was to optimize sampling of urinary Clara cell 16 kDa protein (CC16) and alpha 1-microglobulin (A1M) and test them as biomarkers of changes in kidney and airway function.

For optimized sampling of urinary CC16 and A1M, timed or creatinine-corrected first morning samples are preferable. This is the time of day when the variation in excretion due to influence of urine flow and daily activities is low. For all urinary CC16 sampling, men should discard the first 100 ml to wash out post-renal excretion of CC16 before collection.

Urinary and serum CC16 were tested as biomarkers of changes in the permeability of the respiratory epithelium. This was examined after exposure to wood smoke for 4 hours and compared with a session of filtered clean air for 4 hours. Exposure to wood smoke significantly increased serum CC16 after 20 hours and there was tendency towards increased urinary CC16.

Both urinary CC16 and A1M are sensitive to changes in tubular function and were analysed together with another tubular biomarker, retinol-binding protein (RBP), in the urine of small children with urinary tract infection (UTI). Clara cell 16 kDa protein was significantly higher in children with upper UTI compared with children with lower UTI. Also, the association between decreased uptake of dimercaptosuccinic acid (DMSA) and increased excretion of CC16 was strong. There was no difference in A1M excretion between upper and lower UTI.

In patients with community-acquired pneumonia (CAP), urinary CC16 was increased compared with the control group. By contrast, serum CC16 was decreased in patients with CAP. The increase in urinary CC16 was probably due to fever-related proteinuria since urinary A1M and RBP were also increased. The decrease in serum CC16 could be explained by increased renal excretion. Also, asthma, chronic obstructive pulmonary disease (COPD) and smoking (known to decrease serum CC16) were common among patients with CAP.

In conclusion, this thesis demonstrates that both CC16 and A1M are useful biomarkers of tubular renal function. There is also a possibility to use urinary CC16 as an airway biomarker in screening studies of air pollution under the right conditions, using optimal sampling methods.

Key words: biomarker, Clara cell 16 kDa protein (CC16), alpha 1-microglobulin (A1M), ambient air pollution, renal function, pyelonephritis, children

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Original papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I–V):

I. Lena Andersson, Per-Arne Lundberg and Lars Barregard. Methodological aspects on measurement of Clara cell protein in urine as a biomarker for airway toxicity, compared with serum levels. *J Appl Toxicol 2007 Jan; 27: 60–66*

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III. Lars Barregard, Gerd Sällsten, Lena Andersson, Ann-Charlotte Almstrand, Pernilla Gustafson, Marianne Andersson and Anna-Carin Olin. Experimental exposure to wood smoke: effects on airway inflammation and oxidative stress. *Occup Environ Med 2008; 65: 319–324*

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V. Lena Andersson, Kristoffer Strålin and Lars Barregard. Clara cell 16kDa protein in patients with community-acquired pneumonia. *Manuscript*

List of abbreviations

A1M – alpha 1-microglobulin
BAL – broncho-alveolar lavage
BALF – broncho-alveolar lavage fluid
BMI – body mass index
B2M – beta 2-microglobulin
CAP – community-acquired pneumonia
CCSP – Clara cell secretory protein
CC10 – Clara cell 10 kilo Dalton protein
CC16 – Clara cell 16 kilo Dalton protein
CRP – C-reactive protein
CV – coefficient of variation
DMSA – dimercaptosuccinic acid
ELISA – enzyme-linked immunosorbent assay
HIV – human immunodeficiency virus
HMWP – high-molecular-weight protein
IgA – immunoglobulin A
kDa – kilo Dalton
LIA – latex immunoassay
LMWP – low-molecular-weight protein
LoD – limit of detection
LPS – lipopolysaccaride
NAG – *N*-acetyl-β-d-glucosaminidase
PAH – polycyclic aromatic hydrocarbons
protein HC – human complex-forming glycoprotein
RBP – retinol-binding protein
r_p – Pearson's correlation coefficient
r_s – Spearman's rank correlation coefficient
RSD – relative standard deviation
UTI – urinary tract infection

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1 Introduction

The research in occupational and environmental medicine aims to increase the knowledge about different biological, chemical, physical and psychological factors in our environment. Much of this research identifies and prevents unnecessary health risks in our society. Two important environmental exposures, for this thesis, and their effects on human health are exposure to air pollution by inhalation, and heavy metals through our daily ingestion. These environmental exposures primarily affect the lungs, the circulatory system, the nervous system, and the kidneys. One way to study the effect of environmental exposure in humans is through use of biomarkers. In addition to using various biomarkers in research, method studies are needed to increase the knowledge on variability in biomarkers within and between individuals.

Biological markers (biomarkers) have been defined by Hulka (1991) as "cellular, biochemical or molecular alterations that are measurable in biological media such as human tissues, cells or fluids". More recently, the definition has been broadened to include biological characteristics that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic intervention. In this thesis, the definition of "biomarker" is a specific biological feature such as the levels of a certain protein like Clara cell 16 kDa protein (CC16) or alpha 1-microglobulin (A1M) in the body, which can be measured to indicate the progression of a condition, such as changes in kidney and airway function (Mayeux, 2004).

1.1 Clara cell 16 kDa protein

Clara cell 16 kDa protein is produced by the epithelial Clara cells – non-ciliated cells that are mainly present in the human respiratory bronchioles (Boers *et al.*, 1999). The name of the protein refers to the production site in the Clara cells and the protein mass of 16 kilo Dalton (kDa), determined by mass spectrometry. In biochemistry and molecular biology, when talking about mass of molecules, the term "Dalton (Da)" is used. There are several different names for CC16 in the literature, common ones of which are "uteroglobin", "urinary protein 1", "Clara cell 10 kDa protein (CC10)" and "Clara cell secretory protein (CCSP)". The production of CC16 is most abundant in the respiratory epithelium, but some also takes place in nasal mucosal epithelial cells (Benson, 2005), the male urogenital tract (Bernard & Hermans, 1997), the endometrium, the foetal lung, the foetal kidney (Hermans & Bernard, 1999), amniotic fluid (Bernard *et al.*, 1994a) and the female urogenital tract (Laing *et al.*, 1998). The highest concentrations of CC16 are found in the broncho-alveolar lavage fluid (BALF) (Yoneda, 1978); lower concentrations of CC16 are found in the prostate, the endometrium, and the kidney (Hermans & Bernard, 1999). Clara cell 16 kDa protein has been used as a biomarker in several studies of acute exposure to air pollutants, as described in a review by Lakind *et al.* (2007).

1.2 Alpha 1-microglobulin

The small lipocalin protein alpha 1-microglobulin, 27 kDa, is more often called "human complex-forming glycoprotein (protein HC)". Alpha 1-microglobulin is thought to act in an immunosuppressive way but the exact function in humans is still unknown. Its main production site is the liver. In blood, A1M is found in both free and bound form. Where it is bound, it is bound to immunoglobulin A (IgA) (Akerstrom *et al.*, 2000). As described by Penders & Delanghe (2004), A1M has been used in several studies of patients with renal diseases as a biomarker of tubular dysfunction. Due to its small size, A1M is filtrated by glomeruli and about 99% is reabsorbed by proximal tubuli and catabolized. Normally, only small amounts of A1M are excreted in urine. The proximal tubular function can be examined by determination of A1M and other low-molecular-weight proteins (LMWPs) in urine since increased excretion is a result of compromised reabsorption in proximal tubules.

1.3 The respiratory epithelium

The essential exchange of oxygen and carbon dioxide between pulmonary capillary blood and atmospheric air is carried out in the lungs (Figure 1). The exchange occurs across the alveolar-capillary membrane. Air enters the body and passes through the larynx and trachea. The trachea is divided into two main bronchi, giving rise to the lobar and segmental bronchi, all with a ciliated epithelial lining. The bronchioli and the respiratory bronchioli are the smaller airways between the bronchi and the alveoli. Here, the epithelium contains ciliated cells together with goblet cells that secrete mucus and, among other substances, also CC16. The alveoli are lined by an epithelial layer made up of two cell types, alveolar type I and alveolar type II cells (Kryger, 1981).

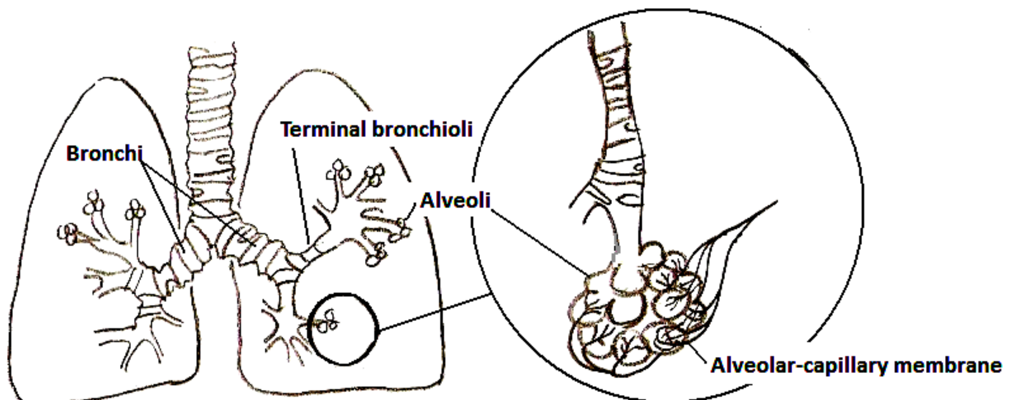

Figure 1. *The respiratory tract with the alveolar-capillary membrane. The amount of CC16 that leaks into serum depends on the permeability of the respiratory epithelium.*

The first defence to inhaled pulmonary irritants lies within the function of the pulmonary epithelial barrier which is more than a mechanical barrier, also being part of the immune system. Inhaled toxic irritants can cause cell damage in the respiratory epithelium as well as to the immune process of eliminating irritants from the respiratory tract. It is not fully understood whether this cell damage is faster than the repair of cells (Plopper *et al.,* 1980; Diamond *et al.*, 2000).

1.3.1 The function of Clara cell 16 kDa protein

Increased permeability of a damaged respiratory epithelium is thought to increase the amounts of CC16 in serum. Clara cell 16 kDa protein has been thought to be able to diffuse through the air-blood barrier of the lung epithelium because of its small size (Bernard *et al.*, 1997a). However, this theory is questioned since different studies imply that CC16 has a role in the inflammatory process and therefore inhaled irritants may up-regulate CC16. Both anti-inflammatory and immunomodulatory qualities have been attributed to CC16 (Jorens *et al.*, 1995; Shijubo *et al.*, 2003) since it has inhibited phospholipase-A2 (Levin *et al.,* 1986) and the cytokine production in T-cells in in vitro experiments (Hung *et al.*, 2004). Furthermore, CC16 may be able to handle oxidative stress in the respiratory tract, and has been reported to have anti-tumour qualities (Broeckaert & Bernard, 2000). The human CC16 gene has been related to asthma in different studies. Proteins, such as CC16, that have survived evolution (i.e. that are found in many species) are thought to be important for survival (Hashimoto *et al.,* 1996; Candelaria *et al.*, 2005). Knockout mice are often used to explain the role of a specific protein, and animals deficient in CC16 are less well equipped to repair damages due to oxidative stress (Mango *et al.,* 1998; Johnston *et al.*, 1999). Furthermore, when allergic inflammation was induced to both wild-type control mice and CC16-deficient mice, the inflammation and production of T-cell cytokines were increased in CC16-deficient mice (Chen *et al.*, 2001).

1.3.2 Serum Clara cell 16 kDa protein as an airway biomarker

Serum CC16 can increase for several reasons including increased leakage across the pulmonary epithelium due to increased epithelial permeability, up-regulated CC16 due to anti-inflammatory qualifications, and decreased renal clearance. Arsalane *et al.* (2000) induced lipopolysaccaride (LPS) in rodents and found increased serum CC16 concentrations without any increased production of CC16, which supports the theory of increased leakage of the pulmonary epithelium. However, the increased serum CC16 concentrations were reversible and had returned to normal levels 24 hours after exposure. It is debated whether LPS cause epithelial cell damage (Meyrick & Brigham, 1986; Meyrick *et al.*, 1986; Johnston *et al.* 1999). In one study, acute exposure to fire smoke has been reported to increase serum CC16 concentrations; however, these were back to normal after 1 week (Bernard *et al.,* 1997b). Studies of ozone exposure have reported increased serum CC16 concentrations (Broeckaert *et al.*, 2000; Blomberg *et al.* 2003) while others have reported unchanged serum CC16 concentrations (Bernard *et al.*, 2005). No associations between lung function and serum CC16 concentrations were found after exposure to ozone (Blomberg *et al.*, 2003) or after exposure to fire smoke (Bernard *et al.*, 1997b).

Broeckaert *et al.* (2000) exposed mice to ozone, and report an increase in serum CC16 concentrations as well as albumin in BALF. Another study investigating the effect of tobacco smoke found increased serum CC16 concentrations at acute exposure (Van Miert *et al.*, 2005). Long-term exposure to tobacco smoke has been found to decrease serum CC16 by approximately 30%. This is explained by the reduction in CC16-producing cells (Shijubo *et al.*, 1997; Robin *et al.,* 2002). In mice, CC16 concentrations in serum increased after acute exposure to high concentrations of trichloroamine, but not to low concentrations (Carbonnelle *et al.*, 2002). In children, acute exposure to chlorine gas increased serum CC16 concentration and led to respiratory distress and reduced lung function (Bonetto *et al.*, 2006). After administration of chemicals toxic to Clara cells, Hermans *et al.* (1999) found increased serum CC16 concentrations, decreased BALF CC16 concentrations and increased BALF albumin concentrations in rats. Exercise can increase the renal excretion of CC16 and thereby possibly decrease serum CC16; furthermore, exercise could possibly also increase the respiratory epithelial permeability and then also increase serum CC16 (Nielsen, 2003).

1.3.3 Urinary Clara cell 16 kDa protein as an airway biomarker

Both serum and urinary CC16 can be measured using enzyme-linked immunosorbent assay (ELISA) techniques and are therefore accessible as biomarkers. The use of urinary CC16 is non-invasive and can be self-administered. Changes in CC16 after different types of exposures have been measured in both BALF and serum in several studies, with different results as mentioned above, and in one study even in urine (Timonen *et al.*, 2004). In their study, Timonen *et al.* (2004) found an association between increases in urinary CC16 and increases in daily ambient concentrations of ultrafine and fine particles. The participating patients had coronary artery disease. Ambient particulate air pollution has been associated with adverse health effects and has been shown to increase mortality and morbidity in pulmonary and cardiovascular disease. Inflammatory processes are suspected to play a key role in the pathomechanisms leading from deposition of particles to exacerbation of diseases. The study by Timonen *et al.* (2004) examined 1,352 urinary samples and this type of screening is possible with the use of urinary samples. Still, in performing such a study it is valuable to investigate how urinary CC16 is excreted and whether it is possible to control other influencing factors in order to further use this protein as a urinary marker for airway exposure.

1.4 The renal system

1.4.1 Primary urine

Urine mostly contains water, dissolved salts, and metabolic waste products. Water-soluble foreign substances are filtered in the kidney and eliminated to the urine in order to protect the body from harmful substances. Therefore, many hydrophobic compounds become water-soluble by different processes in the liver.

Urine production involves several steps. First, the blood enters the nephron, and large numbers of blood vessels, where it is filtered and primary urine is formed (Greger & Windhorst, 1996). The primary urine is gathered in the glomerulus that is connected to the tubule system where the reabsorption of water, essential amino acids and vitamins takes

place (Figure 2). The approximate cut-off limit of glomerular filtrate of serum proteins is 60 kDa (Christensen & Gburek, 2004). The magnitude of glomerular filtration rate with normal kidneys is 180 L/24 hours. Renal filtration is a way for the body to control homeostasis, i.e. regulate the balance of salt, pH and humidity through reabsorption or elimination (Greger & Windhorst, 1996).

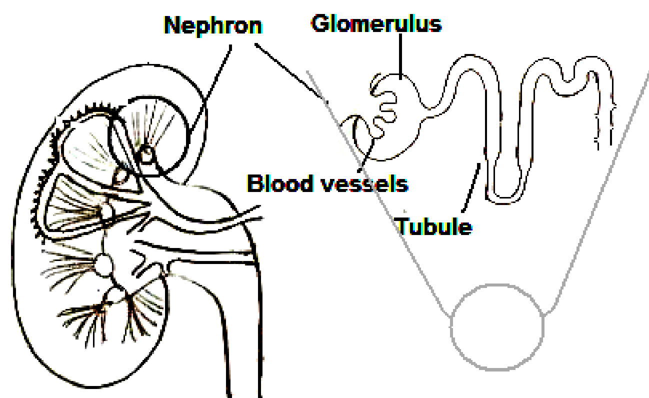

Figure 2. *The nephron where primary urine is formed, and water, important amino acids and vitamins are reabsorbed.*

1.4.2 Reabsorption

The proximal tubule is the most vulnerable segment of the nephron. The solute transport process requires a lot of oxygen. According to Maack *et al.* (1979), the proximal tubules reabsorb 90–95% of all filtered albumin and at least 99% of filtered LMWP. Increased urinary excretion of LMWP <40 kDa occurs when the proximal tubule has diminished capacity to reabsorb and catabolize proteins. Long-term exposure to cadmium or lead may cause tubular kidney damage and A1M is a known marker of early renal impairment after exposure to cadmium or lead (Friberg, 1985; Alfven *et al.*, 2002; Penders & Delanghe, 2004).

1.4.3 Proteinuria

High excretion of proteins is called "proteinuria" and can be divided into two categories, glomerular and tubular proteinuria, with different physiological properties. When reabsorption of proteins in the proximal tubule by receptor-mediated endocytosis is disrupted, proteinuria results. Furthermore, disruption of the glomerular filtration barrier can also lead to proteinuria, with proteins (>40 kDa) found in the glomerular ultrafiltrate (Bakoush *et al.*, 2001). Some of these high-molecular-weight proteins (HMWPs) may damage the proximal tubules and thus increase the renal damage (Eddy *et al.*, 1988; Remuzzi & Bertani, 1998).

1.4.4 Tubular receptors

Two important receptors of the proximal tubule are megalin and cubilin. Alpha 1-microglobulin is reabsorbed by the megalin receptor (Christensen & Gburek, 2004) while CC16 is thought to be reabsorbed by a complex of the cubilin and megalin receptors (Burmeister *et al.*, 2001). These receptors are also found in the respiratory epithelium in alveolar type II cells, the male reproductive tract, intestine, uterus, oviduct and eye (Fisher & Howie, 2006). In the lysosomes, reabsorbed protein is degraded while the receptors return to the cell membrane by receptor recycling. This allows the body to reabsorb vitamins and essential amino acids and, most of all, to clear primary urine of proteins.

1.4.5 Tubular renal biomarkers

Both A1M and CC16 have been used in studies of tubular function and A1M is considered a sensitive biomarker for decreased tubular function. In the field of environmental medicine, interest in biomarkers for early detection of nephrotoxicity related to cadmium exposure is high, and both A1M and CC16 have been used frequently (Penders & Delanghe, 2004). Groups exposed to cadmium had higher urinary A1M excretion and with A1M it was possible to classify workers with a body burden of >15 µg cadmium/g creatinine, which was superior to the other markers in the study. The association between cadmium exposure and the markers A1M and the renal lysosomal enzyme *N*-acetyl-β-d-glucosaminidase (NAG) was stronger than the associations with other common renal markers such as retinol-binding protein (RBP) and beta 2-microglobulin (B2M) (Jung *et al.*, 1993; Fels *et al.*, 1994). Despite the intruding factors in conjunction with urinary CC16 when excreted by men, several studies have shown increased urinary CC16 in cadmium-exposed workers, mostly men (Bernard *et al.*, 1987; Nortier *et al.*, 1997; Vaidya *et al.*, 1997; Prozialeck *et al.*, 2007). Other exposure studies of heavy metals like lead and mercury have shown associations between high blood values of metals and increased urinary CC16 (Bernard *et al.*, 1995; Fels *et al.*, 1998). Furthermore, diabetes patients have been screened with both A1M and CC16 and both biomarkers have been useful for predicting diabetes (Ayatse & Kwan, 1993; Bernard *et al.*, 1994b). Kabanda *et al.*, (1995) evaluated the use of CC16 in Chinese herbs nephropathy and found it to be of the same relevance as RBP for this condition. In a study of human immunodeficiency virus (HIV)-infected patients, 80% had increased urinary albumin, RBP and CC16 and it was suggested that RBP and CC16 were more sensitive for renal impairment (Kabanda *et al.*, 1996). Alpha 1-microglobulin has been a useful biomarker after exposure to carcinogens like dinitrotoluene directed to the tubular system (Bruning *et al.*, 2002), when screening for Balkan nephropathy (Cvoriscec, 2000) and idiopathic membranous nephropathy (Bazzi *et al.*, 2001). In different studies of urological disorders such as vesico-ureteral reflux, outflow disease of the upper tract, and partial ureteral obstruction, A1M has been shown to be a useful diagnostic tool. Everaert *et al.* (1998a; 1998b) found A1M useful for distinguishing between subjects with neurogenic bladder disease and pyelonephritis, and linked the increase in A1M to a decrease in uptake of dimercaptosuccinic acid (DMSA). Alpha 1-microglobulin excretion was elevated in patients with acute pyelonephritis compared with patients with cystitis.

Since DMSA scintigraphy involves radiation, alternative methods (e.g. sensitive biomarkers) that can distinguish between upper and lower urinary tract infection (UTI) would be valuable. This thesis investigates CC16, which has not previously been used for this purpose.

1.5 Correction with creatinine

When biomarkers in urine are used, the urine flow or extent of dilution must be adjusted for. There are different ways to do this, such as expressing the excretion rate in mass per unit of time, e.g. mass/24 hours, and correcting the excreted mass with the specific gravity of each sample or with the excreted mass of creatinine. Each urine sample is unique, depending on circadian rhythm, and the influence of urine flow on clearance may become evident. In a 24-hour sample, on the other hand, daily fluctuations are evened out and this sampling would therefore be more suitable than a spot sample. Nonetheless, a biomarker is more useful if sampling is convenient, self-administered and easy to handle. Therefore, correction with the excreted mass of creatinine or specific gravity is frequently used, even though it is associated with difficulties. Creatinine excretion is influenced by diet, body size, exercise, urine flow, gender, age, and diurnal variations (Boeniger *et al.*, 1993). Creatinine is mainly eliminated by glomerular filtration. Creatinine is a small amine produced in the metabolism in muscle cells and is consequently related to muscle mass. Since creatinine can be affected by urine flow and dilution, it is valuable to examine the effect of correction with creatinine on the variation when expressing the excretion of a protein as mass/mass creatinine. No data were found on how correction with creatinine affects the variability of CC16 and A1M compared with when they are expressed as mass/24 hours.

1.6 Post-renal excretion

There is a difference between genders in CC16 excretion after puberty (Bernard *et al.*, 1993). The male prostate produces CC16 which is washed out with the urine. No previous data have been found on this portion size. Without a wash-out before sampling, urine samples from men would not be useful in studies using urinary CC16 as a biomarker. Therefore, in order to use CC16 as a biomarker in urine, this portion needs to be defined.

1.7 Biomarkers in epidemiology

Biomarkers of all types have been used by generations of epidemiologists, physicians, and scientists studying human disease. There are different types of biomarkers: biomarkers of exposure, which are used in risk prediction, and biomarkers of effect, which are used in screening and diagnosis and monitoring of disease progression. Biomarkers used in risk prediction, in screening, and as diagnostic tests are well established, and offer distinct and obvious advantages (Mayeux, 2004). Biomarkers sensitive to toxic exposure levels are widely usable if they also are assessable, non-invasive, and easy to collect, and can be used at a reasonable analysis cost. The use of biomarkers in epidemiology can facilitate screening of large populations and the benefit of screening is prevention before detection or onset of symptoms.

The optimal biomarker should be simple to handle. All sampling should be as non-invasive as possible, easily self-administered, and uncomplicated. Meeting all these criteria, urinary biomarkers are of great interest. With optimal sampling with regard to creatinine correction, the effects of gender, age, body mass index (BMI), circadian rhythm, temperature, infections, and exercise, a spot urine sample is very useful.

2 Aims of the thesis

The overall aim of this thesis was to optimize urine sampling of Clara cell 16 kDa protein and alpha 1-microglobulin, and test these low-molecular-weight proteins as biomarkers of changes in kidney and airway function.

The more specific aims of this thesis were to address the following questions:

1. How do urine flow, age, gender, and the time of the day affect the excretion of CC16 and A1M?

2. How do various spot urine samples of CC16 and A1M, corrected for creatinine or specific gravity, reflect the 24-hour excretion?

3. Does exposure to wood smoke affect healthy subjects' serum and urinary CC16?

4. Does lower or upper urinary tract infection affect urinary CC16 and A1M excretion?

5. Does pneumonia affect the serum and urinary CC16 levels?

3 Materials and methods

3.1 Study populations

Subjects in ***Paper I*** consisted of two groups of volunteers aged 21–57 years among the staff of the Department of Occupational and Environmental Medicine in Gothenburg and the students of the Sahlgrenska Academy at the University of Gothenburg. All subjects were non-smoking, and had no known diabetes, hypertension, and airway or kidney diseases. Group A consisted of ten male and ten female subjects aged 22–57, mean 35, years, while Group B consisted of 13 female and eleven male subjects aged 21–57 years, mean 37 years. The study was performed between September 2004 and October 2005 and was approved by the Regional Ethical Review Board in Gothenburg. All subjects had given their informed consent.

Subjects in ***Paper II*** were 29 volunteers aged 23–56 years among the staff of the Department of Occupational and Environmental Medicine in Gothenburg and students of the Sahlgrenska Academy at the University of Gothenburg. The 14 female and 15 male subjects were non-smoking, and had no known diabetes, hypertension, and airway or kidney diseases. The study was performed from September to October 2006 and was approved by the Regional Ethical Review Board in Gothenburg. All subjects had given their informed consent.

Subjects in ***Paper III*** were 13 healthy volunteers aged 20–56 years among the staff of the Department of Occupational and Environmental Medicine in Gothenburg. There were six men and seven women. The exposure to filtered and wood smoke-impacted air took place in the year 2005 at the SP Technical Research Institute of Sweden. The study was approved by the Regional Ethical Review Board in Gothenburg. All subjects had given their informed consent.

Subjects in ***Paper IV*** consisted of two groups of infected children, one with UTI and one with other infections. The UTI patient group comprised 52 children <2 years of age, 26 boys (12 days – 1 year old) and 26 girls (1.5 months – 1.9 years old), with first-time UTI. The control group consisted of 23 children, 16 girls and 7 boys (2.4 months – 2.8 years old, median 1.8 years), with a non-UTI infection, negative urine culture and elevated serum C-reactive protein (>20 mg/L). All subjects were recruited between 2004 and 2005. Children with known urigenital or anorectal malformations or neurological diseases were excluded. To enlarge the control group, 14 of the 23 control children were recruited 3 years later, in 2008. To participate in the study, the parents had given their informed consent.

Subjects in ***Paper V*** were from another study, by Stralin *et al.* (2004), performed in 1999–2002, in patients with community-acquired pneumonia (CAP). Subjects who had had both serum and urinary samples taken were selected for our study. A total of 34 female patients with CAP (age range 20–79 years) and 24 female controls with orthopaedic diagnoses (age range 20–79 years) were selected for the study and it was not possible to relate any new results to any specific individual. For this procedure, a renewal of the informed consent was not necessary, according to the Regional Ethical Review Board in Gothenburg.

3.2 Study design

All subjects in ***Paper I*** completed a questionnaire about age, smoking habits, and possible diseases or medication. The evaluation of CC16 excretion in urine was based on urine data on all subjects of both groups. Group A provided all their urine samples during one 24-hour period and gave the first two spot urine samples the next morning (Table 1).

Table 1. *Urine (U) and serum (S) sampling for determination of Clara cell protein in Group A and Group B.*

	Group A			Group B	
	Day 1		Day 2	Day 1	Day 2
	Women	Men			
Morning	U	U†	U	U, S	U, S
Noon	U	U†	U	U, S*	U, S*
Afternoon			U	S	S
Evening			U		
Next Morning				U, S	U, S

**Serum samples taken from 13 of the 24 subjects for the study of serum CC16 diurnal variation. Morning samples were taken about 8 am, noon samples about 2 pm and afternoon samples at about 5 pm.*
†The urine portion was collected as 4 small portions.

For the examination of post-renal excretion, men in group A divided their spot urine samples into four portions (Figure 3). All portions were 50 mL portions, except for the last one which often contained more urine. The CC16 concentration was analysed for each portion. Group B provided three spot urine samples and three serum samples on 2 separate days. These samples were used for comparisons of urinary and serum CC16. In group B, 13 subjects provided one extra serum sample.

***Figure 3**. Cups and bottle for urine samples.*

Also, in ***Paper II***, the participants filled out a questionnaire about age, smoking habits, and possible diseases or medication, and provided spot urine samples at six fixed time points over 24 hours on 2 separate days, Figure 4. In ***Papers I and II***, all samples were analysed fresh and then frozen for stability testing.

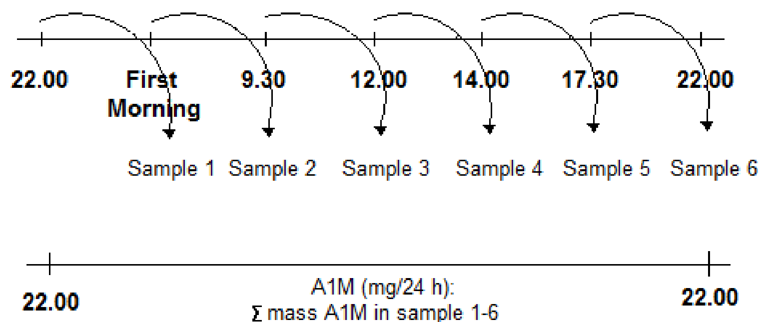

Figure 4. *Each subject provided all their urine for 24-hours as spot samples at fixed time points according to the time line. The excreted amount of alpha 1-microglobulin (A1M) was expressed as mg/h in each spot sample. Additionally, a 24-hour value of total excreted A1M (mg/24 h), was calculated from sample 1-6.*

In ***Paper III***, the subjects were exposed to filtered air for 4 hours on one occasion and then to wood smoke for 4 hours, 1 week later. Both sessions took place in a chamber specially designed for this purpose (Sallsten *et al*., 2006). At both sessions there were two 25-minute periods of light exercise. Blood, urine, and breath samples were taken before and after the exposure sessions, according to a schedule (Barregard *et al*., 2006). The filtered air and wood smoke sessions were identical except for air quality. The experiment was carried out in two sessions (sessions 1 and 2), with seven and six subjects, respectively. During the exposure sessions, subjective symptoms were registered using a self-administered questionnaire.

Subjects in ***Paper IV*** were recruited from patients attending the hospital's emergency ward when the study team were available. All subjects, both UTI patients and controls, had their urine samples analysed for urinary CC16, A1M, RBP, creatinine and CRP. Serum samples were analysed for CRP. A DMSA scintigraphy was performed on all patients.

All samples in ***Paper V*** were anonymized. The serum samples were analysed for CC16 and urine samples were analysed for CC16, A1M, RBP and creatinine. Typically, the samples in the control group were taken between 9 am and 11 am while the samples in the patient group were taken between 11 am and 9 pm. Serum CC16 was corrected for daily variation. The samples from both groups were divided into suitable time intervals. The diurnal variation was approximately calculated after addition of 1 µg/L to the samples taken between 9 am and 11 am, and of 2 µg/L to the samples taken between 11 am and 9 pm.

3.3 Analyses

3.3.1 Sandwich enzyme-linked immunosorbent assay

The sandwich ELISA (Figure 5) measures the amount of a protein present between two layers of antibodies (specific for that protein). The first antibody captures the protein and the second detects the protein. The protein to be measured must contain at least two sites capable of binding to the antibody, since at least two antibodies act in the sandwich. The sample does not have to be purified in order to use a sandwich ELISA, which naturally is an advantage. Sandwich ELISAs are very sensitive compared with assays using only one antibody. It should be noted that antibodies can detect antigens other than proteins, as described in this example.

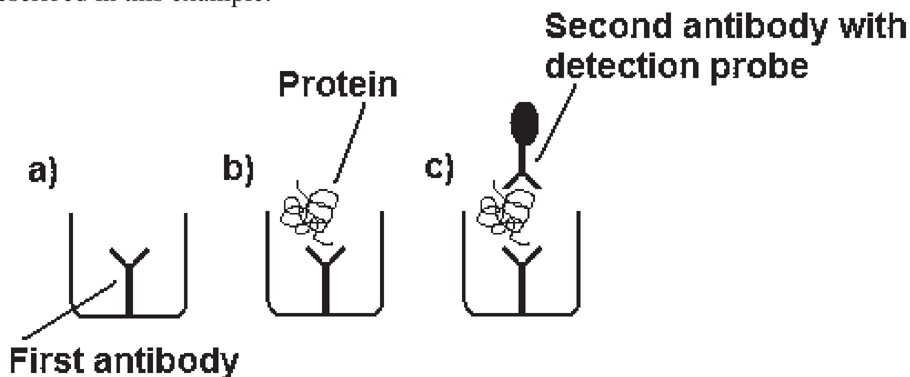

Figure 5. *a) Specific antibodies for the protein of interest are attached to the surface of each well b) The first antibodies bind specifically to the protein of interest in the added sample. All other proteins are washed away before a second antibody is added. c) This second antibody has a detection probe attached to it. When a substrate is added, colour changes are determined by a spectrophotometer.*

3.3.2 Clara cell 16 kDa protein

Analyses of both serum and urinary CC16 were performed using the BVG-RD191022200 Human Clara Cell Protein ELISA 96 test (BioVendor Laboratory Medicine, Inc, Brno, Czech Republic). After 96 μL of dilution buffer were added to each well, 4 μL of each standard and sample were added and incubated for 1 hour in microtiter wells coated with rabbit polyclonal antibodies against human CC16. After washing, biotin-labelled antibodies against CC16 were added, followed by thorough washing and addition of Streptavidin-horseradish-peroxidase. After a 1-hour incubation and a final washing, the conjugate reacted with H_2O_2-tetramethyl-benzidine. The reaction was stopped by adding an acidic solution before absorbance was measured at 450 nm. The absorbance is proportional to the concentration of CC16. Two different calibrators with target values in the ranges 5.0–9.4 μg/L and 15.8–23.9 μg/L were provided in each kit and used at the start and the end of the assay in every run. The results were always within the accepted range. The coefficient of variation (CV) between analyses, as calculated from duplicate analyses of the calibrators, was 11%. For ***Paper V***, an in-house calibrator was also used, and the results were always within the accepted range of 6.5–8.0 μg/L. This ELISA kit has been produced for serum CC16 and in order to use it for urinary CC16, a validation was performed. To examine the linearity of dilution, 163 samples were

analysed with two different dilutions. The samples were diluted over the range of the assay standard curve. Linear regression analysis of samples versus the expected concentration yielded average correlation coefficients of r_s=0.85 for urine. Furthermore, a good correlation was observed between urine samples analysed with ELISA and samples analysed using a latex immunoassay (LIA), r_s=0.95. These 30 samples were compared after analysis at the Université catholique de Louvain, Brussels, Belgium. The LoD was 0.1 µg/L.

3.3.3 Alpha 1-microglobulin

Analyses of A1M were performed using the alpha 1-microglobulin ELISA Kit K 6710 (Immundiagnostik AG, Bensheim, Germany). After washing of the 96-well plate, 100 µL NaCl and 10 µL of standard or pre-diluted sample solutions were incubated for 1 hour. All wells were precoated with rabbit polyclonal antibodies against human A1M. After washing, peroxidase-labelled antibodies against A1M were added. After 1 hour of incubation and a final washing, the conjugate reacted with H_2O_2-tetramethylbenzidine. The reaction was stopped by adding an acidic solution before absorbance was measured at 450 nm. The absorbance is proportional to the concentration of A1M.Calibrators with target values in the range of 0.09–0.28 mg/L, provided in each kit, were used at the start and end of the assay in every run; and the results were always within the accepted range. The CV between analyses, as calculated from duplicate analyses of the calibrators, was 11%. For duplicate analyses of samples performed on four occasions within 6 months (***Paper II***), the median CV was 17 (13–38%). The limit of detection (LoD) was 0.1 mg/L.

3.3.4 Retinol-binding protein

Analyses of RBP were performed using the RBP4 ELISA Kit K 6110 (Immundiagnostik AG, Bensheim, Germany). Samples were prediluted 1:10 in the dilution buffer provided. Thereafter, 100 µL of samples and standard were added to each prewashed well and incubated for 1 hour. All wells were precoated with rabbit polyclonal antibodies against human RBP. After washing, peroxidase-labelled antibodies against RBP were added. After 1 hour of incubation and a final washing, the conjugate reacted with H_2O_2-tetramethylbenzidine. The reaction was stopped by adding an acidic solution before absorbance was measured at 450 nm. The absorbance is proportional to the concentration of RBP. A calibrator with target value in the range 1.3–4.5 µg/L, provided in each kit, was used at the start and end of the assay in every run and the results were always within the accepted range. The CV between analyses, as calculated from duplicate analyses of the calibrators, was 12%. The LoD was 0.1 µg/L.

All samples were normally diluted, according to the manual of each kit. Repeated analyses of samples of concentrations below or above the standard curve were performed with a different dilution.

3.3.5 Other analyses

In ***Paper IV***, urinary CRP was determined by a commercial ELISA from Immundiagnostik (K 9710s; Immundiagnostik AG, Bensheim, Germany). These analyses were performed at the Department of Clinical Immunology, at Sahlgrenska Academy, University of Gothenburg. Serum CRP was determined by a nephelometric method

(NycoCard; Axis-Shield PoC AS, Oslo, Norway) at the Department of Clinical Chemistry, at the Sahlgrenska University Hospital and Academy, Gothenburg. Urinary creatinine was determined with an enzymatic method (CREAplus R1, R2, Roche/Hitachi, Roche Diagnostics, GmbH, Mannheim, Germany) at the Department of Clinical Chemistry at the Sahlgrenska University Hospital and Academy, Gothenburg.
For further information about the analysis of nitric oxide and malodialdehyde, see ***Paper III***.

3.4 Statistical methods

In general, data were not normally distributed except for serum CC16. The tests performed were generally two-sided (p-values <0.05 were considered statistically significant).
Values below the LoD were replaced by the value of the LoD divided by the square root of 2 (Hornung and Reed 1990). Statistical calculations were performed using SAS for Windows, version 9.1 (SAS Statistical Software, SAS Institute, Cary, NC, USA).

In ***Papers I and II***, the CV was used as a measure of the within-individual variation. For CC16 and A1M, the first morning urine (excretion rates and creatinine-corrected concentrations) was used and for serum CC16, morning samples were used on different days in each subject. The total variability was partitioned within and between subjects in serum CC16 and log-transformed urinary CC16. Associations between the 24-hour excretion and the excretion rates, creatinine and specific weight-corrected concentrations were estimated by linear regression and Pearson's correlation coefficient (r_p) for both urinary CC16 and A1M. Comparison of matched serum and urinary CC16 samples was made by the same technique. Further, the difference between gender and sampling time for A1M was tested with *t*-tests on log-transformed data. The urine flow rate effect on A1M excretion was tested using Wilcoxon's signed-rank test on Pearson's correlation coefficients. To test the effects of time, flow rate, body weight and gender on A1M excretion, a mixed-effects model was used.
In ***Paper III***, within-individual differences in urinary CC16 before and after the different sessions were tested using Wilcoxon's signed-rank tests. Associations between biomarkers were assessed using Spearman's rank correlation coefficient (r_s). Changes in serum CC16 during wood smoke exposure were compared with changes in serum CC16 during the clean air session using a mixed-effects model. Separate analyses were performed at each time of sampling (directly after exposure, 3 hours after exposure, and in the morning of the next day).

In ***Papers IV and V***, Kruskal-Wallis and Wilcoxon's signed-rank test were used for group comparisons. Associations between biomarkers and age were assessed using the Spearman rank correlation coefficient. Also, in ***Paper IV***, stepwise logistic regression was used for evaluation of the effect of temperature, urinary CC16, urinary RBP, urinary A1M and serum CRP on the DMSA classes.

4. Results

4.1 Papers I and II

4.1.1 Post-renal excretion

The first portion (0–50 mL) was 3–200 times higher in CC16 concentration compared with the other portions (Figure 6). Statistically, there was a significant difference in CC16 concentration between the second (50–100 mL) and third portion (100–150 mL) but not between the third and fourth portions. This led us to conclude that at least the first 100 mL of urine must be discarded before the CC16 originating from the prostate could be considered eliminated or at least satisfactorily diminished.

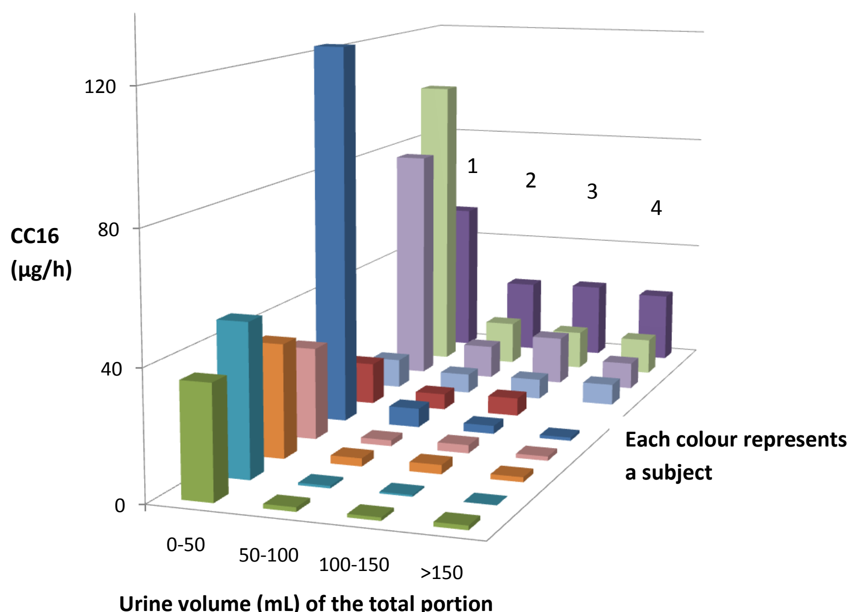

Figure 6. *The bars represents the CC16 excretion for 10 subjects, who provided four urine portions, 0-50 mL, 50-100 mL, 100-150 mL and >150 mL. Each colour represents a subject.*

4.1.2 Gender and diurnal variation

Even after the first 100 mL was discarded from each male urine sample, there was a difference in CC16 excretion between men and women. The difference was statistically significant, with men excreting a median of more than six times as much CC16 (µg/24 hours) compared with women. There was a difference also with A1M, with men in median excreting 1.9 times more A1M (mg/24 hours) than women. For both CC16 and A1M, a diurnal difference was observed (Figure 7). There was an increase in excretion of both CC16 and A1M in the middle of the day compared with the morning samples. The difference in CC16 excretion between the first morning and the evening samples was statistically significant, with low CC16 excretion in the morning sample compared with the day and evening samples. This was also true for the A1M, where excretion in the first morning sample was statistically significantly lower than during the rest of the day.

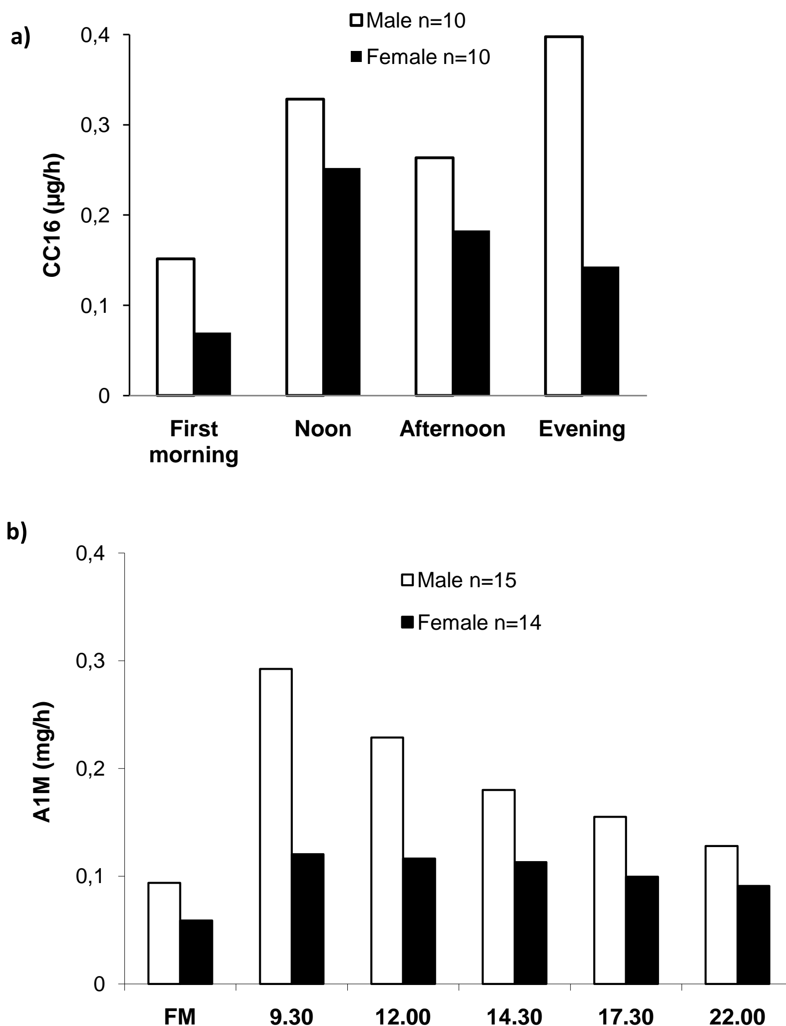

Figure 7. a) Mean values of Clara cell 16 kilo Dalton protein (CC16) excretion in female and male subjects at different times of the day. b) Mean values of alpha 1-microglobulin (A1M) excretion in female and male subjects at different times of the day.FM = first morning urine

4.1.3 Urine flow

Alpha 1-microglobulin is affected by urine flow, especially in men. The associations at the six different measuring occasions of the day tended to be positive. The same calculations were not made for CC16, but they were made for creatinine, and the associations at different measuring occasions of the day tended to be positive (data not published).

4.1.4 Best sampling time

For men, the best association between the total 24-hour CC16 excretion and the separate excretion rates was observed in first morning samples, $r_p>0.91$ ($r_s>0.89$). This was also true for A1M ($r_p>0.75$; $r_s>0.69$). All associations were statistically significant except the association for the afternoon samples for CC16, and the 10 pm sample for A1M. For women, all associations between the total 24-hour excretion and the separate excretion rates were statistically significant, with $r_p>0.75$ ($r_s>0.57$) for both CC16 and A1M. Associations between the total 24-hour CC16 excretion and creatinine or specific gravity-corrected CC16 concentrations were best in first morning samples for both men and women ($r_p>0.85$; $r_s>0.80$). The associations between 24-hour A1M excretion and creatinine or specific gravity-corrected A1M concentrations were all statistically significant in men ($r_s=0.60$). In women, the associations were lower ($r_s>0.35$). For both CC16 and A1M, the correlation coefficients for creatinin-corrected samples were better than those corrected for specific gravity when both methods were compared with the total 24-hour excretion.

4.1.5 Stability

The results of ***Papers I and II*** show that both A1M and CC16 can be stored for 6 months at -20°C, without loss of concentration. Further calculations have been performed and both proteins are stable for 2 years at -20°C, without loss of concentration (data not published).

4.1.6 Variability

The variability in CC16 and A1M excretions is high between individuals compared with that within individuals. The total variability for CC16 within healthy individuals was 10% and 90% between individuals; for A1M, the figures were 20% and 80%, respectively. Variability within individuals, expressed as relative standard deviation (RSD), was 63% between days for urinary CC16. For serum CC16, this figure was 17%. Calculated for each gender, the variability within individuals in urinary CC16 decreased to 59% for men and 42% for women. The corresponding figures for A1M were 54% for men and 32% for women.

4.2 Paper III

The participants reported mild irritation to the eyes after wood smoke exposure. Several biomarkers showed signs of diurnal variation and significant differences in levels before and after exposure to wood smoke, as presented in Table 2. Nitric oxide increased in exhaled air and at alveolar level, 3 hours after exposure. Serum CC16 increased 20 hours after exposure. There was a tendency towards an increase in urinary CC16, 20 hours after exposure; however, this was not significant ($p=0.08$). Further, in breath condensate, malondialdehyde increased just after exposure, 3 hours post-exposure and at 20 hours post-exposure but was less pronounced 3 hours post-exposure.

The exposure of wood smoke was not comparable in the two different sessions, and when calculations were performed separately for each session, the increase in serum CC16 was not significant in round 2. Serum CC16 was positively associated with plasma fibrinogen on all occasions and also between the morning excretion of urinary CC16 and plasma fibrinogen.

Table 2. *Biomarkers of effects on airways before and after exposure to clean air and wood smoke, respectively; results based on 13 subjects for exhaled breath, breath condensate, and blood sample, and 10 subjects for urinary samples.*

	Before	**After clean air**			**Before**	**After wood smoke**		
Sample No.	1	2	3	4	1	2	3	4
Exhaled air:								
Nitric oxide (ppb)	1.2 (0.47-2.8)	1.8 (0.54-4.7)	1.1 (0.33-2.0)	2.3 (0.66-4.1)	1.1 (0.13-2.9)	1.7 (0.44-3.3)	**1.8**[a)] (0.73-2.5)	1.1 (-0.21-3.1)
Breath condensate:								
Malodialdehyde (μmol/L)	0.012 (0.003-0.029)	0.003 (0.003-0.010)	0.003 (0.003-0.025)	0.003 (0.003-0.021)	0.003 (0.003-0.011)	**0.003**[a)] (0.003-0.026)	0.005 (0.003-0.11)	**0.007**[b)] (0.003-0.03)
Serum and urine:								
Serum CC16 (μg/L)	7.4 (2.0-12)	5.9 (2.2-9.5)	6.5 (2.2-10.3)	7.1 (3.7-14.5)	6.8 (4.2-13.4)	5.7 (2.7-10.3)	5.8 (2.9-11.2)	**8.1**[b)] (3.8-13.1)
Urine CC16 (μg/h)	0.02 (0.00-1.3)	0.08 (0.01-3.4)		0.03 (0.00-2.0)	0.01 (0.00-1.2)	0.07 (0.00-1.6)		0.05 (0.00-2.5)

a) Significant increase of the 0.05 level and b) at the 0.005 level.
CC16, Clara cell 16 kDa protein

4.3 Paper IV

In 36 out of 52 patients, DMSA scintigraphy was abnormal on account of decreased uptake. Associations between serum CRP and the other urinary proteins and temperature were positive and statistically significant. Urinary CRP and CC16 were positively associated with all other proteins, but not with temperature.

Compared with the control children, urinary CRP was significantly higher in children with UTI; however, there was no association with DMSA class.

In children with UTI, both urinary RBP and CC16 (Figure 8) were significantly higher compared with the control children, and were also positively associated with DMSA class, i.e. renal involvement. This was also true for serum CRP.

The influence of temperature on the degree of renal involvement according to DMSA class was examined using a stepwise logistic regression model where urinary CC16 and serum CRP were shown to be predictors of DMSA class without the influence of temperature.

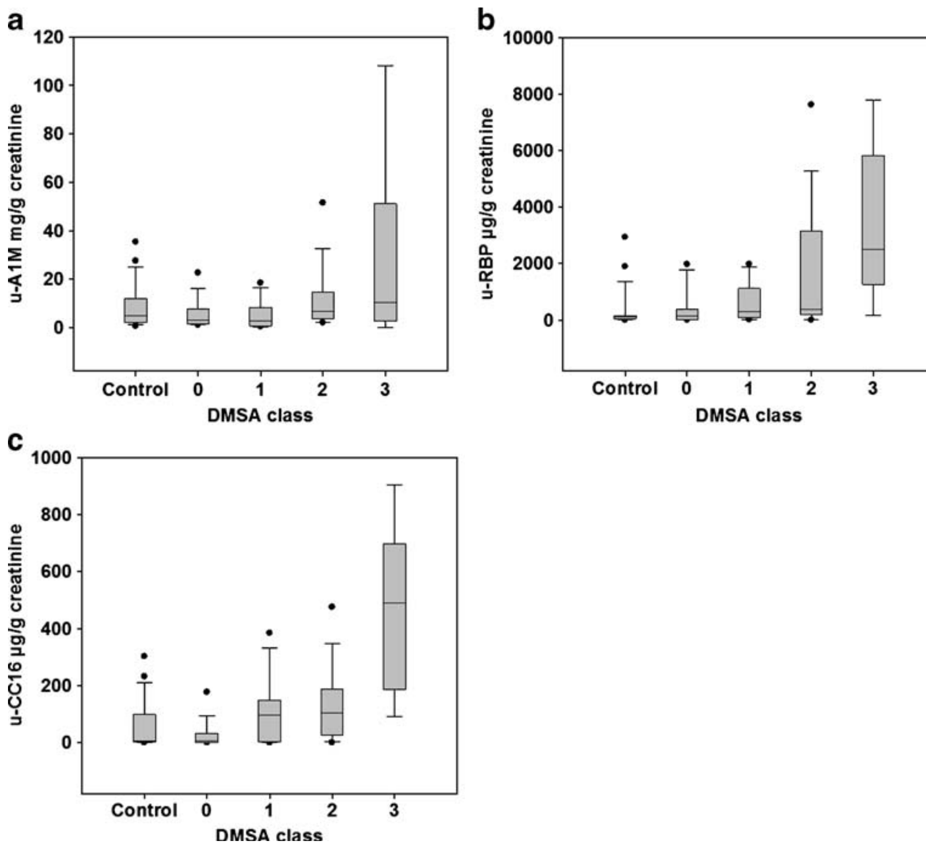

Figure 8. *Levels of alpha 1-microglobulin (u-A1M) (a), retinol-binding protein (u-RBP) (b) and Clara cell protein (u-CC16) (c) in patients with acute UTI, categorized into dimercaptosuccinic acid (DMSA) scintigraphy classes 0–3, and in controls. Median values and 10, 25, 75 and 90 percentiles are given. DMSA classes: 0 normal kidney function, 1 uptake abnormal with relative function ≥45%, 2 relative function 40–44% irrespective of uptake, 3 relative function <40% irrespective of uptake.*

4.4 Paper V

In patients with CAP, urinary CC16 was significantly increased compared with the control group. However, serum CC16 was statistically lower compared with the control group. Within the patient group, subjects with asthma, chronic obstructive pulmonary disease (COPD) or smoking habits had a tendency of lower serum CC16 ($p=0.1$), while subjects without asthma, COPD or smoking habits had similar median serum CC16 concentrations as the control group. This was true also when data were adjusted for age. After serum CC16 concentrations were corrected for time differences, CAP patients still had lower serum CC16 concentrations than the control group. The increase in urinary CC16 was not related to any increased permeability of the respiratory epithelium from CAP; rather, it was caused by fever-related proteinuria. The CAP patients had high urinary excretion of LMWPs compared with the control group. Positive associations between serum CC16 and urinary CC16 were found in both groups.

5 Discussion

This thesis reports both the potential use of urinary CC16 as a marker for environmental effects on the respiratory epithelium and the use of CC16 and A1M as markers for disturbed reabsorption in renal proximal tubules.

5.1 Optimal sampling

Firstly, regarding the use of any of these LMWPs in larger studies, the possibility to use only spot urine samples was tested.

5.1.1 Urine flow, gender and diurnal variation

We concluded that timed first morning urine samples or creatinine-corrected first morning spot samples would be most suitable for both urinary CC16 and A1M. The low excretion of CC16 and A1M in healthy women was noticeable. For female subjects, many of the CC16 values were below the LoD. This made the analysis of the associations between corrected spot samples and the 24-hour excretion more difficult. However, there was no difference in serum CC16 between genders (Shijubo *et al.*, 1997). Therefore, the most likely explanation for the difference in CC16 excretion between genders is the influence of remaining post-renal excretion. In subjects with normal circadian rhythm, first morning urine is less affected by urine flow and daily activities. With the increase in urine flow over the day, reabsorption decreases and consequently there is an increase in excretion of these LMWPs. While creatinine correction can greatly reduce variability in results from random urine specimens, it is not perfect. Urinary creatinine excretion rates can vary throughout the day depending on urine flow. Subjects who have drunk large amounts of liquids will tend to excrete urine with a low creatinine concentration (<0.5 g/L) while the urine of subjects who have been exercised without liquid replenishment will have a high creatinine concentration (>3 g/L). It is important to know that very low or high urine flow may contribute to misleading values when protein excretion is expressed as mass excreted protein per mass creatinine.

5.1.2 Post-renal excretion, and stability

This thesis reports that for urinary CC16 measurements, men should discard the first 100 mL of urine to wash out the post-renal excretion before collection. Another important factor is stability. The stability of A1M in frozen urine without preservatives is satisfactory for use within 2 years; CC16 is stable even for longer periods.

5.1.3 Variability

The variability of a biomarker has to be considered and the variability between individuals is large both for CC16 and A1M. To make group comparisons, large populations are needed. Variability within individuals is usually related to exposures or conditions unique to the individual and, to a smaller extent, to laboratory errors. For urinary CC16 and A1M, analysis of duplicate samples was considered satisfactory overall. However, the CV reported in Paper II will increase the within-individual variability for A1M. In the experimental study of ***Paper III***, the low within-individual variability is beneficial. Consideration of the sources of variability in the measurement of a biomarker decreases the potential for misclassification of the exposure (Mayeux, 2004). Variability between individuals in urinary CC16 can be problematic in cross-

sectional studies when urinary CC16 is used as a biomarker of effects on the respiratory epithelium.

5.2 Urinary Clara cell 16 kDa protein as a respiratory biomarker

With optimal urine sampling of CC16, urinary CC16 could probably be used as an experimental biomarker of the respiratory epithelium. This was tested in ***Paper III*** with exposure to wood smoke. In contrast, urinary CC16 was not appropriate as a biomarker for patients with pneumonia according to the results presented in ***Paper V***. Furthermore, decreased renal tubular function can increase urinary CC16, and it would be preferable to measure a tubular-specific biomarker, in combination with urinary CC16. The association between first morning serum and urinary CC16 samples, r_p=0.76, found in Figure 5 of ***Paper I***, supports the possibility to use urinary CC16 as a respiratory marker.

5.2.1 Urinary Clara cell 16 kDa protein and wood smoke exposure

When 13 subjects were exposed to wood smoke, serum CC16 increased after 20 hours and there was a tendency towards increased urinary CC16 (although only 10 of the 13 subjects managed to provide their urine samples). Timed urine samples were used and therefore any bias from creatinine correction was avoided. During the wood smoke exposures in ***Paper III***, the subjects performed exercise during both sessions, fresh air and wood smoke, and even though exercise has been shown to increase serum CC16, the increase in serum CC16 was most likely caused by acute exposure to wood smoke. There was a difference in particulate matter between rounds 1 and 2, with higher particulate matter in round 1, which also gave increased serum CC16. As also reported by Sallsten *et al.* (2006), the wood smoke in the chamber contained carbon monoxide, particulate matter (inorganic ash material, condensable organic compounds, and carbon-containing particles) and a wide range of gaseous organic compounds. Gustafson (2009) also report findings of formaldehyde, acetaldehyde, benzene, 1,3-butadiene and polycyclic aromatic hydrocarbons (PAHs) as significant constituents in wood smoke emissions. Boutin *et al* (1998) found increased CC16 in broncho-alveolar lavage (BAL), serum and urine in rats instilled with benzo[a]pyrene. Therefore it is not unlikely that wood smoke induces inflammation of the respiratory epithelium and thereby causes either an upgrade in the anti-inflammatory protein CC16 or damage to the epithelium, increasing permeability and leakage of CC16 to serum.

This thesis did not aim to establish the physiological role of CC16 in the airways. With optimal sampling urinary CC16 may be an interesting alternative to serum CC16 in exposure studies of large populations.

5.2.2 Urinary Clara cell 16 kDa protein and pneumonia

The hypothesis that patients with pneumonia would have elevated serum CC16 was not confirmed by ***Paper V***. Instead, urinary CC16 was increased in patients with CAP. Fever is known to cause proteinuria and this was most likely the reason for the high excretions of urinary CC16 and the other two LMWPs, A1M and RBP. The significantly lower serum CC16 in CAP patients could be due to various factors. Increased excretion of LMWPs may lead to decreased CC16 due to an increased renal clearance. Furthermore, among CAP patients many subjects (n=22) with CAP had conditions (smoking, asthma and COPD) that are associated with lower serum CC16. Clearly, the possible damage that CAP could cause did, however, not

exceed the effects from the other factors. Sampling was not optimal due to daily variations and serum CC16 was corrected for different time of sampling in order to minimize the influence from daily variations in serum CC16. However, only small differences were obtained by these calculations and daily variations cannot explain the difference in serum CC16 between the two groups.

5.3 Clara cell 16 kDa protein and alpha 1-microglobulin as renal markers

This thesis gives additional information about the renal handling of both CC16 and A1M, valuable for clinical and epidemiological studies. As a tubular marker, A1M is already widely used both in occupational and in environmental medicine as well as in screening of diabetes patients (1.4.5). The knowledge gained in this thesis relates to the difference in excretion between genders, as well as daily variations, best time of day for sampling, and the influence of urine flow. Urinary CC16 seems to be a sensitive biomarker for tubular function, equal to RBP and A1M, as reported in ***Papers IV and V***. This was also suggested in Tomlinson *et al.* (1990), a study investigating these three proteins and B2M in urine samples from children.

5.3.1 New prospects for urinary Clara cell 16 kDa protein as a biomarker

With support of the findings of ***Paper IV***, further evaluations of the combined use of urinary CC16, RBP and CRP in small children with different infections would be interesting. The search for new, less invasive methods that can distinguish between upper and lower UTIs is of current interest. This could allow screening on a large scale, which is impossible with radiological techniques (DMSA). Urinary tract infection in small children (<2 years of age) is often hard to detect since the specific symptomatology is often absent.
In studies with sufficient patient material, it may be possible to do cut-off calculations for these biomarkers. It may not be recommended to adjust these urine samples with creatinine due to developmental growth of muscle mass and the associated increase in creatinine formation. Pearson *et al.* (2009) observed a diurnal variation. These authors report that creatinine excretion was related to age in children of 2–11 years, and recommend correcting for dilution with specific gravity instead.

5.4 Validity

The group sizes in this study were generally small and larger groups would increase the validity. Therefore, some of these findings require follow-up studies to further support them. The healthy subjects in ***Papers I–III*** represent volunteers among employees and students at the University of Gothenburg and it may be questioned whether they represent the general population. No differences were seen between these 86 healthy subjects and 43 healthy children of another study by Tomlinson *et al.* (1990), regarding mean creatinine-corrected values of the measured proteins. Furthermore, the values from the analysis of healthy subjects in ***Papers I–III*** are within the given range, according to the ELISA kit manuals. This indicates that subjects in these papers have values similar to those of the general population. For urinary CC16, no normal values are given, since the commercial kit used in this thesis is intended for serum CC16 tests.

In ***Papers I–III***, the self-administered sampling may increase variation both within and between individuals due to the influence of diurnal variation on urinary CC16. Subjects tend to

make mistakes and misunderstand instructions. The patients in ***Papers IV and V*** had their samples taken when it was suitable for the hospital staff. Therefore, these samples were not taken according to a set time schedule and this factor will increase the variability in these studies due to daily variations.

In ***Paper III***, the sequence of the sessions was not randomized. This weakness was the result of time constraints, since if wood smoke (but not clean air) induces long-lasting inflammatory effects, these could have affected a control session 1 week after wood smoke exposure. It seems, however, very unlikely that exposure to clean air could affect the response to wood smoke 1 week later.

The urine samples in ***Paper V*** involved very small amounts. In addition, many of these patients had high values, above the highest standard supplied in the assays. Samples with high values were diluted and reanalysed. These results can be considered to have greater variability due to several dilution processes with very small amounts of urine. When samples are diluted, another bias is added to the final analyses.

For analysis of urine from men, we followed the normal procedure described in the kit manual for serum. For women, the urine samples often needed to be analysed a second time, less diluted than described in the kit manual. This factor increases the bias in urine samples from women. The precision was generally 6–12% (CV), which is acceptable. External reference samples give information about the accuracy of the commercial ELISA kit. External reference samples were added to the analyses for ***Papers IV and V*** and the results indicated that the accuracy of the kits was satisfactory. The previous analyses could therefore be considered substantiated.

In ***Papers IV and V***, samples were used after a longer time of storage (-20°C, mean 4 years), which could contribute to misleading results, especially for A1M which has been reported to have less stability. In ***Paper V***, risk factors for pneumonia, such as smoking, asthma and COPD, were not given for the control group. Therefore, comparison between these groups, concerning the risk factors, cannot be made.

5.5 Limitations and future perspectives

Though it may be possible to use urinary CC16 as a biomarker of respiratory epithelial permeability, there are several limitations. The sampling needs to be easy and self-administered. The fact that male subjects have to discard the first 100 mL of their urine sample makes the use of urinary CC16 more complicated and still there could be CC16 originating from the prostate in the samples. The fairly large variation between individuals could mask a possible increase in urinary CC16 used as an airway marker in cross-sectional studies.
As mentioned in the review of Penders & Delanghe (2004), A1M has been considered useful to distinguish between subjects with neurogenic bladder disease and pyelonephritis and has successfully been used as a biomaker of renal effects of low-level cadmium exposure. From the results of ***Paper IV***, A1M was not as sensitive as CC16 and RBP to distinguish between upper and lower UTI. A limitation for A1M as a marker may be the relatively short stability in frozen samples. Furthermore, when validating CC16 as a biomarker of the airways, one would like to

know the true relationship between health and changes in CC16 concentrations – the relative impact of damaged epithelium, up-regulation by inflammation, or loss of Clara cells.

In conclusion, these urinary microproteins have advantages such as non-invasive early detection of renal abnormalities. However, if used for clinical purposes, international standardization is necessary. The sensitivity of the immunoassays makes measurement of CC16 suitable in clinical diagnostics. There is no post-renal excretion in boys before puberty and urinary CC16 could be valuable as a biomarker both for the permeability of the respiratory epithelium and for tubular function in children. Perhaps CC16, in combination with other LMWPs, would also be helpful for distinguishing between lower and upper UTIs.

6 Conclusions

- Gender, urine flow and time of day have significant effects on both Clara cell 16 kDa protein (CC16) and alpha 1-microglobulin (A1M), while the effect of age is not that strong. First morning urine is less affected by urine flow and daily activities.

- Associations between 24-hour excretion and excretion rates in spot samples were strong and significant for both CC16 and A1M at most times of the day, especially in the morning. This was also true for the association between 24-hour excretion and specific gravity or creatinine-corrected values. Correction for the dilution with creatinine is recommended over correction with specific gravity for A1M and CC16. For CC16 the use of first morning spot samples corrected for creatinine is recommended. For creatinine-corrected urinary A1M spot samples, all times of the day are strongly and significantly associated with the 24-hour excretion.

- With optimal sampling methods, urinary and serum CC16 were tested as biomarkers of changes in the permeability of the respiratory epithelium. After exposure to wood smoke in healthy subjects, serum CC16 increased significantly after 20 hours. For urinary CC16, there was a tendency towards increasing levels after 20 hours.

- The excretion of the small protein CC16 was significantly higher in children with upper urinary tract infection compared with children with lower urinary tract infection. With support from larger studies and in combination with other biomarkers, CC16 may be useful in the clinical assessment of children with urinary tract infection. Alpha 1-microglobulin could not distinguish between upper and lower urinary tract infection.

- Community-acquired pneumonia caused increased urinary CC16. However, serum CC16 was decreased and the increase in urinary CC16 was explained by proteinuria, probably as an effect of fever.

7 Populärvetenskaplig sammanfattning

Det finns ett stort antal biomarkörer som används för bedömning av miljöexponering eller effekter på människa av sådan exponering. Andra har nyligen studerat möjligheterna att använda Claracellprotein (CC16) i blod som biomarkör för att detektera lungpåverkan orsakad av höga halter luftföroreningar. Det har visat sig att CC16 i blod kan stiga som tecken på lungpåverkan vid exponering för luftföroreningar. Eftersom CC16 även utsöndras via njurarna kan urinprov eventuellt användas istället för blodprov vilket skulle underlätta studier av större populationer. Små proteiner som utsöndras via njurarna används ofta för att påvisa njurpåverkan. Alfa 1- mikroglobulin (A1M) är ett litet protein som redan har använts i flera studier av hur njuren påverkas vid exponering för tungmetaller och vid diabetes. När man skall mäta en biomarkör i urinprov bör man ha kunskap om hur den påverkas av urinflöde, dygnsvariation, ålder och kön. Detta är viktigt när man studerar hur utsöndringen av biomarkörerna skiljer sig bland friska personer jämfört med lung- och njursjuka patienter.

Vi fann att det är skillnad, i utsöndring av CC16 och A1M, mellan män och kvinnor. Det finns en naturlig förklaring till att män utsöndrar mer CC16 eftersom CC16 inte bara produceras i lungan utan även i prostata hos män efter puberteten. När CC16 skall användas som biomarkör för lungpåverkan vill man inte ha någon påverkan av CC16 från prostata. Genom att utesluta de första 100 ml innan provtagning undviker man påvekan av utsöndring från prostata. Vidare fann vi också att det var skillnad i utsöndring av både CC16 och A1M beroende av vilken tid på dygnet man tog prov. På morgonen är utsöndringen låg, under dagen är den högre och på kvällen minskar utsöndringen igen. Även urinflödet påverkade utsöndringen av proteinerna. Vid ett högt urinflöde utsöndrades generellt mer protein. Detta har inte så stor betydelse om man samlar in all urin under 24 timmar, men ofta är det mer behändigt att använda sig av ett enkelt urinprov och sedan korrigera för utspädningen. Det kan man göra genom att mäta utsöndrad mängd kreatinin eller den specifika vikten i urinen och sedan uttrycka proteinhalten som mängd per gram utsöndrad kreatinin eller genom justering för specifik vikt. Den här korrigeringen fungerar bäst om urinflödet inte påverkar utsöndringen. Därför rekommenderar vi att man använder sig av första morgonurinprov, då urinflödet är lågt och därmed påverkar utsöndringen av CC16 och A1M i mindre utsträckning. Utspädningskorrigering med hjälp av kreatinin verkar vara lite säkrare än korrigering med specifik vikt om man ser till överensstämmelsen med den totala utsöndringen av CC16 respektive A1M under 24 timmar.

För att testa om CC16 i urin går att användas som biomarkör för lungpåverkan vid exponering för luftföroreningar testades detta i ett experimentellt försök. Efter att 13 försökspersoner exponerats för rök från vedeldning steg CC16 i blod efter 20 timmar och det var en tendens till ökning av CC16 i urin. För att se hur CC16 och A1M utsöndras hos njursjuka undersökte vi barn med urinvägsinfektion. Hos barn med urinvägsinfektion visade det sig att utsöndringen av CC16 ökade med ökad njurskada. Normalt mäter man graden av njurskada med scintigrafi, en bild av hur mycket av radioaktivt inmärkt bärnstenssyra som tagits upp i njurarna. Om det visar sig att det går att använda en kombination av bra biomarkörer, bl a CC16 som markörer för njurskada kan man förhoppningsvis minska användningen av scintigrafi. Vi testade också vår hypotes att patienter med lunginflammation borde ha en lungpåverkan som skulle kunna ge förhöjt CC16 i blod och urin. Det visade sig att patienterna hade förhöjd utsöndring av CC16 i urin men inte i blod. Den egentliga orsaken till den förhöjda utsöndringen av CC16 i urinproven tros därför vara en njurpåverkan på grund av feber hos patienter med

lunginflammation. För detta talar också den förhöjda utsöndringen av A1M som är en biomarkör för njurpåverkan.

Sammanfattningsvis är både CC16 och A1M intressanta markörer för njurpåverkan t ex vid urinvägsinfektioner hos små barn och vid epidemiologiska studier av miljöexponering som kan påverka njurarna. Det rekommenderas att använda första morgonurinprov som korrigeras för utspädningsgrad med hjälp av kreatinin för både CC16 och A1M. Män skall utesluta de första 100 ml av urinprovet. Som luftvägsmarkör är CC16 användbar i blod men i urin behöver man även ta hänsyn till njurfunktion och studiedesignen får därför avgöra vilket som är mest fördelaktigt.

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