ANTTI ROINE

Novel Spectrometric Methods in Detection of Prostate Cancer and Urinary Tract Infection

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ACADEMIC DISSERTATION
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This is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning.

- Winston Churchill

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1 LIST OF ORIGINAL PUBLICATIONS

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II Häkkinen MR, **Roine A**, Auriola S, Tuokko A, Veskimäe E, Keinänen TA, Lehtimäki T, Oksala N, Vepsäläinen J. Analysis of free, mono- and diacetylated polyamines from human urine by LC-MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci. 2013;941:81-9.

III **Roine A***, Veskimäe E*, Tuokko A, Kumpulainen P, Koskimäki J, Keinänen TA. Häkkinen MR, Vepsäläinen J, Paavonen T, Lekkala J, Lehtimäki T, Tammela TL, Oksala NKJ. Detection of prostate cancer by an electronic nose: a proof-of-principle study. Journal of Urology 2014;Feb 25. (Epub ahead of print).

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IV **Roine A**, Saviauk T, Kumpulainen P, Karjalainen M, Tuokko A, Aittoniemi J, Vuento R, Lehtimäki L, Oksala NKJ. Rapid and accurate detection of urinary pathogens by mobile IMS-based electronic nose: a proof-of-principle study. (submitted)

2 ABBREVIATIONS

BPH	Benign prostatic hyperplasia
CC	Calibration curve quality control
CFU	Colony forming unit
CRC	Colorectal cancer
CV	Cross validation
DHT	Dihydrotestosterone
DiAcSPD	N^1,N^8-diacetylspermidine
DiAcSPM	N^1,N^{12}-diacetylspermine
DRE	Digital rectal examination
ERSPC	European Randomized Study of Screening for Prostate Cancer (trial)
HFBA	Heptafluorobutyric acid
IS	Internal standard
LOOCV	Leave-one-out cross validation
LC-MS	Liquid chromatography - mass spectrometry
MDS	Multidimensional scaling
ME	Matrix effect
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSU	Mid-stream urine
PCA	Primary component analysis
PCa	Prostate cancer
PLCO	Prostate, Lung, Colorectal and Ovarian (Cancer Screening Trial)
PSA	Prostate specific antigen
PUT	Putrescine
QC	Quality control
SRM	Selected reaction monitoring

SPD	Spermidine
SPM	Spermine
TRUS	Transrectal ultrasound
VOC	Volatile organic molecule
UTI	Urinary tract infection

3 ABSTRACT

Prostate cancer (PCa) is globally the second most common cancer in men and an important source of cancer mortality. Large number of them are indolent diseases that do not even cause symptoms in patient's lifetime, while there is a significant proportion of highly aggressive life-threatening cancers. Prostate specific antigen (PSA) - although sensitive in detection of PCa - is unable to effectively discriminate between these two groups, which leads to unnecessary biopsies and overdiagnosis. Therefore, novel methods for the diagnosis of PCa are needed.

Urinary tract infection (UTI) is a common infectious disease associated with significant costs and morbidity. Its diagnosis relies on time-consuming bacterial culture. Dipstick urinalysis is the only widespread on-site method for the diagnosis of UTI. Its performance is lacking. A rapid on-site tool able to accurately detect bacterial pathogen of would improve treatment of UTI.

Polyamines are essential molecules for growth of bacteria and eukaryotic cells. Their role in cancer is well-documented. Elevated levels in biofluids are associated with cancer, making them potential tumor markers. Liquid chromatography – mass spectrometry (LC-MS) is a highly sensitive and accurate method for the quantification of polyamines in biofluids, but it is not feasible for clinical use.

Canine studies have shown the feasibility of olfactory cancer detection. Electronic nose (eNose) is the artificial counterpart of mammalian olfaction. It allows the use of olfactory information in a clinically applicable form. Its effectiveness in both cancer and UTI detection has been successfully demonstrated.

The aim of this thesis was to test eNose's ability to detect PCa *in vitro* (Study I) and *in vivo* (Study III). We also developed a new LC-MS method for detection of polyamines from urine (Study II), and attempted to discriminate common UTI pathogens with eNose (Study IV).

In Study I we compared a PCa cell line LNCaP to a benign prostatic hyperplasia (BPH) cell line EP-156T with an eNose. The eNose discriminated these cell lines, and LNCaP seemed to modify the smell of its medium. In Study II we developed a new LC-MS method for the simultaneous detection of 14 polyamines from the urine of both healthy individuals and PCa patients. We discovered two polyamines - diacetylputrescine and diacetylcadaverine - which have not been previously documented in human urine. In an unpublished research we showed eNose's ability to detect polyamines in mM concentrations. In Study III we analyzed the urine samples of 50 PCa patients and 15 BPH patients. The eNose discriminated between these groups with a performance equaling existing PCa markers. In Study IV we demonstrated eNose's ability to discriminate between four common bacterial pathogens of UTI with high accuracy.

Together these results suggest that an eNose is able to detect PCa from urine. Polyamines may contribute to smell of cancer, since the eNose detected similar concentrations of polyamines in both aqueous solutions and in the urine of cancer patients. The eNose also discriminated between bacterial pathogens with high accuracy, which suggests that it may be able to detect UTI from urine. These results call for further studies of eNose's capabilities regarding the detection of cancer from urine as well as detection of infections.

4 TIIVISTELMÄ

Eturauhassyöpä on suomalaisten miesten yleisin syöpä ja tärkeä syöpäkuolleisuuden aiheuttaja. Suuri joukko kasvaimista on indolentteja muotoja, jotka eivät edes aiheuta oireita potilaan elinaikana. Toisaalta on olemassa hyvin aggressiivisista, henkeä uhkaavista syövistä koostuva alaryhmä. Prostataspesifinen antigeeni on herkkä testi havaitsemaan eturauhassyövän, mutta se ei kykene erottamaan aggressiivisia ja indolentteja syöpiä, mikä johtaa tarpeettomaan koepalojen ottamiseen ja ylidiagnostiikkaan. Näin ollen eturauhassyövän diagnostiikassa on olemassa tarve uusille työkaluille.

Virtsatieinfektio on yleinen tulehdussairaus, johon liittyy merkittäviä kuluja ja sairastuvuutta. Sen diagnostiikka perustuu aikaa vievään bakteeriviljelyyn. Pikadiagnostiikassa ainoa laajasti käytetty menetelmä on virtsan liuskatesti, jonka suorituskyky on puutteellinen. Pikatesti, joka kertoisi taudinaiheuttajan, parantaisi virtsatieinfektioiden hoitoa.

Polyamiinit ovat solujen ja bakteerien kasvulle välttämättömiä molekyylejä. Niiden roolia syövissä on tutkittu laajasti ja kohonneita pitoisuuksia on todettu elimistön nestetiloissa, mikä mahdollistaa niiden käyttämisen syöpämerkkiaineina. Nestekromatografia- massaspektrometria (LC-MS) on herkkä ja tarkka menetelmä polyamiinipitoisuuksien määrittämiseen mutta, sen soveltuvuus kliiniseen käyttöön on huono.

Koirakokeet ovat osoittaneet, että on mahdollista havaita syöpä hajuaistiin perustuen. Elektroninen nenä (eNose) on nisäkkään hajuaistin keinotekoinen vastine. Sen on osoitettu kykenevän havaitsemaan syöpiä sekä virtsatieinfektion.

Tämän väitöstutkimuksen tavoitteena oli osoittaa eNosen kyky havaita eturauhassyöpä solumallissa (Tutkimus I), sekä virtsasta (Tutkimus III). Lisäksi kehitimme uuden menetelmän polyamiinien mittaamiseksi virtsasta (Tutkimus II) ja tutkimme eNosen kykyä havaita yleisimpiä virtsatieinfektioiden aiheuttajia (Tutkimus IV).

Tutkimuksessa I vertasimme eturauhassyöpälinjaa LNCaPia ja hyvänlaatuisen liikakasvun mallia EP-156T. eNose erotti nämä solut toisistaan ja LNCaP näytti muuttavan elatusnesteen hajua. Tutkimuksessa II kehitimme uuden LC-MS menetelmän 14 polyamiinin samanaikaiseen havaitsemiseen terveiltä ja eturauhassyöpää sairastavilta potilailta. Löysimme kaksi molekyyliä, diasetyylikadaveriinin ja diasetyyliputreskiinin, joiden olemassaoloa ihmisen virtsassa ei ole aiemmin dokumentoitu. Julkaisemattomassa tutkimuksessa olemme todenneet eNosen kyvyn havaita polyamiineja vedestä millimolaarisina konsentraatioina. Tutkimuksessa III analysoimme 50 eturauhassyöpää ja 15 prostatan hyvänlaatuista hyperplasiaa sairastavan henkilön virtsanäytteen eNosella. eNosen erotuskyky oli vertailukelpoinen kirjallisuudessa esitettyihin käytössä oleviin syöpämerkkiaineisiin. Tutkimuksessa IV osoitimme eNosen kyvyn erottaa neljä yleisintä virtsatieinfektion aiheuttajaa.

Tuloksemme osoittavat eNosen kyvyn havaita eturauhassyöpä virtsasta. Polyamiinit osallistunevat syövän hajun muodostumiseen, sillä eNose havaitsee polyamiineja vesiliuoksista samanlaisina pitoisuuksina kuin niitä esiintyy LC-MS:llä mitattuna eturauhassyöpäpotilaiden virtsassa. eNose erottelee bakteereita korkealla herkkyydellä ja tarkkuudella. Tällä perusteella on mahdollista, että eNose kykenee havaitsemaan virtsatieinfektion suoraan virtsasta. Nämä tulokset vaativat lisätutkimuksia eNosesta niin syöpien kuin infektioiden toteamisessa.

5 INTRODUCTION

Prostate cancer (PCa) is globally the second most common cancer in men. It is biologically unique since majority of men develop PCa as their age increases. PCa mortality is relatively low in relation to its incidence. This is explained by the fact that large proportion of PCa cases are of indolent nature and never develop into a clinical disease (Albertsen et al. 2005; Popiolek et al. 2013). There is, however, a population that develops highly aggressive cancers requiring prompt intervention (McGuire et al. 2012). This creates certain challenges for diagnostic tools, since the simple detection of cancer is insufficient - one also has to determine the aggressiveness of the cancer.

Prostate specific antigen (PSA) is highly sensitive yet unspecific marker for PCa (Thompson et al. 2005). Its introduction resulted in a tremendous increase of PCa incidence, which in turn led to a large number of low-risk PCa diagnoses (Penney et al. 2013). Histologic examination is the golden standard for the determination of the aggressiveness of the tumor with a good correlation for an untreated patient's survival. Transrectal ultrasound guided (TRUS) biopsies, however, are not without risks, and the unspecificity of PSA to PCa in general as well as to aggressive cancer leads to a larger patient population being exposed to potential detriments caused by biopsies (Feliciano et al. 2008). These are key questions in the discussion concerning PCa screening that has been able to reduce PCa specific mortality, but has no effect on overall mortality (Schröder et al. 2012). Based on these factors, there is a need for novel, non-invasive tools that - alone or combined with existing methods – are able to predict PCa outcomes more accurately.

Urinary tract infection (UTI) is one of the most common infectious diseases globally, with significant morbidity rates and

financial burdens (Foxman 2002). It may also be a confounding factor regarding PCa diagnostics, since it can elevate PSA levels.

Polyamines are molecules necessary for the growth and function of nearly all living organisms, and also have key role in carcinogenesis (Soda 2011). The prostate's polyamine metabolism differs from that of other organs, and changes in polyamine metabolism seem to have a pivotal role in the development of metastatic PCa (Smith et al. 1995). Increased polyamine contents in blood and urine are associated with many malignancies (Russell et al. 1971). To date, no analytical technology has enabled the simultaneous determination of large patterns of both acetylated unacetylated polyamines from urine.

Canine studies have demonstrated the feasibility of olfactory detection regarding several types of cancer. Since the use of canines is difficult to implement in clinical practice, there is a need for alternative methods. An electronic nose (eNose) is a device that mimics the working principle of mammalian olfactory system. It is capable of detecting volatile molecules that olfaction is based on. It has been used in detecting several types of cancer (Alphus D Wilson & Baietto 2011).

In this thesis, eNose's ability to detect PCa is demonstrated both *in vitro* and *in vivo*. A novel method for detection of large array of acetylated and unacetylated polyamines without preparatory derivatization is also presented. Finally, the ability of eNose to discriminate bacterial UTI pathogens is demonstrated. Together, these studies lay foundation for a novel approach to PCa as well as other malignancies and diseases.

6 REVIEW OF THE LITERATURE

6.1 Prostate Cancer

6.1.1 Epidemiology

The prevalence of PCa is hard to estimate reliably due to a high number of asymptomatic cases; therefore, our knowledge is largely based on autopsy studies that detect both symptomatic and indolent PCas. Differences in autopsy prevalence are minor, whereas incidence and mortality rates differ greatly (Haas et al. 2008). The worldwide incidence of PCa is about 900,000/year, second only to lung cancer (LCa). In developed countries, including Finland, it has surpassed LCa, with USA having the highest incidence. PCa has the fifth place in developed countries, being surpassed by lung and gastrointestinal cancers (Jemal et al. 2011). PCa mortality is highest in Scandinavia and Africa and lowest in Asia. The large differences in incidence and mortality raise questions, bearing in mind the roughly equal worldwide autopsy prevalence. For example, there is large difference in incidence of PCa between Japan and USA (12.6 vs 124.8 per 100000), but a proportionally smaller difference in mortality (5.7 vs 15.8) (Haas et al. 2008). The large differences in PCa incidence can be explained by different diagnostic activities, i.e. the incidence rate is high in countries where PCa is actively screened. Since both countries have equally advanced healthcare, the different mortality rates cannot be attributed to differences in standards of care - an argument that could be used in case of developing countries. This leaves us with the following dilemma: are we treating PCa in

patients who don't actually benefit from the treatment? This is a central question regarding PCa screening, and it will be discussed further in another chapter.

6.1.2 Pathogenesis

Growth of PCa is strongly dependent on androgens (Huggins 1978), especially testosterone which is produced mainly by the testes but also through peripheral conversion of adrenal steroids (Horton & Tait 1966). The exact mechanism of prostatic carcinogenesis is unknown, but polyamines – which will be described later in more detail - seem to have a significant role in it (Feldman & Feldman 2001).

Studies on natural history of early PCa show that a large number of localized cancers neither develop into a metastatic disease nor cause patient's death (Albertsen et al. 2005; Popiolek et al. 2013). A certain proportion of cancers that are initially considered low-risk cases will nevertheless send metastases. The authors speculated that this accumulation of mutations allows the cancer to progress. Since the histological grading of PCa rarely progresses (Popiolek et al. 2013), the spread of low-risk cancers may be triggered by environmental factors.

6.1.3 Early detection

Localized PCa rarely causes symptoms. A localized cancer may cause hemotospermia, hematuria, voiding symptoms such as pain while urinating or perineal discomfort. These symptoms are nonspecific, and can also result from prostatitis, benign prostatic hyperplasia (BPH) or UTI, creating a challenge in differential diagnosis (Madersbacher et al. 2004). Advanced disease can cause pain from metastases in bones or systemic symptoms of cancer such as unintentional weigh loss (Darves-bornoz et al. 2013). The diagnosis of PCa currently relies on digital rectal examination

(DRE), prostate-specific antigen (PSA) and histological examination of TRUS –guided biopsies (Heidenreich et al. 2011).

DRE appears to be highly specific and also has a high negative predictive value, but it also has low sensitivity and positive predictive value (Hoogendam et al. 1999). Therefore, both positive and negative findings require further examinations.

PSA is a protease secreted by prostatic epithelial cells (Catalona et al. 1991). Its concentration in serum is elevated in PCa, but also in benign conditions such as BPH or prostatitis (Catalona et al. 1991). PSA is a continuous parameter and, and thus no threshold for PCa suspicion can be defined (Thompson et al. 2005). Actually, it has been shown that high grade PCa cases i.e. those with Gleason score of 7 or higher but with low preoperative PSA, had poorer outcomes than the ones with higher PSA levels (McGuire et al. 2012). Therefore, PSA analysis is incapable of detecting aggressive PCa in a significant number of patients. A number of nomograms designed for pre- and post-biopsy risk evaluation have been introduced to assist physicians. The only widely studied model applicable for a typical primary care physician is the PCPT-CRC –calculator, which is based on US screening study. Its European counterpart is not well-suited for primary care, since it requires TRUS finding (Caras & Sterbis 2014), which is unavailable to a typical primary care physician. Therefore, several modifications of PSA have been introduced. Free/total PSA refers to the percentage of PSA bound to plasma proteins; the lower the ratio, the higher the risk of PCa (Ito et al. 2003). PSA density i.e. the ratio of PSA in comparison to prostate volume (Sfoungaristos & Perimenis 2012), PSA density of transition zone, age-specific reference ranges, PSA kinetics that include PSA velocity and PSA doubling time (Vickers & Brewster 2012) were also introduced. Only free/total PSA has contributed to the diagnostic performance of PSA by avoiding unnecessary biopsies (Kobori et al. 2008).

A newer, promising biomarker is the messenger RNA of prostate cancer gene 3 (PCA3), which can be isolated from post-

DRE urine samples (Groskopf et al. 2006). It has been proven superior to free/total PSA in predicting biopsy outcomes, and seems to correlate with Gleason scoring, thus possibly assisting in discrimination of indolent and aggressive PCa cases (Haese et al. 2008). The DRE requirement is this methodology's drawback, since it makes the method more invasive than a urine sample alone. Since DRE has been indicated as a barrier for PCa screening (Nagler et al. 2005), a method that would work without DRE would be preferable.

6.1.4 Prostate Cancer Screening

As a common and potentially lethal cancer, PCa is a candidate for screening. Views on screening vary among different organizations. The European Association of Urology currently recommends against systematic screening due to inconclusive evidence of its effects (Heidenreich et al. 2011). American Cancer Society has taken a more flexible stance, placing a heavier emphasis on patient's informed decision-making. It recommends screening opportunities to be offered to men at age 50 years, or at the age of 40 for high risk individuals. Men with life expectancy of less than 10 years should not be offered PCa screening. They recommend annual visits for patients with PSA over 2.5-4.0 ng/ml and biannual visits for patients with PSA less than 2.5 ng/ml. Men with PSA over 4.0 ng/ml should be referred for further evaluation. A PSA of 2.5-4.0 ng/ml represents a grey area, wherein individualized risk assessment takes place. (Smith et al. 2010)

The harms of PCa screening include anxiety caused by false positives of PSA (Fowler et al. 2006) and a risk of hematoma and infection caused by biopsies (Roberts et al. 2002). Even though complication rates have remained the same, the introduction of PSA has greatly increased the number of biopsies, leading to more patients exposed to complications (Roberts et al. 2002). The rise of resistant bacterial species has also made infectious complications more troublesome (Feliciano et al. 2008).

Current recommendations are based on two large-scale, randomized screening trials: the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial and the European Randomized Study of Screening for Prostate Cancer (ERSPC, to which Finland has also contributed). These trials' key goal was to determine whether screening with PSA testing and DRE (although DRE was excluded from ERSPC during the study (Wolf et al. 2010)) could reduce both cancer and overall mortality. So far, the findings of the two studies are conflicting. To date PLCO has failed to demonstrate a reduction in either PCa specific or overall mortality (Andriole et al. 2009), which may be caused by significant screening in control population. The more robustly designed ERSPC demonstrated a reduction in PCa specific mortality, but no difference in overall mortality after 11 years of follow-up (Schröder et al. 2012). The reduction in PCa mortality comes at the cost of significant overdiagnosis (Draisma et al. 2003).

6.1.5 Diagnosis

TRUS guided transrectal or perineal biopsies are the golden standard of PCa diagnosis. The European Association of Urology currently recommends plasma PSA and DRE finding as a basis of selecting patients for biopsies, with consideration for patient's biological age, comorbidities and the therapeutic consequences. (Heidenreich et al. 2011)

Eight to twelve biopsy cores are recommended. Biopsies are focused on lateral and posterior parts of peripheral gland, where most cancers are located (Sánchez-Chapado et al. 2003). If no biopsies turn out to be positive, then clinical suspicion (high and/or rising PSA, suspicious digital rectal examination finding) or certain histological features (multifocal high-grade intraepithelial neoplasia (PIN) or atypical small acinar proliferation) may warrant repeat biopsies. (Heidenreich et al. 2011) There is no consensus of optimal repeat biopsy timing. Repeat biopsies within one year have been suggested in case of high-grade intraepithelial neoplasia (Merrimen et al. 2009).

PCa diagnosis is made by a histologic examination of biopsy material. A Gleason score is given to each biopsy based on the differentiation of cancer. Scoring is based on histological assessment of the tumor's architecture, ranging from 1 to 5; a score of one highly resembles normal tissue, while a tumor of five has no recognizable gland architecture left. The scoring is expressed as a sum of the two of the most prevalent architectures. Scoring essentially tells the reader how well differentiated the PCa in question is. The disadvantage of Gleason scoring lies in its dependency on the experience and competence of the evaluator, making it a non-objective indicator of PCa's aggressiveness. Staging is conducted with combination of biopsies, local ultrasound imaging and MRI. Patients with PSA over 20 undergo a bone scan in order to detect possible metastases (Heidenreich et al. 2011; Epstein 2010). See table 1 for TNM staging of PCa.

Table 1. TNM Staging of PCa (Adapted from Sobin & Wittekind 2002).

Stage	Sub-stage	Definition
T1		**Tumor undetectable by DRE or imaging**
	T1a	Incidental histological finding ≤5% of tissues resected during TURP
	T1b	Incidental histological finding >5% of tissues resected during TURP
	T1c	Tumor identified by needle biopsy
T2		**Confined within prostate**
	T2a	Tumor involves half of lobe or less
	T2b	Tumor involves over one lobe but not both lobes
	T2c	Tumor involves both lobes
T3		**Tumor extends through the capsule but has not spread to other organs**
	T3a	Extracapsular extension
	T3b	Tumor invades seminal vesicles
T4		**Tumor invades adjacent structures other than seminal vesicles**
	T4a	Tumor invades bladder neck and/or external sphincter and/or rectum
	T4b	Tumor invades levator muscles and(or is fixed to pelvic wall)
Node		**Regional lymph nodes**
	NX	Regional lymph nodes cannot be assessed
	N0	No regional lymph node metastasis
	N1	Regional lymph node metastasis
Metastasis		**Systemic spread**
	MX	Distant metastasis cannot be assessed
	M0	No distant metastasis
	M1a	Non-regional lymph nodes
	M1b	Bones
	M1c	Metastasis at other sites

6.1.6 Treatment

The treatment chosen for PCa depends on the patient's overall condition (life-expectancy, comorbidities) and on the risk-profile of PCa in question. The most important factor, i.e. aggressiveness is mainly defined by the following three factors: PSA, Gleason score and staging.

Active surveillance is a practice aiming to reduce overtreatment of low-risk PCas. Patients with clinically confined (T1-T2), low Gleason (< or =6), cancer in three or fewer biopsies, sub-50% cancer involvement in aforementioned biopsies and PSA<10 ng/ml are suitable for active surveillance. For these patients, no primary treatment for PCa is given. Instead, the cancer's progression is followed via PSA and periodically repeated biopsies. If histological progression is evident, or if PSA progresses significantly (doubling time of 2-4 years, PSA>10 ng/ml), patient is treated with either surgery or radiotherapy. (Heidenreich et al. 2011)

Radical prostatectomy is the treatment of choice for patients who have localized disease and are ineligible for active surveillance. Nerve-sparing surgery is the preferred method for patients with organ-confined disease and normal erectile function. Pelvic lymphadenectomy is performed to all patients with intermediate- and high-risk patients. Adjuvant radiotherapy is offered to patients with high risk of failure in radical prostatectomy (positive margins and/or seminal vesicle invasion), and low PSA levels.

Radiotherapy is nowadays considered an option in low-risk PCa cases, even for young patients who refuse surgery. It can be conducted by either intensity modulated radiotherapy or transperineal brachytherapy. Neoadjuvant and adjuvant androgen deprivation therapy are typically used in locally advanced and high-risk cancer cases. The recommended duration of therapy is up to 3 years. (Heidenreich et al. 2011)

Patients with a local PCa treated with curative intent typically receive a 10-year follow-up. PSA is measured quarterly during the first year, biannually until three years have passed, and annually afterwards. If PSA either rises over 0.2 ng/ml after radical prostatectomy or 2 ng/ml above nadir - lowest PSA level achieved during treatment (Critz et al. 1996) - after curative radiation therapy, then residual or recurrent cancer should be suspected (Heidenreich et al. 2011).

6.1.7 Concluding Remarks

PCa is globally the second most common cancer in men and important cause of cancer mortality. DRE, PSA and nomograms are the tools of a general practitioner, whereas TRUS-guided biopsies are reserved for specialist practice. Biopsies are associated with increasing infectious complications. A significant number of diagnosed cancers would never cause a clinical disease, while treatment may reduce patient's quality of life without any corresponding benefit. Due to these factors, there is a great need for novel, non-invasive diagnostic methods that both contribute to the current tools in terms of PCa patients' risk stratification and are suitable for primary care use.

6.2 Urinary Tract Infection

UTI is a condition in which bacterial pathogens enter the urinary tract and cause an infection. It can range in severity from mild to life-threatening, and it also inflicts significant costs on society. UTI is also a significant confounding factor for diagnostic tools that utilize urine as sample material. Prostatitis is, in addition to BPH, perhaps the best-known benign cause of temporarily elevated PSA levels. Prostatitis is, however, rarely caused by bacteria, with the exception of bacterial prostatitis as a complication of prostate biopsies (Karlovsky & Pontari 2002). UTI can also elevate blood-based PSA (Bell et al. 1998), thus being a confounding factor for PCa diagnostics. It also affects urinary polyamine levels and may confound their use in diagnostics. Polyamines are covered in chapter 6.3.

The following review of diagnostics and therapy is based on the guidelines of European Association of Urology for the management of urinary and male genital tract infections (Grabe et al. 2008), if not stated otherwise.

6.2.1 Epidemiology

UTI is classified into two subtypes: lower UTI, which affects urethra and bladder, and upper UTI which affects ureters and kidneys, i.e. pyelonephritis. UTIs cause a significant financial burden on society. In the US, the direct costs if UTI – which include physician visits, medications and hospital expenses - exceed 600 million dollars, but the indirect expenses generated by sick leaves, travel costs and morbidity are even higher (Foxman 2002). UTI cases account for seven million annual physician office visits in the US alone.

6.2.2 Pathogenesis

Pathogens can enter urinary tract either hematologically, via lymphatic system, or most commonly by directly ascending through urethra. Pathogens usually originate in the gastrointestinal tract. Insertion of a catheter into the urethra increases the risk of UTI in proportion to catheterization's duration. UTIs of hematological or lymphatic origin are uncommon and have a different set of pathogens (*Staphylococcus aureus*, *Candida spp.*, *Salmonella spp.*, *Mycobacterium tuberculosis*). Bacterial etiologies are discussed in detail in the next chapter.

UTIs are classified as either uncomplicated or complicated, depending on the factors that impair natural resistance such as age, diabetes, spinal cord injury, or catheterization. An uncomplicated UTI requires a pathogen of high virulence, whereas a complicated UTI can be caused by bacteria of relatively low virulence. This results in a different spectrum of pathogens. (Grabe et al. 2008; Ronald 2003)

6.2.3 Bacterial Etiology

80% of all uncomplicated UTIs are caused by *Escherichia coli*, a bacterium originating in the gastrointestinal tract. *Staphylococcus saprophyticus* causes 10-15% of the cases and the rest are mostly caused mostly by *Klebsiella* species or *Enterococcus faecalis*. *E. coli*, *Klebsiella spp.* and *E. faecalis* all originate in the gastrointestinal tract whereas *S. saprophyticus* can be found in female genital tract. (Ronald 2003)

A Complicated UTI has a more diverse etiology, depending on predisposing factor. *E. coli* is still the most common pathogen in most populations. Elderly patients have virtually no UTIs caused by *S. saprophyticus* but have more infections with multiple pathogens. Diabetics have a two- to threefold risk of contracting *Klebsiella*- and group B streptococci infections in comparison to the non-diabetic population. Catheterized patients and patients with

anatomical abnormalities of the urinary tract are more prone to *Pseudomonas* and *Proteus Mirabilis*. (Ronald 2003)

6.2.4 Diagnostics

The significant presence of bacteria in urine is the cornerstone of UTI diagnosis. In a mid-stream sample of urine (MSU), $>10^3$ colony-forming units (CFU) of bacteria/ml is considered significant in uncomplicated cystitis, as is $>10^4$ CFU bacteria/ml in acute uncomplicated pyelonephritis in women. The threshold is higher in a complicated UTI (10^5 CFU bacteria/ml in MSU in women, and 10^4 CFU bacteria/ml in MSU in men or in straight catheter urine in women). Any bacterial finding in suprapubic bladder puncture specimen should be considered significant. If 10^5 CFU bacteria/ml of the same strain is acquired in MSU more than 24 hours apart and no symptoms are defined, then the condition is defined as an asymptomatic bacteriuria.

Urinalysis conducted by dipstick strips is an on-site method available in practically every healthcare unit. It reveals the presence of leukocytes (pyuria), red blood cells (hematuria) and nitrites in urine. It is effective in ruling out an infection, but positive results need to be confirmed either by a high pre-test probability or by culture (Devillé & Yzermans 2004).

The clinical distinction between lower and upper UTI relies on symptom analysis and blood tests. Fever, flank pain and nausea indicate pyelonephritis as do leukocytosis and elevated C-reactive protein (CRP) in blood tests.

Bacterial culture is the only diagnostic method that can provide detailed information about bacteria (bacterial strain, antibiotic susceptibility). However, it is relatively costly and taking about 24 hours, is not suitable for on-site diagnostics. Bacterial culture is not recommended for acute uncomplicated cystitis, but is necessary for patients whose symptoms do not resolve or recur within two weeks.

In recent years, the introduction of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) has offered a rapid and inexpensive method for micro-organism identification. It is able to identify the bacterial species in approximately 90% of samples, and the genus (a taxonomic rank above species i.e. E. coli belongs to genus of Escherichia) in 98% of samples taken from bacterial cultures. The misidentification rate is about 1%. The MALDI-TOF MS has also been employed in microbial typing and antibiotic susceptibility testing, where it has demonstrated its ability in discriminating methicillin-susceptible and –resistant strains, thus drastically reducing the time required for identifying methicillin resistant strains (Murray 2012). The disadvantage of MALDI-TOF is that - although economical in high-volume centers, where a high throughput can be fully utilized - its high fixed cost makes it unfeasible for small-volume laboratories, i.e. primary health care.

6.2.5 Treatment

A lower UTI is treated with oral antibiotics for 5-7 days. An upper UTI is treated with oral antibiotics, although in moderate to severe cases a therapy is initiated intravenously and then followed by oral therapy for a total duration of 7-21 days, depending on underlying cause. Predisposing factors such as urinary obstruction should treated to avoid relapses.

6.2.6 Concluding Remarks

UTI causes millions of physician visits annually. The diagnosis of UTI is based on both clinical picture and urinalysis, which is performed by dipstick testing for leukocytes and nitrate. Dipstick testing is capable of reliably ruling out infection, but positive findings attained through it re-quire confirmation. In order to achieve this, a bacterial culture of urine has to be performed, which takes about 24 hours. A rapid identification of cultured bacteria can be achieved by MALDI-TOF MS. However, this method is

only feasible in the largest primary care units, and is also currently incapable of identifying bacteria directly from urine.

There is therefore a demand for inexpensive, rapid tools that can be used for identifying cultured bacterial species in smaller laboratory units, as well as for quick, on-site confirmation of UTI directly from a urine sample.

6.3 Polyamines

6.3.1 Overview of Metabolism

Polyamines are organic molecules that are present in nearly all living organisms. They contain two (putrescine (PUT)), three (spermidine (SPD)) or four (spermine (SPM)) amino groups in a hydrocarbon chain (Shah & Swiatlo 2008). Positively charged amino groups interact with negatively charged structures - most importantly phosphate groups in nucleic acids (Shah & Swiatlo 2008). Polyamines play a role in DNA synthesis, stabilization and transcriptional regulation. They have been recently found to have an effect on DNA complexation, where affinity rises according to the number of amino groups (Kabir & Suresh Kumar 2013). Polyamines also participate ion channel regulation and protein phosphorylation (Gerner & Meyskens 2004; Soda 2011). These roles make polyamines essential for cell growth and differentiation. In addition to synthesis in human cells, they are acquired from diet and synthesized by the bacteria of the gastrointestinal tract (Kalač & Krausová 2005).

Putrescine, spermidine and spermine are the most common polyamines in mammals. Ornithine – which is produced from arginine by arginase in urea cycle and S-adenosylmethionine - is an essential substrate for polyamine metabolism. Ornithine decarboxylase (ODC) converts ornithine to polyamine precursor

PUT. By addition of 3-aminopropyl group from S-adenosylmethionine by SPD synthase and SPM synthase, SPD and SPM are produced, respectively.

Intracellular polyamine pool is carefully regulated. In addition to restricting synthesis, polyamine pool can be constrained via acetylation, which prevents interaction of anionic sites of polyamines with cellular molecules, thus effectively deactivating the polyamine (Casero & Pegg 1993). Spermidine/spermine N^1-acetyltransferase (SSAT) is responsible for this action, producing either N^1-acetylspermidine (N^1-AcSPD) or N^1-acetylspermine (AcSPM). These can be further acetylated into N^1,N^8-diacetylspermidine (DiAcSPD) or N^1,N^{12}-diacetylspermine (DiAcSPM), respectively. Acetylated polyamines and diamines can be extracted from the cell by diamine exporter (DAX). Whether acetylated polyamines have role beyond waste product remains unknown. (Casero & Pegg 1993; Gerner & Meyskens 2004) N^1-acetylpolyamine oxidase (APAO) is capable of reversing acetylated polyamine into putrescine or spermidine, depending on the substrate. Mammalian spermine oxidase (SMO) can directly convert spermine to spermidine, although physiologic role of this function is currently unclear. Dysregulation of polyamine metabolism has also been documented in connection to inflammation, renal failure, diabetes and stroke. (Park & Igarashi 2013) A summary of polyamine metabolism and key enzymes is represented in figure 1.

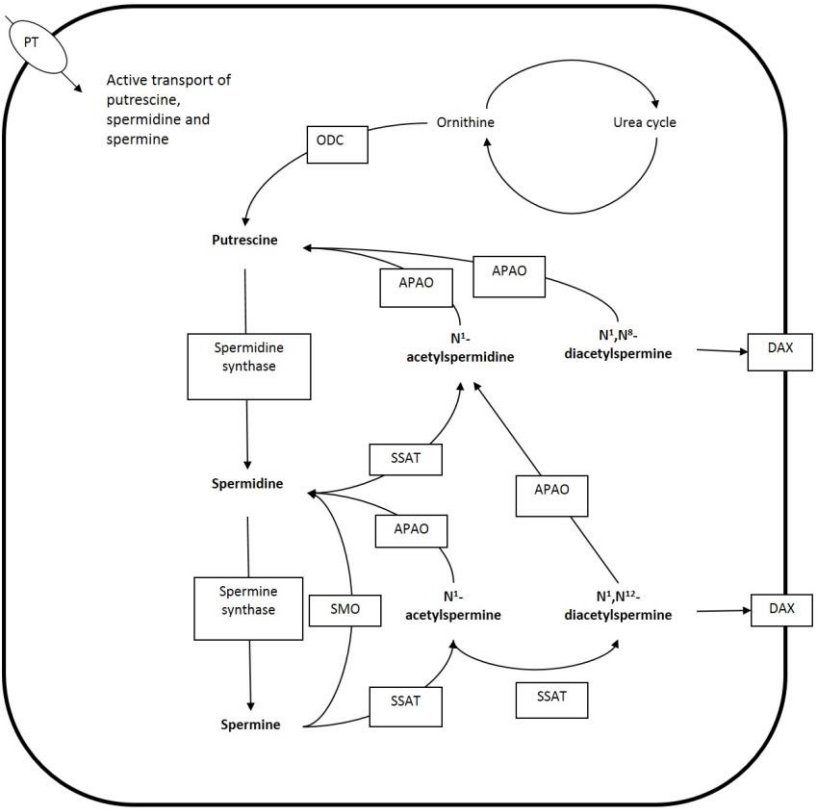

Figure 1. Polyamine metabolism. Ornithine from urea cycle is metabolized into putrescine by ornithine decarboxylase (ODC). Putrescine in turn is metabolized into spermidine and finally spermine by spermidine synthase and spermine synthase respectively. Spermine and spermide can be deactivated by spermidine/spermine N^1-acetyltransferase (SSAT), resulting in N^1-acetylspermidine and N^1-acetylspermine. Another round by SSAT produces N^1,N^8-diacetylspermidine and N^1,N^{12}-diacetylspermine. Acetylpolyamine oxidase (APAO) is responsible for reversing acetylation, resulting in deacetylated or monoacetylated polyamines. Spermine oxidase SMO) is able to directly reverse spermine to spermidine. Acetylated polyamines are extracted from the cell by diamine exporter (DAX). (Adapted from Gerner & Meyskens 2004 and Soda 2011)

6.3.2 Polyamines in oncogenesis

Polyamine concentrations are elevated in all tissues with active proliferation. This is also true for cancer characterized by increased cellular proliferation. Dysregulation of polyamine biosynthesis and degradation has been documented in various cancers (Gerner & Meyskens 2004). The role of polyamine metabolism is particularly well-documented in familial adenomatous polyposis, a well-known condition, which predisposes an individual to the development of colorectal carcinoma. The function of adenomatous polyposis coli (APC), a tumor suppressor gene, is lost in familial adenomatous polyposis. This defect leads to overexpression of MYC oncogene (He et al. 1998). ODC has been proven to be a direct transcriptional target of MYC oncogene (Bello-Fernandez et al. 1993). APC also regulates ODC regulator antizyme, which controls the concentration of intracellular ODC (Erdman et al. 1999). Other oncogenes linked to polyamine metabolism include k-ras, which downregulates the expression of SSAT, resulting in decreased polyamine acetylation (Gerner & Meyskens 2004).

Polyamines are essential for angiogenesis occurring in tissue healing, as well as in tumor growth. Hypoxia and subsequent core necrosis can develop in rapidly growing cancers due to insufficiency of the blood supply. Hypoxia causes malignant cells to increase the intake of polyamines from extracellular space, and it also downregulates expression of CD44 and E-cadherin adhesion molecules (Tsujinaka et al. 2011), thus weakening the cancer cell's connection to extracellular matrix and finally releasing the cell. ODC overexpression was directly linked tumor invasion in a rat model (Kubota et al. 1997). Polyamines seem to also have a role in suppressing immune response towards cancer cells (Kano et al. 2007), possibly by decreasing expression of adhesion molecules in peripheral blood mononuclear cells and decreasing cytotoxicity towards cancer cells (Kano et al. 2007). Interestingly,

host macrophages produce DiAcSPM in response to SPM and low glucose, environment often occurring with tumorigenesis, suggesting that acetylated polyamines play a role in anti-tumor response (Hamaoki & Nagata 2006). The significant role of polyamines in cancer biology has led to development of several therapeutic agents, which affect the different steps of polyamine metabolism with the goal of depleting the polyamine pool. These agents have now entered clinical phase II clinical trials; so far, no significant breakthroughs have been reached. The most clinically successful agent is difluoromethylornithine (DFMO), which appears to be effective in combination with cyclooxygenase 2 inhibitor sulindac in the prevention of postoperative recurrence of colon cancer (Sporn & Hong 2008).

Elevated concentrations of polyamines in cancerous urine were first documented by Russell et al. in analysis of 24 h urine samples by LC-MS (Russell et al. 1971). The highest concentrations were found in ovarian teratomas and the lowest in cancers of the central nervous system. Polyamine concentration in urine varies throughout the day, and is also affected by body mass, circadian rhythm, menstrual cycle and sex (Pöyhönen et al. 1990) as well as by dietary changes (Uusitupa et al. 1993), which makes their use as clinical markers challenging. Urinary polyamine levels are also altered in various non-cancerous diseases, including certain hereditary muscular dystrophies (Rudman et al. 1980), cystic fibrosis (Russell et al. 1979), liver cirrhosis (Cecco et al. 1992), and rheumatoid arthritis (Furumitsu et al. 1993). Plasma polyamines seem to be more sensitive, since only high-volume cancers produce sufficiently high concentrations in urine to be specific to cancer (Löser et al. 1990; Byun et al. 2008).

Russell et al. already noted different mobilities of polyamines in electrophoresis prior to hydrolyzation suggesting that polyamines are also excreted as conjugated compounds (Russell et al. 1971). Van den Berg described a gas chromatography – mass spectrometry (GC-MS) method that did not require hydrolysis of the polyamines, allowing for the quantification of both free and acetylated polyamines in urine (van den Berg et al. 1986). A

remarkable finding of this study was the discovery of a diacetylated polyamine DiAcSPM, which seemed to be specific to cancer. To date, elevated polyamine levels have been documented in urine, plasma and ascites fluid of cancer patients (Teti et al. 2002), and more sensitive methods have shown small concentrations of DiAcSPM in healthy patients (Hiramatsu et al. 1995). Urinary diacetylated polyamines perform better in comparison to their unacetylated and monoacetylated counterparts also in small volume cancers such as breast cancer, surpassing the performance of currently used biomarkers (Hiramatsu et al. 2005). The enzyme activities of polyamine metabolism can be estimated by calculating ratios of a substrate and the product of selected enzyme such as PUT/SPM-ratio or SPD/N-AcSPD, or the ratio of two stereoisomers (N1-AcSPD/N8-AcSPD) (Suh et al. 1997). It has been suggested that different malignancies have different and characteristic polyamine profiles and that polyamine ratios add another variable to the puzzle that can be used to distinguish different types of cancer, such as solid and hematological tumors (Suh et al. 1997). Androgens and polyamine metabolism are closely linked in PCa pathogenesis, which will be discussed in-depth in the next chapter. Hormonal status and polyamines seem to be linked in breast cancer as well (Byun et al. 2009). Buyn et al. compared the serum polyamine levels of pre- and postmenopausal breast cancer patients, finding elevated concentrations in premenopausal breast cancer patients, but not in postmenopausal. Surgery returned polyamine levels similar to the controls.

6.3.3 Polyamines in the prostate and their role in prostate cancer

Historically, the prostate and its secretions have had an important role in polyamine research. The first observation was in 1678 when crystals were found in human semen (Lewenhoeck 1677); they were later identified as SPM. (Gerner & Meyskens 2004) Prostate

tissue has one of the highest polyamine concentrations found in any human tissue. Rat models have shown that androgens induce SSAT, thus inducing the whole polyamine metabolism; they also proved that the addition of SSAT inhibitor delays the progression of androgen-induced PCa (Basu et al. 2009).

SPM seems to have a central role in the pathogenesis of PCa. Dunzenkopfer and Russell reported elevated SPM levels in BPH in comparison to normal prostate tissue (Dunzendorfer & Russell 1978). This is expected in proliferating tissues, which makes the discovery of decreased polyamine levels in PCa surprising (van der Graaf et al. 2000). Even more surprisingly, SPM inhibits the growth of PCa *in vitro* - an effect is lost in more poorly differentiated metastatic diseases (Smith et al. 1995). This is supported by the finding that - in comparison to other cancers - PCa is uniquely insensitive to ODC inhibition by DFMO (Kee et al. 2004). Simoneau et al observed decreased prostate volume and SPM content in prostate biopsy tissues of healthy patients treated with DFMO (Simoneau et al. 2008). Despite - or unaware of – the *in vitro* findings, they launched a PCa chemoprevention with the drug. In light of the *in vitro* findings, it is unsurprising that the clinical trials failed (Schipper et al. 2003). Schipper hypothesized in his review that the failure may have been due to the intake of polyamines from the extracellular pool. It can be argued that the use of DFMO is unfeasible despite the availability of extracellular pool (Schipper et al. 2003). These works show that the role of polyamines is anything but clear. Strikingly different polyamine metabolism in BPH, and local and metastasized PCa suggest that polyamines have a pivotal role in the pathogenesis of PCa and - more importantly - in the development of an aggressive, metastasizing form. This suggestion is also supported by the correlation of the SPM to citrate -ratio in magnetic resonance spectroscopy, and the Gleason score (Selnæs et al. 2013). Differences in the SPM contents of high- and low grade cancers have also been documented (Giskeødegård et al. 2013).

To date, any studies about polyamines in biofluids of PCa patients are sparse, and focus solely on patients with a metastatic

disease. Circulating erythrocyte SPM level was shown to predict poor a response in androgen-deprived PCa (Cipolla et al. 1996). Hiramatsu et al. studied urinary diacetylpolyamines in advanced PCa and in testicular, renal and bladder cancers. In this study, a 90 % discrimination rate for PCa was achieved by using only a combination of DiAcSPM and DiAcSPD. An occasional elevation of DiAcSPM level was documented in BPH but they could often be distinguished from PCa when DiAcSPD was also taken into consideration (Hiramatsu et al. 1997).

6.3.4 Polyamines and Urinary Tract Infection

Little research has been conducted about the influence of UTI on urinary polyamines. The relation between UTI and malignancies is problematic; in particular, the presence of a tumor in the urinary tract can cause an obstruction that predisposes a patient to UTI. Increased polyamine levels in the urine of cancer patients (Russell et al. 1971) may create more favorable conditions for bacterial growth, since polyamines seem to protect the common uropathogen *E. coli* from one modality of innate immunity; the increased oxidative stress in the urinary tract (Chattopadhyay et al. 2003). Furthermore, an asymptomatic bacteriuria is relatively common in women of all ages, and its prevalence increases with age in women, and to a smaller degree in men as well (Hooton et al. 2000; Juthani-Mehta 2007). This increases the difficulty of obtaining a sterile sample from patients with a suspected cancer. Collectively, these factors contribute to the fact that bacteriuria and UTI potentially pose a significant confounding factor when polyamines are employed as tumor markers.

A comparison of free and monoacetylated polyamines in both fresh and incubated urine of healthy persons and UTI patients, taken before and after the treatment (Satink et al. 1989), revealed an increase in the concentration of PUT in association with UTI; it returned to baseline after the therapy. The PUT concentration in urine rose during the incubation suggesting that microbes contribute to the increase in PUT. No changes in other polyamines

were observed in UTI patients. However, it should be noted that diacetylated polyamines were not studied (Satink et al. 1989). This is the only study published about polyamine levels in UTI.

Polyamines increase the virulence of *E. coli* in UTI. A comparison between a wild-type *E. coli* and a polyamine-deficient strain showed that the polyamine-deficient strain quickly perished at an atmosphere with 95 % oxygen content whereas the wild-type strain tolerated these conditions (Chattopadhyay et al. 2003). Oxygen toxicity to the polyamine-deficient strain could be prevented by the addition of PUT or SPD. It was also discovered that the threshold for ozone toxicity was lower in the polyamine-deficient strain in comparison to the wild-type strain (Chattopadhyay et al. 2003). Polyamines were attributed with the role of protecting DNA and enzymes against oxidative stress. Nitric oxide (NO) has a key role in innate immunity. It is converted to reactive nitrogen intermediates that damage genetic material, proteins and lipids. Within hours of the beginning of an infection, the nitrite levels rise and gaseous levels of NO elevate (Bower & Mulvey 2006). NO stress stimulates the production of cadaverine, a diamine produced by ODC from lysine (Pegg et al. 1982) present in *E. coli* found in human urine (Lee et al. 2003), in *E. coli*. A mutation that cripples the production of cadaverine synthesis exposes *E. coli* to the toxic effects of NO, but it is rescued by the addition of polyamines (Bower & Mulvey 2006) In a further study, it was shown that nitrosative stress stimulates *E. coli*'s cadaverine synthesis, thus significantly increasing the virulence of *E. coli* in comparison to the unconditioned strain (Bower et al. 2009).

6.3.5 Concluding Remarks

Polyamines have a significant role in oncogenesis. Elevated concentrations have been documented in both serum and urine of cancer patients. The few studies on PCa have focused on metastatic disease and have had a limited polyamine array. Polyamines were a subject of avid research in a period lasting from the 1960s to the 1980s, but interest in them waned due to the poor

specificity of measurable polyamines for cancer. Recent advances in analytical chemistry have allowed the determination of more cancer-specific mono- and diacetylated polyamines in biofluids, as well as allowing the analysis of a wide range of polyamines in a single run. To date, there are no studies examining polyamines as the indicators of localized PCa. UTI is a potential cause of diagnostic error in a urine-based polyamine analysis. It is also possible that elevated urinary polyamine levels predispose cancer patients to urinary tract infections.

6.4 Liquid Chromatography – Mass Spectrometry

6.4.1 Overview

Chromatography refers to the separation of molecules done by forcing a mixture (mobile phase) through a sorbent (stationary phase). Molecules interact differently with the solid phase and are thus separated by the speed in which they travel through the stationary phase. Due to its working principle, chromatographic method cannot determine either mass or composition beyond some general principles (e.g. the stationary phase can separate molecules of a certain size from the mixture). MS directly measures a parameter known as mass-to-charge ratio (m/z) which is the ratio of the mass and the electrical charge of an ionized molecule i.e. a molecule with a mass of 100 and a charge of +1 has the same m/z of 5 as a molecule with mass of a 200 and a charge of +2. Biological samples contain a large number of different molecules that have confounding effects on the analysis. This phenomenon is known as the matrix effect. It can be reduced by the addition of sample preparation and the optimization of chromatographic parameters. Internal standards are used to estimate the matrix effect and compensate for it (Bird 1989; Collins & Lee 2002; Kang 2012).

See figure 2 for a schematic representation of LC-MS/MS.

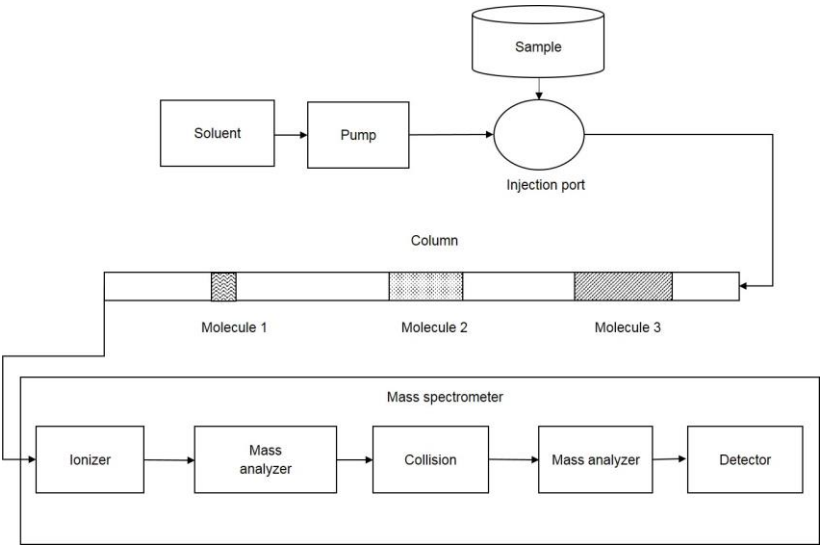

Figure 2. A schematic representation of LC-MS/MS. A sample is injected into the soluent flow pumped through the system. The sample enters column in which molecules are separated based on their interaction with the column's stationary phase. The separated molecules are introduced to a tandem mass spectrometer. The samples are ionized and fragmented in an ionizer, and the ionized molecules are then fed to a mass analyzer, which separates the molecules based on their mobility (determined by mass and charge). Ions then enter a collision chamber where they are bombarded with inert gas molecules. This further fragments the ions, which are then separated in a second mass analyzer and fed to a detector, where they cause a measurable electrical signal (Adapted from Kang 2012 and Bird 1989.).

6.4.2 Sample Preparation

Sample preparation is always a compromise between reduction of matrix effect (ME), sensitivity and complexity of preparation. For example, diluting the sample is a straightforward way to reduce the ME at the cost of diluting the target molecules, which in turn necessitates higher sensitivity in the analysis. When measuring multiple target molecules, an optimal preparation of a singular

molecule can prevent the analysis of another. Complex sample preparation is both costly and causes a bottleneck in the analytic process whereas simple preparation can be automated. Sample preparation can be conducted with techniques relying on precipitation, filtration or extraction to solid or liquid phase. (Kang 2012)

6.4.3 Separation

Separation is achieved in the liquid chromatography phase. In separation, one has to the find optimal compromise between the time consumed in separation and the ability to separate complex samples. The size of particles in the stationary phase of the column is of central importance to the chromatography. The smaller the particle size, the higher the column's separation power. The problem with a smaller particle size lies in the steeply growing back pressure required for a constant flow, which causes technical challenges for construction and required equipment's cost (Kang 2012).

6.4.4 Mass Spectrometry

MS begins by the introduction of a sample by either a direct injection or an infusion through a capillary, allowing for a further separation of the molecules. In LC the sample is first vaporized, whereas in GC it already is in a gaseous form. Vaporization has been a major challenge in regards to combining LC and MS. Sample molecules are then ionized and introduced to a mass analyzer, which sorts the molecules according to their m/z with either magnetic or electric field (Kang 2012).

Tandem mass spectrometry (MS/MS) is a construction in which two mass analyzers are set up in succession, with a field of inert gas between them. The first mass analyzer is used to separate the ionized molecules, which are then fragmented by the collision with the inert gas. The resulting fragments are then fed to the

detector. This technique adds an additional magnitude of accuracy to the analysis, but sacrifices some of the sensitivity, since the second collision spreads energy to the subparts of the original molecule. Generally, the reduction of background noise in MS/MS compensated for the signal reduction, making it an overall superior method (Kang 2012; Pitt 2009).

The detector is the final part of MS; it relies on the electrical current or charge created in the detector by an ion passing the detector or colliding with it. Again, there are different techniques to attain the same basic principle. The performance of the detector is a compromise between accuracy and response time. The detector is the final part of MS and relies on electrical current or charge created in the detector when ion passes or collides the detector. Again, there are different techniques to attain the same basic principle. The performance of detector is a compromise of accuracy and response time.

6.4.5 Internal Standards

The term internal standard (IS) refers to a molecule that behaves in a similar fashion to the target molecule in chromatography but which can be distinguished from it. It is typically either an isotope-labeled target molecule or its structural analog. A known concentration of IS is added to the sample and then measured alongside the actual target molecules. This technique is used to calculate the recovery rate (the proportion of the added internal standard detected in the analysis), which is then used to compensate for the ME in MS. A key challenge of this method lies in the fact that an IS never perfectly mimics the target molecule, which can cause a bias in ME correction. An isotope-labeled IS is created by modifying the target molecule by replacing its atoms with stable isotopes. This results in a different mass, which can then be detected with MS (Kang 2012). An isotope-labeled IS is generally superior to structural analogues, although in some applications structural analogues have achieved equal performance

at a lower cost (Wieling 2002).Detection of polyamines by LC-MS/MS

6.4.6 The Detection of Polyamines by LC-MS/MS

Numerous chromatographic techniques have been developed for the detection of free, mono-, and diacetylated polyamines (Teti et al. 2002; Hiramatsu et al. 1995). Although a number of studies describe the detection of biogenic amines from urine (Kushnir et al. 2002; de Jong et al. 2010; El-Beqqali et al. 2007), only few describe the detection of polyamines from urine with a LC-MS/MS. Buyn et al. described a method for the simultaneous analysis of ten polyamines. Their method relied on extractive carbamoylation, and their assay lacked the polyamines with a highest specificity to cancer, namely DiAcSpm and DiAcSPD. The inability to detect diacetylated polyamines may have been the reason behind the fact that they did not find any significant differences in the urinary polyamines of healthy patients and breast cancer patients (Byun et al. 2008). They, however, documented elevated concentrations of 1,3-diaminoproane, PUT, SPM, and AcSPD in serum (Byun et al. 2008). This led to the exclusive use of serum in their follow-up study, where they compared pre- and postmenopausal breast cancer patients, finding elevated concentrations in the premenopausal cancer patients, but not in the postmenopausal population (Byun et al. 2009). To date, the only method for the detection of underivatized polyamines by LC-MS/MS from urine is by Gosetti et al. They focused heavily on cathecoliamines and their polyamine array included only PUT, SPD and SPM, altogether omitting acetylated polyamines, whose role in cancer applications is paramount (Gosetti et al. 2013).

6.4.7 Concluding Remarks

LC-MS has the potential to be an extremely accurate and sensitive tool for the detection and quantification of molecules. There are a large number of different techniques for each step of the process, which allow for the method's tailoring to suit highly specific needs. The analysis is always some kind of compromise between speed, complexity, and the extent of target molecules analyzed in a single run; a decision which has to be weighted according to the application in mind. The complexity of the instrumentation leads to high costs, and the device's use requires either considerable expertise or extensive automation applied for specific tasks. The use of MALDI-TOF MS in the identification of microorganisms is an example of an application for which completely automated devices are available (Seng et al. 2010).

There is currently no method for reliably and simultaneously analyzing free, mono-, and diacetylated polyamines from urine. So far, no studies describing the presence of DiAcCAD or DiAcPUT have been conducted.

6.5 Olfactory Detection of Cancer

6.5.1 Overview

The concepts of taste and smell in regards to medicine are ancient. Historically, diabetes was diagnosed from the sweet taste of glucose in patient's urine. Ketoacidosis can be suspected from a distinctive smell of acetone in the breath of a diabetic patient. A foul smell is often present in infections such as bacterial vaginitis, UTI and wound infection. The distinct odor of certain diseases is caused by the release of volatile organic compounds (VOC) into the surrounding air, which are then experienced as an olfactory sensation (Alphus D. Wilson & Baietto 2011). To date, the only disease where human olfactory detection has been the subject of scientific communication is *Clostridium difficile* – associated diarrhea. The discussion started with a report of a nursing staff's ability to detect *C. difficile* via olfaction (Burdette & Bernstein 2007). A response (Wilcox 2007) pointed out an earlier study (Johansen 2002), in which nurses had reached fairly high sensitivity and specificity by using a combination of clinical details and smells from samples. In another experiment, nurses attempted to discriminate between *C. difficile* toxin-positive and -negative stool samples. The median correct rate was equal to chance. The author speculated that the positive findings in previous studies were due to a lack of proper blinding (Rao et al. 2013). Interestingly, all these experiments were published as correspondences - possibly reflecting the attitude of medical community towards the use of olfaction in the diagnosis of a disease.

The relation between cancer and olfaction entered medical literature in correspondence to an editor of Lancet, in which

Williams and Pembroke reported a case where a patient's dog had started to pay extraordinary attention to a mole in the said patient's leg, even attempting to bite the mole off. The dog's behavior led the patient to seek medical attention; the mole was excised and identified as a melanoma (Williams & Pembroke 1989). Ten years later, a family dog paid a suspiciously high amount of attention to a skin lesion, which proved unresponsive to topical steroids and antifungal agents. The lesion was excised and found to be a basal cell carcinoma. Curiously enough, the dog lost attention to the skin area afterwards (Church & Williams 2001).

6.5.2 The Use of Dogs in Detection of Malignancies

Inspired by the aforementioned incidents, the first study utilizing dogs in the detection of bladder cancer from urine was carried out. The authors hypothesized that VOCs are directly excreted to urine from a tumor. Six dogs were trained to locate a pot with cancerous urine from a group of 6 pots with urine taken from the controls. They employed both healthy and diseased (kidney stones, UTI, diabetes etc.) controls in order to prevent the dogs from learning to detect cancer based on secondary features, such as inflammation. The dogs succeeded in detecting significantly better than could be attributed to pure chance (Willis et al. 2004). Almost immediately after the publication of the study, another trial on the detection of skin cancer was published. In this trial, two dogs were trained to locate a bandaged malignant lesion from other bandaged areas. The dogs achieved a success rate of 100% (Pickel et al. 2004).

McCulloch et al. collected breath samples by having patients exhale through a tube containing polypropylene wool covered with silicon oil. The tube was sealed for storage after a sufficient number of breaths. In this study, every dog reached a performance level comparable to a computed tomography scan in terms of ruling out cancer. The high performance may be partially due to the use of healthy controls lacking any confounding effects, such as inflammation (McCulloch et al. 2006). Ehmann et al. confirmed

this finding with a relevant control population, which contained smokers and patients with other airway diseases (Ehmann et al. 2012). Amudsen et al. selected 93 consecutive patients referred to a lung clinic due to a suspicion of LCa. Urine samples were also collected in addition to breath samples. The study failed to repeat the success of previous studies regarding either urine or breath. This may be due to deviation from breath collection protocol in comparison to previous studies or to an inadequately low testing frequency, which would have pre-vented the dogs from maintaining their discrimination ability (Amundsen et al. 2013).

Gordon et al. failed to discriminate between prostate and breast cancer and healthy controls from urine. Authors attributed the failure to shortcomings in the dogs' training protocol and to the samples' lengthy freezer storage (Gordon et al. 2008).

Horvath et al. trained a dog to discriminate ovarian cancer from other gynecological tumors and control tissues. They achieved a nearly perfect rate in the detection of malignant ovarian tumors, as well as in the dog's ability to discriminate between ovarian cancer and other gynecological malignancies (Horvath et al. 2008). A similar performance level of cancer detection was achieved with plasma (Horvath, Andersson, et al. 2010). In 2013, Horvath et al. performed another experiment, which displayed the dogs' ability to detect post-treatment ovarian cancer patients, providing anecdotal evidence that dogs may be able to predict cancer relapse (Horvath et al. 2013).

In 2011, Cornu et al. conducted one of the most elegant dog studies to date. The team collected urine from patients referred to an urologist for TRUS prostate biopsies on basis of abnormal PSA or DRE finding. The dog reached excellent sensitivity and specificity of 91%, demonstrating the potential of olfactory detection of PCa (Cornu et al. 2011). This caught the eye of several researchers, and the identification of the VOCs behind the discrimination was called upon (Bjartell 2011; Lippi 2011).

Sonoda et al. expanded the use of canine olfaction to the detection of colorectal cancer from benign conditions (Sonoda et al. 2011). Again, the dog reached a near-perfect discrimination level, superior to that of a fecal occult blood test. The authors noted that there was no correlation with the dog's response and a fecal occult blood test, implicating that the dog did not rely on blood in detecting cancer.

A conclusion of canine studies is represented in table 2.

6.5.3 Concluding Remarks

During the last ten years, the attitudes towards the olfactory detection of cancer have changed considerably, after well-executed trials have been published in respected publications. For the most part, dogs have demonstrated an excellent performance in detecting various cancers, providing further proof about the concept of different cancers' characteristic smells. Clinically relevant study designs have provided strong evidence about the potential of dogs in distinguishing cancer. However, there are several factors that prevent the use of dogs in the clinic. The regular training time for a dog is about 12 months, which includes multiple training sessions per week, making them extremely expensive. Dogs are also prone to distractions and their lifespan is limited. These factors pose a challenge in standardizing the training and performance of dogs that could be considered for clinical use. Replacing canine olfaction with electronic discrimination conducted by an eNose may provide a solution to several of these problems.

Table 2. A conclusion of studies of canine olfaction in detection of cancer

Study	Target	Cases	Controls	Results (sensitivty/spesificity)	Sample material
Willis et al. 2004	Bladder cancer	36 (9 used for testing) localized transitional cell carcinomas	108 (54 used for testing) with urological and systemic diseases	Success rate of 41 % vs 14 % expected by chance	Urine
Pickel et al. 2004	Melanoma	7 cases of melanoma or lentigo maligna	no controls	100 % detection of skin area affected by cancer	Skin odor
McCulloch et al. 2006	Detection of early- and late-stage lung and breast cancers	55 LCas (28 used for testing) 31 breast cancers (6 used for testing)	17 controls (healthy)	LCa: 99 % / 99 % Breast cancer: 88 %/ 98 %	Exhaled air
Gordon et al. 2008	Breast cancer and PCa	57 PCas (11 used for testing) 62 breast cancers (9 used for testing)	186 controls (80 used for testing) with non-related, non-cancerous conditions	Dogs did not perform better than chance	Urine
Horvath et al. 2008	Discrimination of ovarian cancer, other gynecological tumors	31 ovarian cancers (20 used for testing)	100 (100 used for testing) 13 other cancers (8 used for testing)	Ovarian vs other cancers: 100 % /91 % Ovarian vs healthy: 100 %/ 98 %	Tissue specimen

Table 2. Continued

Cornu et al. 2011	Discrimination of PCa and biopsy-negative patients.	33 patients with PCa	33 patients with negative biopsies	91 %/ 91%	Urine
Sonoda et al. 2011	Discrimination of colorectal cancer from benign conditions.	breath: 33 cancers stool: 27 cancers	patients with benign conditions breath: 132 stool: 148	breath: 91 % / 97 % stool: 99 %/ 99 %	Exhaled air, watery stool
Ehmann et al. 2012	Discrimination of LCa from COPD and healthy controls.	60 LCas (25 used for testing)	110 healthy (50 used for testing) 50 COPD (50 used for testing)	71 %/ 93 %	Exhaled air
Amudsen et al. 2013	LCa versus benign lung condition.	63 LCas (8 with other malignancy)	30 benign lung conditions	breath: 5 6%/ 33 % urine: 64 %/ 29 %	Exhaled air Urine
Horvath et al. 2013	Indicating ovarian cancer after treatment (Detection of relapse)	series 1: 42 cancers Series II: 10 cancers	310 healthy	Detection of cancer: baseline 98 %/ 100 % 3 months: 70 %/ 100 % 5 months: 80 %/ 90 %	Serum

6.6 Electronic Noses

6.6.1 Overview

An electronic nose (eNose) is a device designed to mimic the working principles of mammalian olfaction. It consists of an olfactory epithelium responsible for binding the gaseous volatile molecules; associated neural pathways that transmit and preprocess the signals created by the olfactory epithelium; and an olfactory cortex that is responsible for interpreting the meaning of olfaction (Turner & Magan 2004). An important characteristic of the olfactory epithelium are its receptors, which are non-selective and more qualitative than quantitative in nature. eNose's multisensory array is an artificial counterpart for olfactory epithelium; it consists of a number of different non-selective sensors. It is important to notice that the sensitivity of artificial sensor arrays is not limited to that of human or other mammalian olfaction - it can also detect odorless gases. The advantage of a multisensory array lies in its ability to identify gas mixtures without any prior knowledge about the specific molecules or their concentrations (Wilson & Baietto 2009).

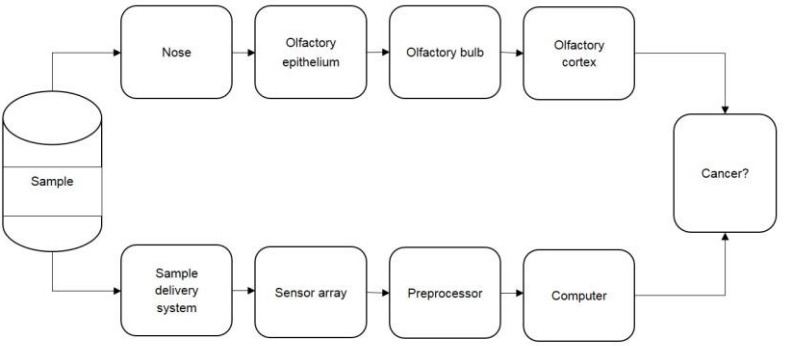

Figure 3. Illustration of the setup of mammalian olfaction and eNose.

The first gas multisensory array was described as early as 1982 (Wilson & Baietto 2009). The sensor array's working principle has remained unchanged since then, but the miniaturization of technology, the associated electronics and the power source has led to contemporary portable devices, which are capable of analyzing the sensor array's signals on-site (Bhandare et al. 2013). Increased computational power has drastically expanded the possibilities of data analysis, allowing the detection of patterns from complex multidimensional data.

First commercial eNoses entered the market in the early 1990s (Turner & Magan 2004). Since then, they have been used for a wide range of applications. A new application is typically tested with the black box approach, in which the discrimination of a given sample set is attempted. If this initial phase is successful, a quantification of the component responsible for discrimination will be attempted in the next phase. The ease of testing new applications has led to prolific experiments in various fields. Initial results gained in controlled laboratory environments have often been better than the ones received in real life situations, which is potentially due to a large number of confounding factors. A sufficient knowledge of the discriminating molecules, their

chemistry and the used sensors is required for avoiding these problems (Bhandare et al. 2013).

6.6.2 The Sample Delivery System

The sample delivery system is responsible for sending volatile compounds to the sensor array. It typically includes some means for refining the sample material, either by filtering compounds that create background noise or by enriching the desired molecules.

The simplest way for delivering a sample to the sensor is via **static headspace extraction**, in which a sample is stored in a sealed container, wherein volatile compounds are allowed to build up and reach equilibrium in the headspace. The headspace is removed with a syringe and injected into the sensor. This process is followed by the cleansing of the sensor with reference gas. Container volume, sample matrix and temperature are the key factors regarding the sampling's sensitivity and reproducibility. It is a fairly labor-intensive method, which exposes the sensor to large changes during sample concentrations (Wilson & Baietto 2009; Chen et al. 2013).

An automating delivery systems reduces the sampling time and standardizes the sample concentration, but is also associated with higher material costs and slower response times. **Dynamic headspace extraction** is a method in which a sample is exposed to a continuous flow of inert carrier gas, which carries the sample molecules to the sensor array. The dynamic headspace never reaches equilibrium due to the constant gas flow, and it also typically contains a lower concentration of sample molecules; these are the key differences in comparison to the static headspace extraction. This feature can be counteracted by using an adsorbent

to bind the target molecules, so that they can be later released and fed to the sensor array (Wilson & Baietto 2009; Chen et al. 2013).

Solid-phase microextraction employs solid material: typically a fiber coated with adsorbing molecules. The fiber is introduced into the headspace, where it begins adsorbing the targeted molecules. Once the fiber has reached equilibrium, it is extracted from the headspace, and the sample molecules are released from the coating to the sensor array via heating. In some cases, a number of fibers with different coatings are used in order to achieve maximum sensitivity to a wider range of molecules (Chen et al. 2013).

6.6.3 The Sensor Array

A number of different technologies can be employed in the sensor array. Each of them possesses characteristics that make them suitable for particular tasks.

Metal oxide semiconductor sensor is the oldest and still the most common eNose sensor. It is based on interaction between the sensor's metal oxide and the surrounding volatile molecules, which affects the sensor's conductivity. The advantage of this technology lies in its stability in different environments. A high operating temperature results in a high power consumption (Chen et al. 2013; Wilson & Baietto 2009; Arshak et al. 2004).

Metal oxide silicon field effect sensor (MOSFET) consists of a source and a drain, which are connected by gate material and insulated with porous, gas sensitive material. The characteristics of the sensor can be altered by varying the gate material, its thickness, and the operating temperature. Compared to metal oxide semiconductor-based sensors, a MOSFET operates in significantly lower temperatures and is also more power-efficient (Chen et al. 2013; Turner & Magan 2004; Arshak et al. 2004; Wilson & Baietto 2009).

Conducting-polymer sensors rely on an array of conducting polymers that have unique physio-chemical characteristics that cause different reactions towards different volatiles. A reaction, e.g. expansion, in the conducting polymer increases the polymer's resistance, thereby creating a measurable signal. The advantages of conducting polymers are their low cost, low power consumption, their ability to work in room temperature and their wide sensitivity. The key challenges of this technology lie in its sensitivity to humidity and in sensor aging caused by the polymers' oxidation (Arshak et al. 2004; Turner & Magan 2004; Wilson & Baietto 2009; Chen et al. 2013).

Optical sensors consist of a light source, the optics and a light detector. They are based on a light crossing a space containing volatile molecules, which alter the light signal's characteristics. Optical sensors are either intrinsic or extrinsic. In intrinsic sensors, the volatiles directly affect the light signal's properties. The optics' fragility results in limited mobility. Extrinsic sensor rely on indicator material that reacts to the volatile molecules, changing the indicator's optical properties. Optical sensors enable the creation of lightweight sensors with wide sensitivity, but they have a short life span due to photobleaching (Arshak et al. 2004; Chen et al. 2013).

Piezoelectric sensors rely on piezoelectric effect, in which a quartz crystal vibrates at certain frequency when an electrical potential is applied to it. The sensor is coated with a gas-adsorbing surface that binds volatile compounds, resulting in an increased mass in the coating, which in turn changes the sensor's oscillation frequency. The advantage of this technology is its high sensitivity, but its complex production makes the reproducibility of sensor arrays difficult (Arshak et al. 2004).

Ion mobility spectrometry (IMS) consists of an ionizer that ionizes the gas introduced to the device. The ion swarm is then allowed to travel through an electronic gradient parallel to the gas flow. Afterwards, ions collide with the detector at the end of the

drift space. The time an ion spends in the drift space is called time-of-flight; this is the parameter measured by an IMS. The factors that affect the time-of flight are collectively known as ion mobility (mass, charge, physical size and shape). A more recent deflection field design uses an electrical field perpendicular to the gas flow, which makes the ions deviate from their path according to their charge and to collide with the detectors placed on detector space's walls. The IMS has several advantages that make it an appealing option for mobile solutions. The sensor has a low power consumption and can be miniaturized as handheld devices. They operate well in atmospheric pressure and can apply ambient air as a carrier gas. They also have high sensitivity and fast response times. The disadvantage of this sensor is its sensitivity to humidity. A combination of an ion mobility spectrometry with a deflection field and metal oxide semiconductors is used in the ChemPro devices employed in this thesis. It is produced by the Finnish company Environics Oy, Mikkeli (Borsdorf & Eiceman 2006; Utriainen et al. 2003).

6.6.4 Data Analysis

Data analysis consists of four stages. Preprocessing includes the compensation for drift and the selection of optimal time points for a further analysis. The dimension reduction aims to compress the data into a minimal number of dimensions without compromising variation, in order to simplify the analysis. The most commonly used technique is the principal component analysis (PCA). It produces a low-dimensional presentation by finding the dimensions with the highest variance. It is best suited for methods producing linear responses. Linear discriminant analysis (LDA) is closely related to primary component analysis, but outperforms PCA in cases where discriminatory information is unaligned with variance. Multidimensional scaling (MDS) is a technique that projects high-dimensional data to low-dimensional space with the aim of maximizing the distance between data points. It is suitable for datasets where the distortion of absolute distances is accepted

in order to preserve the relative distances between data points. In other words, as much topography is pre-served as possible (Maaten 2009; Chen et al. 2013).)

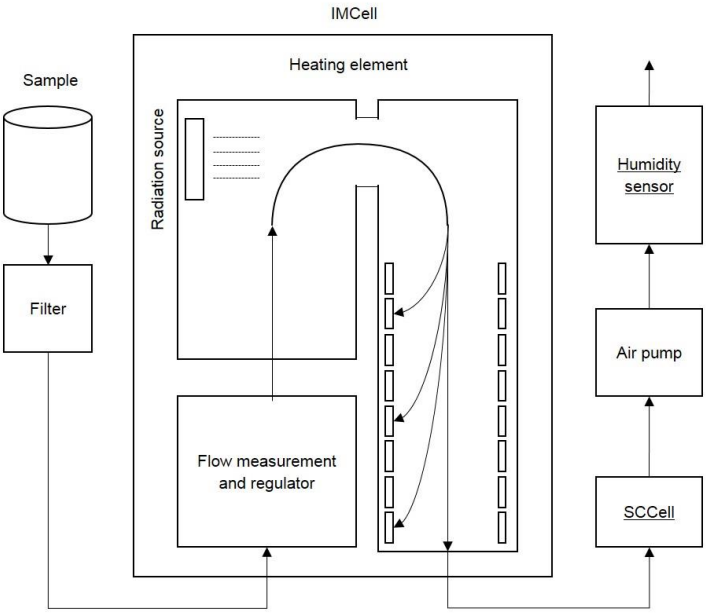

Figure 4. An illustration of the construction of ChemPro 100 IMS-MOS-device. A gaseous sample is fed to the system through dust filter, then introduced to the ion mobility spectrometer which features flow regulation instrumentation. The sample is then ionized by Am-234 – radiation source and deflected by an electrical field into the electrical strip where another electrical field causes ionized molecules to deviate from their path and collide with detectors along the strip, producing an electrical current. This current is then recorded. The remaining molecules exit The IMCell and enter the SCCell. The pump is located near the outlet, creating an aspiration device. A humidity sensor is located before the outlet.

6.6.5 eNoses in Detection of Cancer

Since the first studies about eNose's ability to discriminate bacterial strains were published, the potential of an eNose in the detection of human diseases has been demonstrated in a wide

range of conditions ranging from obstructive sleep apnea (Greulich et al. 2013) to renal dysfunction (Voss et al. 2005) and pregnancy (Bikov et al. 2011). Two of its important applications are the detection of urinary tract infection and of cancer, which are the focus of this literature review. Wilson and Baietto have written an excellent general review about eNoses and their biomedical applications (Alphus D. Wilson & Baietto 2011).

In vitro studies

The exact mechanism behind the distinctive odor of cancer remains unclear. Studies about LCa suggest that it might be partially explained by increased oxidative stress (Phillips et al. 1999). To find out whether any changes are present in a cellular level, a number of studies on eNose and cell lines have been conducted. Chen et al. reported similarities in the VOCs found in the breath of LCa patients and in cultured LCa cells, with higher concentrations of these molecules being present in the breath samples, suggesting that both systemic and cellular changes contribute to VOCs (Chen et al. 2007). ENoses have been able to detect LCa cell lines (Gendron et al. 2007), glioblastoma and melanomas (Kateb et al. 2009), as well as synovial sarcoma and thyroid cancer cells (Bartolazzi et al. 2010). A distinct smell profile proportional to tumor growth phase has also been observed (Pennazza et al. 2011). Together, these studies prove that cancer cells do indeed possess a distinct odor detectable by an eNose. It also seems that this odor is enhanced by the changes caused by systemic response to cancer. No studies of PCa cell lines have been published so far.

Clinical studies

Although alterations of VOCs in the breath of cancer patients was not a novel concept (Gordon et al. 1985), it was the study by Phillips et al. in The Lancet that finally introduced it to a larger audience (Phillips et al. 1999). They used GC-MS to analyze twenty-two VOCs - consisting mainly of alkanes - discriminating

between LCa patients and cancer-free patients with high sensitivity and specificity. Di Natale et al. compared the air exhaled by LCa patients scheduled for surgery to that of the medical staff. Some LCa patients provided another sample post-operatively. They achieved an overall discrimination rate of 90%. Interestingly, post-operative patients shifted towards the healthy population (Di Natale et al. 2003). These results were repeated by Machalo et al. with a larger sample, and - most importantly - a comparison was conducted between LCa and other air-way conditions diseases (Machado et al. 2005). Sensitivity and specificity were comparable to that of contrast-enhanced spiral computer tomography, suggesting that eNoses could provide a new tool for LCa screening. Since the publication of these trials, the number of studies on eNoses and the detection of LCa has rapidly increased. An outline of literature is presented in table 3. Several studies suggest that eNoses might be suitable for LCa screening, yet only Machado discusses the extent of the disease. Only LCa detected at a very early stage has a high survivability rate, due to the applicability of radical operative management in that stage (Henschke et al. 2006; Lim et al. 2010). Only a single study included patients belonging to this particular good-prognosis group (D'Amico et al. 2010). Future trials should focus on patients with early tumors and a good prognosis. If this approach proves successful, then a screening approach could be tested.

Even though the detection of LCa from exhaled breath has been the main research focus, other cancers and sample materials have also been tested. D'Amico described an eNose setup capable of detecting melanoma by comparing the skin headspace over a lesion to that of normal skin area (D'Amico et al. 2008). Horvath et al. have - in addition to the dog studies described earlier – used an eNose to discriminate between ovarian cancer and healthy ovarian tissue (Horvath, Chilo, et al. 2010). A study on the detection of head and neck cancer from healthy controls showed excellent discrimination capability; in this application, an eNose proved superior to GC-MS, mainly because GC-MS was too sensitive to confounding molecules (Hakim et al. 2011). A recent

study proved that an eNose could discriminate between stool samples taken from colorectal cancer and advanced adenoma patients and healthy controls. An eNose was significantly more sensitive in comparison to fecal immunochemical test for occult blood - the current tool for screening colorectal cancer -, suggesting that eNoses should be tested for screening application (de Meij et al. 2014).

Bernabei et al. conducted a preliminary study on the detection of urinary tract cancers from urine samples. They recruited patients with PCa, bladder cancer, BPH, and other urologic conditions (Bernabei et al. 2008). This study provided preliminary evidence about the possibility of detecting urinary tract cancers from urine, although a more detailed description of the patient population would be desirable; currently, many important characteristics such as the clinical staging of cancers and age distribution remain unreported. The study only reports the discrimination between cancer patients and healthy patients, leaving it unclear whether the healthy population only contained healthy reference patients, or also ones with BPH or other benign urologic conditions. The sample analysis was also complicated, using static headspace extraction and the injection of volatiles to the eNose via syringe. Peng et al. attempted to detect PCa - among other malignancies - from exhaled breath (Peng et al. 2010). PCas in the study population were mostly early stage I diseases, with eligibility for radical treatment and good prognoses. Although the reporting was somewhat lacking, as described in the context of LCa, the results suggest that cancer detection from breath is not limited to LCa, but is also possible for PCa, colorectal and breast cancer. Weber et al. used an eNose to discriminate bladder cancer from a urine headspace. They accounted for possible confounding factors by including patients with positive dipstick findings related to non-cancerous conditions (such as menstruation or UTI) in the control population. This varied control group may be the cause for inferior performance compared to that of most eNose experiments and the work of Bernabei. However, the authors noted that while their method's specificity was lower than with conventional urine

cytology, its sensitivity was higher. Unfortunately, the authors did not report a comparison between patients with UTI and normal patients (Weber et al. 2011). A conclusion of studies about eNose and cancer detection are presented in table 3 for LCa, and in table 4 for other cancers.

Table 3. A conclusion of studies concerning the detection of LCa by eNose

Study	Target	Samples	Controls	System	Sample delivery	Sensor	Analysis	Results
Di Natale et al. 2003	Identification of LCa	35 LCa	17 healthy	Libranose	Dynamic headspace extraction	8x quartz microbalance	PLS-DA	90 % discrimination (100% cancer)
Machado et al. 2005	Identification of LCa	28 LCa (14 used for training)	107 healthy or other lung disease (45 used for training)	Cyranose 320	Dynamic headspace extraction	32 conductive polymer sensors	PCA	sens 72 % spes 92 %
Westhoff et al. 2009	Identification of LCa	32 LCa	54 healthy	Prototype	Dynamic headspace extraction	IMS	LDA	100 % discrimination with CV
Dragonieri et al. 2009	Discrimination of non-small cell LCa and COPD	10 LCa	10 healthy 10 COPD	Cyranose 320	Dynamic headspace extraction	32 conductive polymer sensors	CDA	85 % discrimination
D'Amico et al. 2010	Discrimination of LCa, healthy and diseased control	28 LCa	36 healthy 28 other lung disease	Libranose	Dynamic headspace extraction	8x quartz microbalance	PLS-DA	86 % discrimination

sens = sensitivity, spes = specificity

Table 3. Continued

Study	Target	Samples	Controls	System	Sample delivery	Sensor	Analysis	Results
Peng et al. 2010	Detection of lung, colorectal, breast and PCa	30 LCa 26 colon 22 breast 18 prostate	59 healthy	Prototype	Dynamic heaspace extraction	20 gold nanoparticle sensors	PCA	No crossvalidation or numeric results
Dragonieri et al. 2012	Detection of malignant pleural mesothelioma	13 cancers	13 healthy 13 asbestos with asebestos exposure	Cyranose 320	Dynamic heaspace extraction	32 conductive polymer sensors	CDA	sens 9 2% spes 86 % with CV
Chapman et al. 2012	Detection of malignant pleural mesothelioma	22 cancers	42 healthy 18 with asbestos related disease	Cyranose 320	Dynamic heaspace extraction	32 conductive polymer sensors	PCA	sens 90 % spes 88 %

Table 4. A conclusion of other cancers studied with eNose

Study	Target	Samples	Controls	System	Sample delivery	Sensor	Analysis	Results
Bernabei et al. 2008	Discrimination of urinary tract cancers and healthy patients from urine headspace	25 bladder cancer 12 PCa	29 BPH 53 other urologic conditions 18 healthy	ENQBE (prototype)	Static headspace extraction	8 quartz microbalance	PCA, PLS-DA	100 % ill versus healthy
D'Amico et al. 2008	Detection of melanoma from skin headspace	10 melanomas	30 other skin lesions	Prototype	Dynamic headspace extraction	7 quartz microbalance	PLS-DA	80 % discrimination
Horvath et al. 2010	Discrimination of ovarian cancer and normal tissue	23 grade III ovarian cancers	9 samples of normal uvarian and uterine tissue	Unspecified commercial eNose	Dynamic headspace extraction	4 Metal oxide sensors	PCA and neural networks	sens 84 % spes 87 %
de Meij et al. 2014	Discrimination CRC from healthy controls and adenomas from fecal headspace	40 CRC 60 advanced adenomas	57 healthy	Cyranose 320	Dynamic heaspace extraction	32 conductive polymer sensors	PCA, CDA	sens 85 % spes 87 %
Hakim et al. 2011	Detection of head and neck cancer from breath	22 head and neck cancers	40 healthy 25 LC	NA-NOSE (prototype)	Dynamic heaspace extraction	not specified	PCA	sens 100 % spes 92 %
Weber et al. 2011	Detection of bladder cancer from urine headspace	30 bladder cancers	59 (healthy, menstruating and UTI)	NST 3320 Lab emission analyzer	Dynamic heaspace extraction	12 MOS 10 MOSFET	PLS-DA	sens 60 % spes 67 %

sens = sensitivity, spes = specificity

6.6.6 eNoses in the Detection of Urinary Tract Infection.

Vernat-Rossi et al. first attempted to discriminate the bacteria associated with meat spoilage with MOS-based sensors, achieving a fairly high discrimination rate of 86% (Vernat-Rossi et al. 1996). Gibson et al. also achieved good discrimination rate and reproducibility while classifying 12 bacteria and a singular yeast species (Gibson et al. 1997). They employed a plastic bag of 1.5 liters for creating a stable headspace. Schiffman et al. paid considerable attention to discriminating between fungal species, but their experiment also included ten bacterial strains. The performance of Gibson et al. which resulted in a discrimination rate of 83%, could not be fully reproduced (Schiffman et al. 2000). Lykos et al. attempted to discriminate bacteria mixed with human blood, but the small sample provided only anecdotal evidence about the detection of bacteria from blood; especially because no cross-validation was performed (Lykos et al. 2001). Pavlou et al. managed to discriminate cultures of *clostridium* species and *Bacteroides fragilis*, demonstrating the ability of eNoses in discriminating anaerobic bacteria by employing the plastic bag approach of Vernat-Rossi (Pavlou et al. 2002). Kodogiannis worked with the same setup, but put a greater emphasis on the advanced use of neural networks. An overall prediction rate of 94% was achieved (Kodogiannis 2005). There was a break in the use of eNoses in bacterial detection until 2009, when Bruins et al. published their study of MonoNose. The study design was largely similar to previous studies, attempting to discriminate between 52 samples of 11 different bacterial species. The authors studied the possibility of a device-to-device variation by producing a patch of 30 MonoNose devices, which were all operated during the study. They controlled time- and device-independency by measuring each sample on three different days with three different devices. The discrimination rate varied from the 67% of *E. cloacae* to the 100% of *C. difficile*, with the average discrimination rate being 87%. However, they did not conduct a cross-validation, but instead tested a discrimination model created with the same sample. Thus, there is no evidence about the generalizability of their results.

Another drawback in their study was their use of a single sensor, which makes it necessary to rely on time-dimension as the source of variation, which in turn leads to excessively long measurement periods ranging from 12 to 14 hours (Bruins et al. 2009).

A study about the detection of UTI with an eNose was first published by Aanithan et al. in 2001. They employed the Osmetech Microbial Analyzer, an automated eNose device that feeds samples to the sensor with a carousel. The samples are prepared by acidification with hydrochloride and addition of sodium sulfate, which increases the device's sensitivity. The device was initially tested with artificial samples made by infecting commercial human reconstitute urine (HRU), and then with over 500 clinical urine samples received from a local laboratory. The samples were analyzed after an overnight culturing. They reached a high specificity rate of 90%, but also a somewhat disappointing sensitivity rate of 72% (Aathithan et al. 2001). Pavlou et al. attempted a similar approach. After an extraction of eukaryotic cells and 4.5 hours of incubation they placed the MSU sample in a bubbling device and led a gas stream from the sample into the eNose. A discrimination rate of 93%-100% was achieved with the use of neural networks (Pavlou et al. 2002). Kodogiannis et al. experimented with urine samples by using a very similar protocol (filtration, a 5 hour incubation without bubbling). A 100% accuracy was achieved through a fusion of two neural network models (Kodogiannis & Wadge 2005).

These studies demonstrate eNose's potential in detecting bacteria directly from urine after a short period of incubation. The Osmetech Microbial Analyzer attained FDA approval and is now available as a commercial product. In 2010, Osmetech was acquired by a gene technology company GenMark, likely for its genetic testing products. The acquisition apparently proved to be the undoing of the Osmetech Microbial Analyzer, since nowadays no mentions of it can be found in the GenMark website. The factors that led to this result remain unknown. We can only speculate whether the Sub-Prime crisis in 2008 led Osmetech to

financial turmoil, or if a lack of advanced computational methods prevented the device from succeeding commercially.

A conclusion of studies concerning bacterial discrimination and UTI diagnosis is represented in table 5.

6.6.7 Concluding Remarks

Overall, it can be concluded that eNoses are capable of discriminating cultured bacterial strains with high accuracy. A commercial product has already been developed, but it has since then disappeared from the market. There is still an unmet demand for point-of-care devices usable for UTI diagnosis, since every solution described so far is designed for laboratory use. A compact eNose device, combined with a neural network approach, has the potential of being a point-of-care device that could be brought to a patient's home.

Table 5. A conclusion of studies examining discrimination of bacteria by eNose

Study	Target	Samples	System	Sample delivery	Sensor	Analysis	Results
Vernat-Rossi et al. 1995	Discrimination between bacterial strains and meat products	*S. carnosus, S. xylosus, M. varians, S. warneri, S. aureus, S. saprophyticus, S. epidermis* = 42	FOX 2000 Alpha M.O.S.	Dynamic headspace extraction	6 MOS sensors	LDA	Overall classification 86% with CV.
Gibson et al. 1997	Identification of micro-organisms from headspace	*E. Coli, P. aeruginosa, C. freundii, E. aerogenes, B. cereus, K. aerogenes, C. albicans, S. aureus, S. epidermis, S. reading, S. poona, S. gamiarium* Total n=244	Bloodhound BH114 (commercial)	Dynamic headspace extraction	14 conductive polymer sensors	Neural network	Overall classification 93.4 %
Schiffman et al. 2000	Discrimination between bacterial and fungal growth	*E. coli, E. cloacae, group D enterococcus, C. freundii, K. pneumonia, S. agglutin negative, S: aureus. S. fonticola, P. mirabilis, P. aruginosa*	NC State eNose (non-commerial)	Dynamic headpace extraction	15 MOS sensors	LDD, K. nearest neighbors, least squares analysis	Overall classification 82.5 % with LOOCV
Lykos et al. 2001	Discrimination of bacteria mixed with human blood	*E. coli, P. aruginosa, S. aureus, E. faecalis*	IPRO eNose (non-commercial)	Dynamic headspace extraction	Seven undisclosed electrochemical sensors	PCA	n/a
Pavlou et al. 2002	Discrimination of anaerobic bacterial cultures	*Clostridium spp, B. Fragilis* n= 24	Bloodhound BH114 (commercial)	Dynamic headspace extraction	14 conductive polymer sensors	Neural network	Overall discrimination of 94 % with CV

sens = sensitivity, spes = specificity

Table 5. Continued

Study	Target	Samples	System	Sample delivery	Sensor	Analysis	Results
Kodogiannis 2005	Discrimination of bacterial cultures	*E. coli, Citrobacter spp, E. cloacae, E. faecalis, Enterococcus spp, K. oxytoca, K. pneumonia, Lactobacillus spp, Proteus spp. P. mirabilis, S. aureus, P. oeruginosa, S. pyogenes* n=248 (48 used for testing)	Bloodhound BH114 (commercial)	Dynamic headspace extraction	14 conductive polymer sensors	Neural networks	Discrimination of 94 %
Bruins et al. 2009	Discrimination of bacterial cultures	*C. difficile, E. cloacae, E. faecalis, E. coli, K. oxytoca, K. pneumonia, P. mirabilis, P. aruginosa, S. enteriditis, S. typhimurium, S. aureus*	MonoNose	Static headspace extraction	Single MOS sensor	Variance matching	Discrimination of 67-100 %, mean 87 %
Studies concerning detection of detection of UTI from incubated urine:							
Aathithan et al. 2001	Detection of UTI from sample cultured urine	*E. Coli, E. faecalis, S. aureus, Klebsiella spp, S. sapprophyticus, P. mirabilis* RHU: n = 36 clinical n= 534	Osmetech microbial analyzer (commercial)	Dynamic headspace extraction	Multiple polymer sensor array	PCA	Sensitivity 72 % Specificity 90 %
Pavlou et al. 2002	Detection of UTI from urine	*E. coli, P. mirabilis, Staphylococcus spp*, normal n = 70 (25 used for testing)	Bloodhound BH114 (commercial)	Dynamic headspace extraction	14 conductive polymer sensors	Neural network	Overall classification 93-100 %
Kodogiannis et al. 2005	Detection of UTI from urine	*E. coli, Proteus spp*, coagulase-*Staphylococcus* speciesn=40 (14 used for testing)	Bloodhound BH114 (commercial)	Dynamic headspace extraction	14 conductive polymer sensors	Fusion of two neural networks by fuzzy integral	Overall 100 %

sens = sensitivity, spes = specificity

7 AIMS OF THE STUDY

The aim of this study was to evaluate the ability of the eNose to detect PCa, and to explore its performance in the detection of urinary tract infection - a condition likely to affect the device's performance - in a clinical context. The hypotheses were as follows:

1. The eNose is able to discriminate the cultured cell models of benign prostatic hyperplasia (EP-156T) and prostatic carcinoma (LNCaP), and the smell print is secreted to the surrounding medium (**I**)

2. A method for accurate and simultaneous detection and quantification of free-, mono-, and diacetylated polyamines from urine with LC-MS/MS can be developed (**II**)

3. The eNose is able to discriminate between PCa and BPH in patients referred for surgery. (**III**)

4. The eNose is able to discriminate between different urinary pathogens. (**IV**)

8 MATERIALS AND METHODS

8.1 Samples

8.1.1 Study I

Two cell lines were selected for the study: a telomerase-immortalized prostate epithelial cell line, EP-156T, considered as being as benign as possible (Kogan et al. 2006), and a prostate adenocarcinoma line, LNCaP (Horoszewicz et al. 1983). They were cultured according to the manufacturer's guidelines (American Type Culture Collection–LGC Standards, Teddington, UK for LNCaP and V Rotter; Weizmann Institute of Science, Rehovot, Israel for EP-156T cells) in 10 ml of their media in 100 × 20-mm cell culture plates (NUNC International, NY, USA). The samples were collected for analysis when cells were approximately 80% confluent; after 4 days for LNCaP and after 6 days for EP-156T. Their media were collected and frozen (at -80°C; conditioned medium) and the cells were trypzined and collected by centrifugation (200 × g, at room temperature). The cells were then washed once with phosphate-buffered saline and recentrifuged, after which the formed cell pellets were frozen (at -80°C). For the control samples, the cell-free media were incubated, handled and frozen (at -80˚C) in a similar manner to the actual samples (unconditioned medium). These control media (unconditioned media) were then collected and frozen at -80˚C to be stored until the eNose analysis. Samples consisting of 5 ml of sterile water were prepared and used as controls.

8.1.2 Study II

The study population consisted of patients that had been referred to a urologist for operative treatment in Tampere University Hospital (Tampere, Finland) and of normal healthy controls. The criteria for exclusion were the following: a patient's refusal, insufficient material for histopathology, a known malignancy other than PCa, persistent urinary infection, and a urinary catheter in place. The sample consisted of confirmed PCa patients (n = 22, men, aged 49–73) scheduled for a robot-assisted radical prostatectomy and of healthy controls (n = 6, men, aged 22–38 years). The subjects provided a routine morning urine sample. Urine was collected without preservatives, aliquots were taken for a separate analysis for creatinine. The urine samples were stored within two hours of their collection at −70 to 80°C until the measurements.

8.1.3 Study III

The study population was expanded from that of study II to contain 50 biopsy-confirmed PCa patients BPH Patients (n=15) scheduled for a transurethral resection of the prostate were recruited as controls. Benign histology was confirmed from resected prostate tissue. Three of the BPH patients had previously undergone negative prostate biopsies. The healthy control group of Study II was omitted from this study.

All patients in the control group gave urine samples preoperatively, and 9 of them provided another sample 3 months after the operation, for a total of 24 samples. Since the measurement of postoperative prostate volume was not included in the study protocol, we reduced the prostate volume by 40% in the postoperative results. Subjects provided a standard morning urine sample before the operation and 3 months after the operation. No standardization of diet, hydration status or bladder time was conducted. The samples were stored at -70°C until the eNose analysis.

The baseline clinical characteristics of the patients are provided in Table 6.

Table 6. The baseline clinical and pathological characteristics of the subjects in the Study III.

Clinical variables	Confirmed PCa group (n= 50)	Control group (n= 24)
Age		
mean, yr (range)	62 (49-73)	67 (53-72)
Total PSA		
mean, ng/ml (range)	7.0 (2.0–18.0)	3 (0.2-9.0)
Prostate volume		49 (18–100)
mean, ml (range)	37 (15–75)	
PSA density		0.05 (0.02-0.20)
mean (range)	0.18 (0.04-0.70)	
Gleason score (post-op)	9	N/A
6	34	N/A
7	1	N/A
8	6	N/A
9		
pT status		
pT2	27	N/A
pT3	23	N/A
Presence of Gleason ≥ 4	41	N/A

PCa= prostate cancer, PSA= prostate specific antigen, PSA density = Total PSA / Prostate volume

8.1.4 Study IV

The four most common UTI pathogens, *Escherichia coli, Staphylococcus saprophyticus, Klebsiella species and Enterococcus faecalis*, were included in the study. Pure culture samples were conducted in 92 mm x 16 mm polystyrene Petri dishes containing a cysteine lactose electrolyte deficient (CLED) medium. The urine samples for pure cultures were acquired anonymously from patients of Tampere University Hospital, which had been collected and processed by Fimlab laboratories, the provider of laboratory services for Pirkanmaa Hospital District.

The sample contained a total of 101 samples, which included all four pathogens as well as reference samples containing only the CLED agar. A detailed description of the samples is presented in table 7. All samples pathogen-containing samples were marked with an individual identification number and were stored in a refrigerator between measurements (2 - 8ºC).

Table 7. A description of the samples in study IV

Pathogen	Number of samples
Escherichia coli	20
Staphylococcus saprophyticus	19
Klebsiella species	20
Enterococcus Faecalis	21
CLED agar	21

8.2 Analytical techniques

8.2.1 Study II

Creatinine determination

The creatinine concentration of the urine samples was determined enzymatically with a Cobas 6000, C 501 – module (Roche diagnostics GmbH, Mannhein, Germany) in Fimlab laboratory (Tampere, Finland).

LC–MS/MS instrumentation and analytical conditions

Validation was performed according to the FDA guideline with bioanalytical method validation (FDA 2001). LC separation was performed with an Agilent 1200 Series Rapid Resolution LC System. Mass analysis was carried out with an Agilent 6410 Triple

Quadrupole mass spectrometer equipped with an electrospray ionization source. Agilent MassHunter Workstation software (Agilent Technologies, Palo Alto, CA, USA) was used for data acquisition. The employed reversed-phase column was Phenomenex Kinetex C18 (150 mm × 2.1 mm, 2.6 μm) protected with a Phenomenex SecurityGuard Ultra C18 guard cartridge for 2.1 mm ID columns (Phenomenex, Torrance, CA, USA). The eluent flow rate was 200 μl/min, and the eluents were 0.1% Heptafluorobutyric acid (HFBA) in water (eluent A) and 0.1% HFBA in ACN (eluent B). A linear mobile phase gradient was used; the eluent B was increased from 5% to 40% in 10 minutes and held at 40% B for 1.5 minutes. The gradient was then decreased from 40% to 5% of eluent B in 0.1 minutes, and the column was stabilized with a 6.4 minute flow of 5% eluent B for a total run-time of 18 minutes. The column temperature was held at 35ºC and the autosampler temperature at 4ºC. A sample volume of 10 μl was used. A divert valve was used to direct the eluent flow into the mass spectrometer for a timespan ranging from 2.8 minutes to 12 minutes. A positive electrospray ionization (ESI) was used as an ionization source. Nitrogen (temperature300°C) was used as a drying gas at a flow rate of 8 l/min. The nebulizer pressure was 40 psi and the capillary voltage was 4000 V. Detection was performed with multiple reaction monitoring. Four time segments were used during the analyses, their turning points being 2.8, 4.3, 6.9 and 9.5 minutes. Dwell times were 20 ms, and mass resolution (peak width) for MS1 and MS2 quadrupoles was 0.7 FWHM for each transition. Collision induced dissociation (CID) was made with nitrogen. Deuterated internal standards were used in the quantification.

The preparation of standard working solutions for calibration standards and IS

The standard solutions for each polyamine were created for concentrations of 5, 10, 30, 50, 100, 300, 500, 1000, 3000, 5000,

10,000 nM. The IS stock solutions were mixed and diluted with water in order to make a solution with an IS concentration of 1000 nM. Calibration curve quality control (CC) samples were prepared for concentrations of 30, 300, 1000, and 5000 nM.

The sample preparation for polyamine LC–MS/MS determination

The urine samples were thawed at room temperature and centrifuged for 5 min, 13000 rpm. Water and IS were then mixed with the urine samples. 550 µl of the sample was then passed through solid-phase extraction (SPE) cartridges (Phenomenex Strata SAX cartridges, 100 mg/3 ml (Phenomenex, Torrance, CA, USA). The sorbent was conditioned with 1 ml of methanol and equilibrated with 1 ml of water before the sample handling. After the IS spiked urine had been eluted, 450 µl of water was passed through the cartridge into the sample. The samples for LC–MS/MS analysis were then prepared by mixing 100 µl of the SPE treated sample and 25 µl of 10% HFBA, and then centrifuged for 20 min, 13,000 rpm, 4ºC. Diluted urine samples were prepared by water-diluting the urine samples with a 1:10 ratio before the sample preparation. Calibration standards and CC samples were prepared in a similar manner to the urine samples. In addition to the eleven standard levels, the calibration curve included a blank sample that contained neither standard nor IS, and a zero sample which had received only IS.

Quality control samples

The endogenous concentration of polyamines taken from the urine samples of six healthy men was measured. The samples were then pooled by mixing an equal amount of urine from each subject. The resulting pooled urine (QC matrix) was used to prepare quality control (QC) samples in the same fashion as was

done with the calibration samples. The pooled urine QC matrix without added IS was used as a cross-talk check.

8.2.2 Studies I, III and IV

eNose–sensor & measurement chamber used in studies I and III

A commercially available eNose (ChemPro® 100, Environics Inc., Mikkeli, Finland) was used in the study. It is based on an ion mobility spectrometer-type flow-through system and utilizes ambient air as a carrier gas. The commercial application of the device lies in its ability to detect hazardous gases, which makes it usable for military and industrial applications. There are no prior publications about its use in medical applications. The device consists of two sensor clusters which produce measurement data in both relative and absolute scales, an ion mobility cell (IMCell) which contains eight electrode strips producing a 16-channeled output, and a metal oxide-based semiconductor cell which produces two channels of output. The IMCell contains a radiation source of Am-234 5.92 MHq and a heater element for maintaining constant temperature. The metal oxide-based semiconductor cell contains a screen-printed WO_3 gas sensor. In addition to the aforementioned features, the device also contains humidity and temperature sensors and a continuous flow pump. A schematic drawing of the sensor is presented in Figure 4. The IMCell temperature was set at 27.8°C and the flow at 1.05 l/min during the present study's measurements. The eNose was connected to a Windows-based computer containing software (Environics Inc., Mikkeli, Finland) for real-time monitoring and data logging. The measurement chamber consisted of a standard 100 × 20 mm polystyrene cell culture plate, and its cover was built from a standard polystyrene cell culture plate cover, into which three holes were drilled; two holes were meant for air replacement, while a modified 16G intravenous cannula was inserted into the third.

The film from the injection port was removed, and the injection port was used to provide sample air for the eNose through a Teflon® (Dupont, DE, USA) pipe. A similar design has been reportedly used previously. The cover was secured to the plate with parafilm. The covers were recycled throughout the study and washed (with sterile water) and dried between measurements. Each cell culture plate was used only once.

The cell and culture samples were prepared by adding 250 µl of defrosted sample material to 4.75 ml of sterile water, which resulted in a thin fluid film over the plate, thus maximizing the area for evaporation. It was theorized that the hypotonic water would cause the cells to disrupt, thereby maximizing the release of VOCs. The urine samples were also defrosted and 5ml of urine was added to the plate. The sample plate was heated and kept at 37°C with a water poultice, and afterwards kept in a laminar flow cabinet for the measurement cycle's whole duration. A photograph of the device and the measurement setup can be found in Figure 5.

Figure 5. A photograph of the ChemPro 100 device and the setup used in studies I and II. In the front, a ChemPro 100 –eNose is connected to the measurement chamber with a Teflon® pipe.

Each measurement lasted for approximately 15 minutes, and a 10 minute pause was taken between the samples for avoiding carryover. These time intervals had been found sufficient in pre-study experiments. The highest absolute values taken during the measurement period were extracted for a further analysis. Each sample was measured only once, except the conditioned medium samples of Study I, wherein four measurements were conducted in a single sample. Two of the 18 channels gave no response during the measurements and were thus ignored, leaving 16 channels for analysis. The samples were analyzed in a random order. In Study III, the persons performing the measurements were blinded to the patients' statuses.

eNose–sensor & measurement chamber used in study IV

A successor to the eNose used in studies I and III was employed in study IV (ChemPro® 100i, Environics Inc., Mikkeli, Finland). It features an IMCell identical to that of the previous model, but it also has four additional metal oxide-semiconductor cells designed to enhance the device's sensitivity and specificity. The physical proportions of the device are similar to the ChemPro 100 device illustrated in Figure 5. The flow was set at 1.30l/min, and the sensor temperature was 31.5°C on the average.

In this analysis, the data received from the additional sensors was omitted in order to provide a maximal comparability with previous studies; thus, the 18-channeled data was used for the analysis.

The measurement chamber employed in this study was an evolutionary version of the one used in studies I and III. In order to minimize background noise, the received air was brought to the measurement chamber through an activated carbon and molecular sieve before being fed to the sensor.

8.3 Mathematical Analysis

Study I

The analysis methods serve two purposes: visualization and classification. Before the analysis, the data was normalized for emphasizing the smell print profile produced by the eNose, and to reduce possible offset in the responses. Each sample x_i is divided by the sum of the 16 channels' absolute values. Thus, the sum of the absolute values of the normalized sample $x'_i=1$:

$$x'_i = \frac{x_i}{\sum_{i=1}^{16} |x_i(j)|}, \sum_{i=1}^{16} |x'_i| = 1$$

A visualization of the 16-dimensional space requires dimension reduction. Principal component analysis is a linear projection of high-dimensional data to lower dimensional space while retaining the maximum variance of the data (Lee & Verleysen 2007). Multidimensional scaling (MDS) transforms the data to a lower dimension so that the interpoint distances between the data points in the new space are as similar as possible to the original ones. MDS also allows nonlinear transforms; for example, Sammon mapping (Lee & Verleysen 2007), which is used for MDS projection in this study. For visualization, the data is transformed to two dimensions with Sammon mapping. The axes in figure 6 are the directions that optimally maintain interpoint distances.

Study III

Classification uses the measured data for assigning each sample into predefined classes. In this case classifiers that use the eNose

measurements to distinguish the cell lines from water, media and from each other, are identified. Linear discriminant classifiers were used (Seber 1984). Each sample was scaled by dividing each element (16 channels) with the L2-norm of the vector in order to emphasize the smell print profile produced by the eNose. Thus, the L2 norm of each scaled sample equaled one. LDA was used to assign each sample into predefined classes; PCa or BPH.

Pointwise confidence intervals for the ROC curves were computed as bias corrected and accelerated percentiles taken from 500 bootstrap samples (DiCiccio & Efron 1996). The optimal thresholds were identified as the upper leftmost contact points between a straight line with a slope S and the ROC curve. Assuming equal costs for false positive and false negative classes, the slope S is reduced to the ratio N/P; the number of negative samples divided by the number of positive samples (Metz 1978).

Finally, the correlations between the eNose data with the prostate volumes in the BPH group and with the tumor volumes in the PCa group were examined with a multilinear regression model, predicting the prostate size using eNose data. A stepwise regression was performed using the p values 0.05 and 0.1 for including and removing variables from the model, correspondingly 25. The same procedure was applied for examining the correlation between the eNose data and tumor size.

Study IV

A sample analysis was designed to mirror the clinical decision making. In the first phase, we created a classifier, which discriminates the samples to either sterile or infected group. We per-formed additional classification for the infected group with the aim of identifying the bacterial species in question.

Linear discriminant analysis (LDA) and logistic regression (LR) were employed to identify the classifier for the discrimination between sterile and bacterial samples. Just LDA was employed in

the identification of bacterial species, since LR is only suitable for discrimination into two classes.

All results from the studies I, II and IV were validated via leave-one-out cross validation (LOOCV), which tests the generalizability of the classifier by determining a single data point's effect on the classification (Bishop 2006).

9 ETHICAL CONSIDERATIONS

A written, informed consent was acquired from every patient. The study was approved by ethical committee of Tampere University Hospital (code: R10066).

10 Results

10.1 Study I

The eNose discriminated the cell and medium samples from the water samples with a MCR of 1.4–1.7 % using LDA (Table 8). The MCRs of five- and ten- fold cross-validations are the averages of 50 random divisions into groups of five and ten. This was done to compensate for the small sample size. Malignant (LNCAP) and nonmalignant (EP-156T) cell lines were discriminated with a MCR of 2.9–3.6 % (Table 8 and figure 6). We also studied whether cells affect the smell print of their medium by comparing the cell samples with both conditioned and unconditioned media. This was also done in order to rule out the suspicion of differences between cell lines being caused by cell medium remnants. The conditioned media of EP-156T differed from the cell samples (MCR: 5.3 %), whereas the conditioned media of LNCaP did not (table 9). On the other hand, the smell prints of the unconditioned media of both EP-156T and LNCaP, differed from the cell samples, suggesting that cells modify the smell print of their medium. MCRs for LNCaP and EP-156T were 24–26 and 0.0–.90 %, respectively (Table 9, figure 6).

Table 8. Misclassification rates for the classification of cell lines and medium against water, of cell lines against each other, and of cell lines against their respective conditioned and unconditioned media using 16 measurement channels. (Reproduced with the permission of Future Medicine Ltd.)

Classification	Cell line	Whole sample	Leave-one-out	5-fold	10-fold
Cell & medium vs. water	All	0%	1.4%	1.7%	1.6%
Cell vs. cell (2 classes)	All	0%	3.6%	2.9%	3.5%
Cell vs. unconditioned medium	LNCaP	0%	24%	NA	26%
	EP-156T	0%	0%	6.0%	0.9%
Cell vs. conditioned medium	LNCaP	NA	NA	NA	NA
	EP-156T	0%	5.3%	NA	NA

Table 9. Misclassification rates for the classification of cell lines against their respective conditioned and unconditioned media using three dimensions by MDS and PCA. (Reproduced with permission of Future Medicine Ltd.)

Classification	Cell line	Projection type	Whole sample	Leave-one-out	5-fold	10-fold
Cell vs. unconditioned medium	LNCaP	MDS	4.8%	9.5%	10.7%	10.4%
		PCA	4.8%	9.5%	10.3%	9.5%
	EP-156T	MDS	4.4%	4.4%	4.4%	4.4%
		PCA	0%	4.4%	4.1%	4.4%
Cell vs. conditioned medium	LNCaP	MDS	18%	24%	NA	NA
		PCA	18%	30%	NA	NA
	EP-156T	MDS	5.3%	24%	NA	NA
		PCA	5.3%	30%	NA	NA

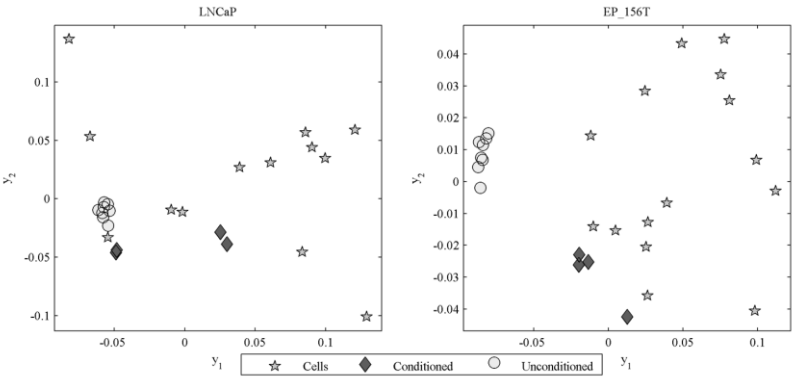

Figure 6. A Sammon projection of samples. Note the clustering of cells, conditioned and unconditioned media. (Reproduced with the permission of Future Medicine Ltd.)

10.2 Study II

Sample preparation

The use of SPE cartridges allowed the extraction of DiAcPUT and DiAcCAD and significantly reduced clogging and contamination of the column.

LC-MS/MS method

HFBA was used as an ion-pairing agent in LC. Multiple columns were tested in order to separate the analytes from each other and the matrix molecules. Phenomenex Kinetex C18 (150 mm × 2.1 mm, 2.6 μm) provided the best performance. The retention times of IS in DiAcPUT and DiAcCAD slightly deviated from the analyte, with deuterated analogs eluting earlier from the column (see Figure 7), indicating that ME is still present and the quantification is only quasi-quantitative. Selected reaction monitoring (SRM) was used in MS/MS.

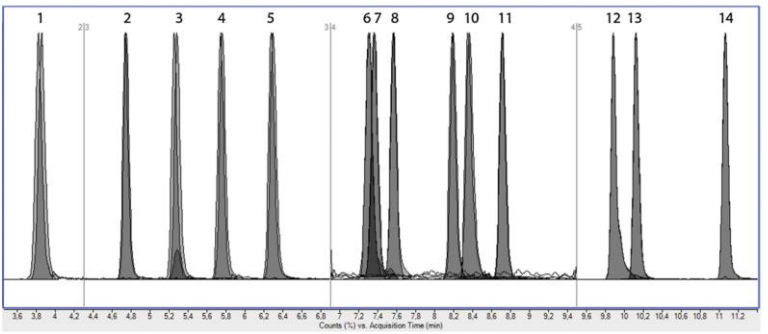

Figure 7. The overlaid chromatograms of 14 polyamines and their deuterated internal standards. All peaks are scaled to the same height. DiAcPUT (peak 1) and DiAcCAD (peak 3) deviate from their deuterated ISs (Reproduced with the permission of Elsevier.).

Method validation

Calibration standards including both blank and zero sample were analyzed before the samples in each analysis batch in order to verify the calibration curves' accuracy and precision. The selection of the best curve fit was based on comparing the sum of standard deviations from their nominal concentrations with the weighing scheme that gave the smallest sum of deviations (1/x2). Correlation coefficients were also calculated. The acceptance value for intra- and inter-run precision error (RSD %) and accuracy were <15% and 85–115% for every standard; with the exception of the lowest calibration point, where the precision error limit was 20%. The calibration ranges for each polyamine are shown in table 10. The detection limit for each polyamine standard in water after the sample preparation was defined as the concentration which had a signal-to-noise value of ≥3 (see Table 10, fmol/injection).

Selectivity and matrix effect

Selectivity and ME were assessed in several ways. Urine samples taken from six healthy individuals - measured without IS added - did not include interfering components in the SRM channel for

the internal standard at the retention time of interest. For DiAcCAD, some of the low concentration urine samples did contain endogenous components that emitting a signal close to the analyte's retention time, and thus some of the samples may not be quantified reliably. In addition, while the SRM transition for DiAcPUT standard (m/z 173–114) provided the best sensitivity, the urine matrix had components interfering with the peak of interest and, therefore, this transition was only monitored for confirmatory purposes. Carry-over was investigated via analyses of the highest standard samples (10000 nM), followed by analyses of the blank samples. The carry-over percentage was determined by comparing the blank samples' peak areas against the standard solutions' peak areas. The mean carry over values (n = 3) of the first and second blank were 0.038 % and 0.014 % for SPM, 0.024 % and 0.006 % for AcSPM, 0.022 % and 0.015 % for SPD, and 0.012 % and 0.002 % for DiAcSPM. The carry-over was below 0.01 % for the remaining analytes. A matrix factor was used to estimate the strength of the ME caused by the urine matrix as shown in Figure 8.

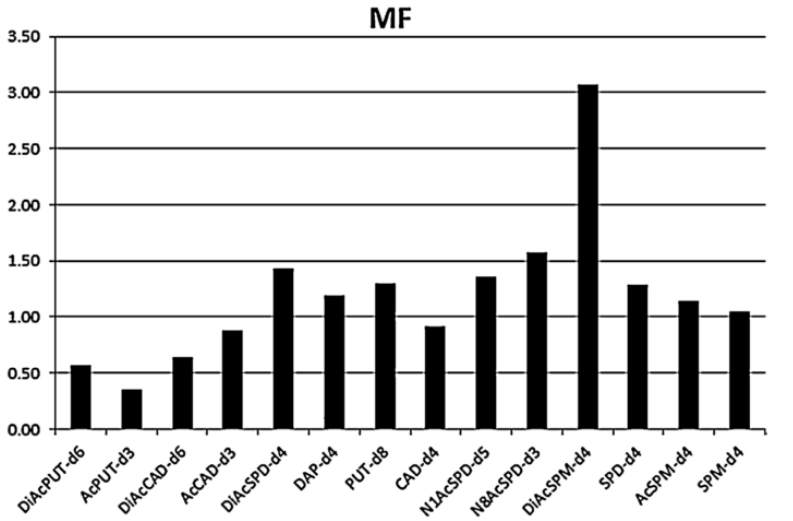

Figure 8. A visualization of a matrix factor for deuterated ISs. (Reproduced with the permission of Elsevier)

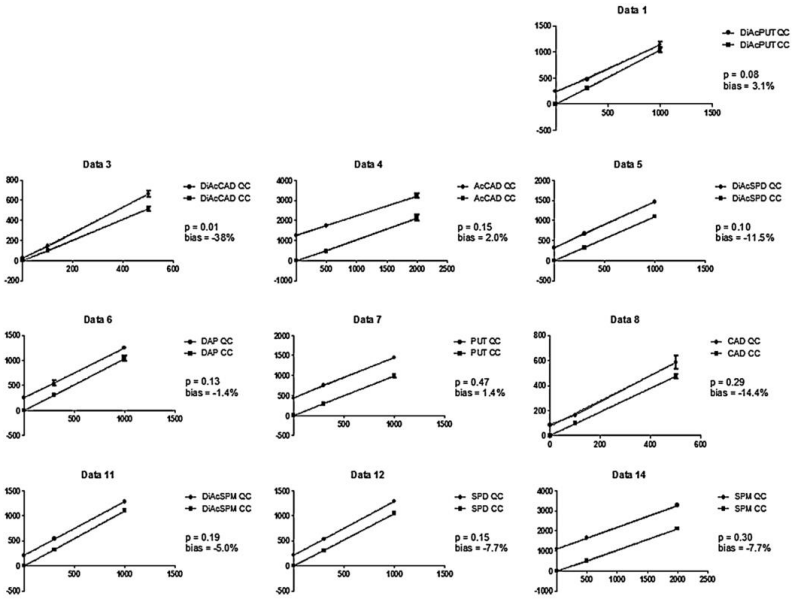

Figure 9. The addition of polyamines to water (CC) or urine (QC) before the sample preparation. Bias (%) is the relative error between the endogenous concentration calculated by applying STD addition to urine or water. The endogenous concentrations of AcPUT, N1AcSPD and N8AcSPD were too high for an STD addition, and the endogenous concentration of AcSPM was too far below the calibration range. Note that the calibration lines are unparalleled in regards to DiAcPUT and DiAcCAD, indicating the presence of ME in the urine. (Reproduced with the permission of Elsevier)

Three samples were made at each concentration level and analyzed afterwards. Standard addition curves were drawn and slopes were calculated (figure 9). If the lines' slopes were parallel, then the IS could compensate for the ME in the samples, making and water based standards usable for quantitation. For DiAcCAD, the slopes differed in urine and water samples, making the results for DiAcCAD no more than quasi-quantitative. A dilution linearity was measured in order to test whether the high concentration samples could be diluted with water without affecting assay accuracy. Dilution linearity was tested with both unspiked QC-urine with a known endogenous concentration and QC-urine spiked with 1000 nM polyamine standards. The unspiked samples were analyzed after 0, 4, 10 or 20-fold water dilution, whereas

spiked samples were analyzed after 0, 5, 10 or 20-fold dilution. The mean back-calculated concentrations, corrected according to dilution, were compared to the theoretical total concentration (measured endogenous plus spiked analyte). There were no significant changes in any analyte's accuracy after an up to 5-fold dilution, and the most abundant urinary polyamines (AcPUT, N^1AcSPD and N^8AcSPD) could be analyzed from the 1:20 diluted samples, if necessary.

Stability studies

The polyamines' stability in water was tested by analyzing solutions of 300 and 3000 nM after a 6 h storage (short term stability) at room temperature, and after a two-month storage (long term stability) at −20°C. The effect of thawing was studied by completing three cycles of freezing during the −20°C analysis. The polyamines' stability in urine was studied by analyzing the samples after a 6 h storage at room temperature, after a two-month storage at −80°C (long term stability), and after three cycles of freezing at −80°C. Polyamines were found to be stable in both urine and water solutions.

Accuracy and precision

QC samples were prepared from the pooled urine of healthy individuals for verifying the accuracy and precision of the study samples. Since cancer patients were expected to yield higher polyamine levels, a 1:1 dilution of the QC matrix was expected to be a sufficient limit of detection. Intra- and inter-batch precision and accuracy were measured by the analysis of QCs over four separate analytical batches; each contained four levels of QCs at low, basal, middle and high concentrations, and each had five replicates. The back-calculated concentrations of QCs were measured against the surrogate matrix calibration line; this was done by using the analyte's relative peak area response to IS against

the theoretical concentration of the calibration standards. The assessment of precision and accuracy was performed by analyzing multiple aliquots of both unspiked and spiked samples of the QC matrix against the surrogate calibration curve. The unspiked sample represented the mean endogenous background concentration. Accuracy was calculated by comparing the mean experimental concentrations of measured QC samples to their nominal values, and the received percentage values were then used as the index. The concentrations' relative standard deviation was used as an index of precision, with an acceptance criteria of 15%. The mean endogenous concentrations in pooled QC-urine are shown in table 10.

Table 10. The calibration ranges, the limit of detection (LOD) and the concentrations of different polyamines in pooled QC-urine. LOD and LLOQ (the lowest calibration point) are measured and validated using water based standards according to the FDA guidelines (FDA 2001). (Reproduced with the permission of Elsevier.)

	Calibration range (nM)	LOD (S/N0>=3, fmol/injection)	QC-urine concentrations (nM) mean ± sd, n=20
DAP	243-8114	71.4	219±36
PUT	103-10250	45.1	485±37
CAD	31-10472	27.6	88±12
AcPUT	25-8396	3.7	12785±488
AcCAD	24-8136	7.2	1041±67
SPD	30-10145	4.5	208±10
DiAcPUT	30-5000	8.8	228±31
DiAcCAD	5-5000	2.6	25±5
N1AcSPD	27-9083	8.0	5828±165
N8AcSPD	30-10145	4.5	4349±150
SPM	32-10552	9.3	1170±39
DiAcSPD	6-11587	1.0	361±12
AcSPM	5-4881	0.9	<<5
DiAcSMP	4-8612	0.8	172±5

The accuracy rates of DiAcPUT and DiAcCAD in QC samples were acceptable in the first two batches (>300 injections), but they deteriorated in the third and fourth batch of the same column, with DiAcPUT showing progressively lower concentrations and DiAcCAD exhibiting higher concentrations. In certain samples, the matrix contains a background compound that interferes with the DiAcPUT and DiAcCAD signals. DAP, CAD and AcSPM concentrations were below the method's quantitation range; these polyamines can therefore only be quantitated if they are present in sufficiently high concentrations. The AcPUT and N1AcSPD concentrations in some samples were above the calibration range, and had to be diluted to 1:10 to be quantifiable. Different LC columns and conditions and different sample preparation methods were studied in order to solve the matrix problems of DiAcPUT and DiAcCAD, as well as the sensitivity problems of DAP, CAD and AcSPM. However, the best compromise between selectivity and sensitivity regarding all the analytes was achieved in the previously used conditions. The sensitivity of the instruments used in this study is inferior to the newer, more sensitive instruments currently available on the market. It might be possible to reach a sufficient sensitivity level for DAP, CAD and AcSPM analysis with this method by using more modern instruments. A higher sensitivity would also allow a further dilution of the samples, which might decrease ME and thus enable the analysis of DiAcPUT and DiAcCAD. Additionally, more modern instruments would allow the use of a narrower window for the selected ions, which both increases selectivity and reduces noise.

Polyamine concentration in human urine

Creatinine normalized and molar concentrations of urinary polyamines are represented in Table 11.

Table 11. Polyamines concentrations (umol/g of creatinine) and (nM in urine) in the urine samples of healthy men and in PCa patients.
N/A = analyzed concentration was below lowest calibration point

	Healthy men (n=6)			**PCa patients (n=22)**		
	Range umol/g creatinine	Range nM in urine	Found in samples	Range umol/g creatinine	Range nM in urine	Found in samples
DAP	N/A-0.19	N/A–349	50%	N/A-4.58	N/A-3216	23%
PUT	0.14-0.59	179-829	100%	N/A-6.27	N/A-4400	95%
CAD	N/A-0.21	N/A-290	83%	N/A-0.59	N/A-411	36%
AcPUT	5.35-9.20	7618-25239	100%	6.01-49.24	2637-27213	100%
AcCAD	0.14-1.52	284-4181	100%	0.13-12.54	158-5608	100%
SPD	0.09-0.15	139-364	100%	0.09-1.35	77-892	100%
DiAcPUT	0.05-0.45	71-881	100%	N/A-1.14	N/A-552	95%
DiAcCAD	0.002-0.026	6-70	100%	N/A-3.24	N/A-922	90%
N1AcSPD	1.67-5.39	2180-13994	100%	1.54-20.03	1069-11310	100%
N8AcSPD	1.40-2.67	2332-6648	100%	1.79-9.11	1031-7203	100%
SPM	0.33-1.04	457-2863	100%	0.06-6.48	74-7013	100%
DiAcSPD	0.14-0.24	189-581	100%	0.12-0.94	60-735	100%
AcSPM	N/A	N/A	0%	N/A-0.014	NA-12	14%
DiAcSPM	0.06-0.17	68-454	100%	0.05-0.39	32-582	100%

10.3 Study III

First, the eNose's ability to differentiate the patients with an operable PCa from the ones with a symptomatic, surgery-requiring BPH was evaluated. The classifier was comprised of all the variables in the 16-dimensional dataset without performing a dimension reduction. A sensitivity of 82%, a specificity of 88% and an AUC of 0.92 were achieved with LDA. These values represent the methodology's potential maximum performance, but are also prone to showing overly positive results in small sample cohorts due to overfitting. LOOCV was performed in order to minimize the effect of overfitting and to model an actual diagnostic implementation more accurately. In the LOOCV-analysis, a sensitivity of 78 %, a specificity of 67 % and an AUC of 0.77 were achieved. The ROC curve is represented in figure 10.

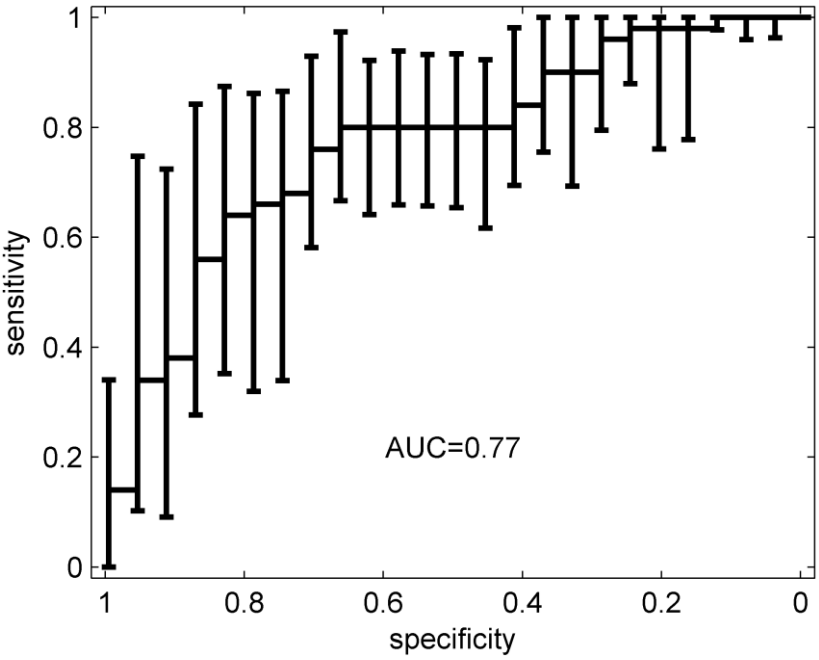

Figure 10. The ROC-curve for the eNose with 95% confidence intervals. (Reproduced with the permission of Elsevier.)

A stepwise regression model for predicting the size of the prostate with eNose measurements resulted in the final model

including only one eNose channel: the number nine. Its correlation coefficient with the size of prostate was 0.34 (p value 0.02 probability of zero correlation) with the size of prostate. The R^2 was 0.11, thus, the model captured 11 % of the total variance. No significant model for predicting the size of the tumor was found.

10.4 Study IV

In the discrimination of sterile and infected samples, the eNose achieved a sensitivity of 95 % and specificity of 97 % using LR. Sensitivity of 90 % and specificity of 96 % were achieved with LDA. These results were validated with LOOCV. Classification is presented in table 12 below.

Table 12. Classification results of bacteria vs sterile samples with LDA and LR. Left columns shows the true class of the sample. Left-hand column shows the true class of samples. Top rows identify used prediction model and how it classifies samples. Both methods achieve near perfect discrimination, LR demonstrating marginally better performance.

		Predicted LDA		Predicted LR	
		Sterile	Bacteria	Sterile	Bacteria
True	Sterile	19	2	20	1
	Bacteria	3	77	2	78

In classification of 5 different bacteria, a LOOCV-validated sensitivity of 95 % and specificity of 96% were achieved using LDA. A 2-dimensional Sammon map –projection is presented in figure 11 to demonstrate the clustering of bacterial samples. Both sterile and *Klebsiella spp* samples were all classified correctly. Three *E. coli* samples were classified as sterile. The discrimination

between *S. saprophyticus* and *E. faecalis* was more challenging, with six *S. saprophyticus* samples classified as *E. faecalis*, and five *E. faecalis* as *S. saprophyticus* samples. One *S. sapropyticus* and *E. faecalis* were misclassified as *Klebisiella spp*. Classification results are presented in table 13 and illustrated in Figure 11.

Table 13. The identification of bacterial species and sterile samples. The left-hand columns identify the true classification of the samples. The top row shows the discrimination by LDA. *S. saprophyticus* is most commonly misclassified and also often confused with *E. faecalis*. Overall discrimination is very high.

		Predicted LDA				
		Sterile	*S.Sapro-phyticus*	*E.Coli*	*Klebsiella spp*	*E. faecalis*
True	Sterile	20	0	1	0	0
	S.Saprophyticus	0	12	0	1	6
	E.Coli	3	0	17	0	0
	Klebsiella spp	0	0	0	20	0
	E. faecalis	0	5	0	1	15

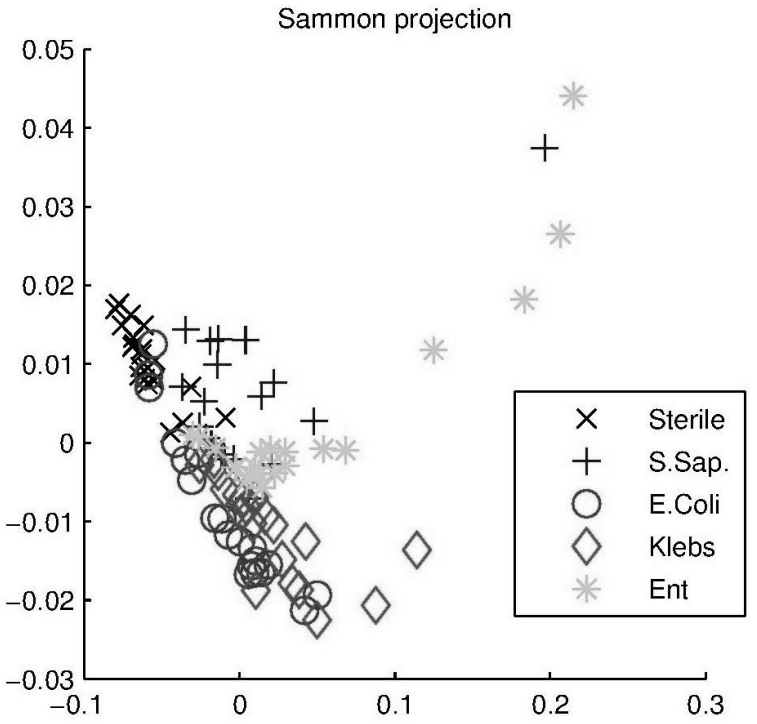

Figure 11. A Sammon projection of the dataset used in this study. The different species cluster in their own areas, with some overlapping in the 2-dimensional projections.

Unpublished data

In an unpublished research, we have shown that putrescine, spermidine and spermine can be detected from water in micromolar concentrations by eNose (ChemPro 100). We have discovered that increasing pH from 10 enhances the discrimination rate.

11 DISCUSSION

The results of this study show that an eNose is capable of discriminating BPH and PCa cells *in vitro*, and that this smell print is excreted to the medium (Study I). The discovery that the smell prints in PCa and BHP patients' urines differ is in line with these results (Study III). In an unpublished study, we have also documented an eNose's ability in detecting polyamines at micromolar concentrations similar in magnitude to concentrations found in urine of healthy volunteers and cancer patients. We have developed a method for the accurate simultaneous detection of fourteen poly-amines from urine, two of which (DiAcPUT and DiAcCAD) have not previously been detected in human urine (Study II). Lastly, we have demonstrated eNose's ability to rapidly discriminate cultures of common bacterial uropathogens with high sensitivity and specificity (Study IV).

Our *in vitro* findings are in line with previous cell culture studies (Gendron et al. 2007; Kateb et al. 2009; Pennazza et al. 2011). This is the first time a discrimination of urologic cell lines was attempted with an eNose. EP-156T was created by infecting histologically normal prostate epithelial cells with a retrovirus encoding human telomerase reverse transcriptase. The enzyme elongates the chromosomal ends of cell DNA, preventing normal death (Kogan et al. 2006). This makes EP-156T a closer model to luminal epithelial cells, since - unlike commercial prostate epithelial cell lines - it retains expression of androgen receptors and PSA (Sobel & Wang 2006). LNCaP represents a typical androgen-sensitive metastatic PCa (Horoszewicz et al. 1983) and is therefore an excellent model for this thesis. Comparing benign cell line to metastatic PCa line is analogous to clinical setting, where we aim to find aggressive cancer that need radical treatment amongst a large population of relatively low-risk cancers.

The source of the VOCs that make the detection of PCa from urine possible remain unknown. The discovery about cultured prostate cells modifying the smell print of their medium (Study I) supports the hypothesis of prostate cancer cells excreting volatile compounds directly into urine. On the other hand, PCa can also be detected from exhaled air (Peng et al. 2010), suggesting that there may be systemic factors involved, which also affect smell print of urine. A similar pattern is also observed in colorectal cancer, which can be diagnosed from the VOCs in breath (Altomare et al. 2013) and also from fecal samples (de Meij et al. 2014). The fact that tumor size did not correlate with the eNose signal suggests that a systemic reaction might be behind the smell of cancer. Since PCa group was fairly homogenous, with a large majority of cancers having a Gleason grade of 7, it is possible that our analysis is confounded by tumor location in relation to the urethra, i.e. a large peripheral tumor may elicit a weaker signal than a smaller tumor proximal to the urethra. One hypothesis holds that the smell of PCa is caused by prostatic secretions. This hypothesis is supported by a recent study in which eNose discriminated patients from initial part of the urine stream but not from midstream sample (Asimakopoulos et al. 2014). The hypothesis could be further tested by collecting urine samples after DRE, which increases the content of prostatic secretions especially in the first 30-50ml of urine. This technique is used in some PCA3 protocols (van Gils et al. 2007). Our discovery about eNose's ability to detect polyamines in aqueous solutions provides an alternative hypothesis regarding the origin of distinct smell of cancer. Since similar concentrations of polyamines are found in urine, it is possible that eNose responds to these volatile molecules. However, it should be noted that no significant differences between healthy persons and patients with PCa were found in the small sample used to demonstrate the feasibility of LC-MS/MS method; this indicates that eNose's discrimination capabilities may be explained by other factors as well. The association of odor and elevated polyamine concentrations has been previously demonstrated in connection to bacterial vaginosis (Yeoman et al. 2013), a disease known for its characteristic "fishy

smell". This aspect has never been the subject of discussion in publications made about the olfactory detection of cancer from urine.

The LC-MS/MS method described in this thesis allows the analysis of the widest polyamine array to date. Since the evidence about the significant role of polyamines in PCa and - most importantly - in aggressive metastatic PCa is mounting (Smith et al. 1995; Giskeødegård et al. 2013), there is a demand for accurate and practical methods for polyamine analysis. Urine is preferable to blood as a sample material, since it can be obtained noninvasively. No statistically significant differences between healthy controls and PCa patients were noted in this small preliminary sample. In future, we are planning to employ the newly developed LC-MS/MS methodology in the determination of polyamines from the same patients that were recruited for Study III. Rat studies suggest that DiAcPut has anti-tumor activity mediated by the immunity system (Bowlin et al. 1990), suggesting that DiAcPUT might have potential as a tumor marker. No studies describing DiAcCAD were found in a literature search, suggesting that this is the first time such a molecule is studied as a marker for PCa. Since acetylated polyamines are more specific to malignant diseases than their unacetylated counterparts (van den Berg et al. 1986), it is possible that these newly described molecules will have a role as cancer markers.

In comparison to existing biomarkers for PCa, the eNose's AUC of 0.77 is comparable to the 0.678 reported in literature for PSA (Thompson et al. 2005). AUCs for PCA3 range from 0.66 to 0.746 (Haese et al. 2008; Groskopf et al. 2006; Marks et al. 2007), suggesting a similar performance. Nevertheless, these comparisons should be interpreted with great caution since the mentioned studies were conducted in a setting where patients referred for biopsies were studied. In our study, the histologic material from BPH patients was acquired with TURP from the central zone of the prostate, not the peripheral zone which harbors most cancers (Chen et al. 2000). This means that some TURP patients may actually suffer from an undiagnosed PCa. According

to the current data, an eNose cannot match the performance of dogs in the detection of PCa (Cornu et al. 2011). There are two possible explanations. First, a canine olfactory apparatus is probably superior to the artificial sensor arrays, and the implementation of biologic receptors may be the answer, as suggested by Strauch et al., who recently published a study on the detection of cancer cells in vitro via a fruit fly's antenna (Strauch et al. 2014). We have already taken steps to enhance eNose's performance by improving its sample delivery system, with the main goal of reducing excessive humidity in the system. This improved setup was employed in Study IV, and it displayed an excellent performance in the discrimination of bacterial cultures. In future, we aim to reanalyze the samples of Study III with this new setup.

The fact that different bacterial species produce distinct smell prints also points out the issue that bacteriuria may be a confounding factor in the detection of PCa and other cancers from urine. Evidence about PCa's association with the bacterial colonization of the prostate can also be found (Krieger et al. 2000); the presence of *Propionibacterium acnes* in the TURP material is associated with an increased risk of developing PCa (Alexeyev et al. 2006). These factors necessitate a trial investigating the effect of the presence of bacteria on the smell print of urine. Previous studies on the detection of bacteria in urine have employed an incubation period to detect bacteria (Aathithan et al. 2001; Kodogiannis & Wadge 2005). This suggests that - at least with previous devices - the effect of bacteria on the smell print of urine might be negligible. The fact that the composition of urine has a large physiological variance may require the use of more complex discrimination algorithms, which are more adept at ignoring these confounding factors. The neural network is one possibility; this approach has been used successfully in previous trials on detection of UTI from urine (Aathithan et al. 2001; Kodogiannis & Wadge 2005). A surprising discovery was made in Study IV; while most bacterial cultures were discriminated from sterile plates as well as from each other with a very high overall accuracy, the *S. saprophyticus* and *E. faecalis* strains were challenging to discriminate

between. No studies on the release of VOCs by *S. saprophyticus* were found in our literature review. In a systematic review comparing the VOCs of different bacterial species, *Staphylococcus aureus* and its relative *S. saprophyticus* had fewer shared VOCs than *Klebsiella spp* and *E. Coli*, which were never confused (Bos et al. 2013). *S. saprophyticus* seems to form two clusters, only one of them overlapping with *E. faecalis*. This could be explained by two subspecies: *saprophyticus* and *bovis*. Unlike *spp saprophyticus*, *spp bovis* produces nitrogen dioxide, a volatile compound that is likely to be detected by an eNose. A LC-MS analysis of headspace would be required to confirm this hypothesis.

Since 80% of uncomplicated UTIs are caused by *E. coli*, the prediction of the pathogen holds only a limited value and uncomplicated cystitis can be treated based on clinical picture alone (Grabe et al. 2008; Ronald 2003). However, complicated and recurring UTI necessitates a bacterial culture (Grabe et al. 2008). Clinically, the knowledge of a causative agent is secondary to its resistance status, which directs the selection of an antimicrobial agent (Grabe et al. 2008). Dutta et al. have demonstrated eNose's ability in discriminating between methicillin-resistant and -susceptible *Staphylococcus aureus* species in swabs (Dutta et al. 2005), suggesting that discrimination of bacteria on basis of antimicrobial resistance is feasible, provided that a sufficiently advanced statistical analysis is used. Antimicrobial resistance is a very challenging subject, since it is caused by multiple mechanisms that are not completely understood (Davies & Davies 2010). This further highlights the potential of eNose technology being combined with modern data analysis. To date, no studies have attempted to discriminate resistant bacterial species from urine. This is an area that should be given a high priority in the future.

The major advantages of eNose technology in comparison to LC/GS-MS approaches are the unit's is relatively low cost, and - more importantly - the nearly negligible upkeep expenses. The cost of a ChemPro 100i unit is approximately 10 000 € and the company reports operating expenses of only 0.30 € per hour. The cost of disposable culture plates is approximately 0.10 €. The

average measurement rate 2.5 samples per hour, which results in a cost of 0.22 € per measurement, which compares favorably to the cost of a PSA analysis – typically approximately 5 €. The greatest expenses in eNose analysis derive from need for labor, since samples are analyzed manually. In future, we aim to automate the sample preparation and analysis, and to develop a measurement protocol that minimizes the measurement time.

This thesis is subject to a number of limitations. The system mechanical design of the eNose used in Studies I and III is vulnerable to noise caused by moisture. This problem was addressed in Study IV, where we used activated carbon filtration for the sample chamber's air intake. An experimentation with an enhanced measurement chamber should be undertaken while keeping in mind the risks of excessive filtration, which can negatively affect the dis-crimination process by catching cancer-specific VOCs. Therefore, experiments with a standardized sample set are necessary. The limitation of Study II in the fact that, DiAcCAD and DiAcPUT cannot be quantified accurately due to ME. In future, different internal standards should be tested in order to better compensation for the ME. The method's sensitivity is not sufficient for quantifying concentrations of DAP, CAD and AcSPM in some samples. On the other hand, the quantification range does not allow a simultaneous analysis of AcPUT and N1AcSPD in some samples without dilution. These limitations are mostly due to the available instrumentation. More advanced instruments would offer a higher sensitivity, which in turn allows further dilution of samples for reducing the ME. Another prospect lies in experimenting with different ISs, such as C-13 or N-15 labeled molecules, which may allow for a better compensation for matrix effect with DiAcCAD and DiAcPUT analysis. A significant limitation of Study III is that preoperative and postoperative urine samples taken from the BPH patients had to be pooled to gain an adequate control population. Since TURP reduces the volume of prostate tissue and lowers PSA (Fonseca et al. 2008), the inclusion of postoperative samples may shift the population, deceiving the eNose into detecting the effect of surgery alone. Our analysis showed no correlation between prostate volume and eNose signal

regarding PCa patients. Since no data of the post-operative prostate volume vas available for TURP patients, we resorted to using an estimated reduction of 40 % (Fonseca et al. 2008). This estimation is confounded by the fact that TURP volume varies from 20 to 80 % of the preoperative volume (Fonseca et al. 2008) presumably due to the presence of middle lobe and the size of the prostate. The surgeon's experience also plays a significant role, since operation time is limited by the steep rise of complications in prolonged procedures, which necessities a refined technique for resecting a sufficient amount of tissue in limited time (Reich et al. 2008). Study IV is by nature a preliminary study. Bacterial cultures, although all originating from independent urine samples taken from patients have little variation in comparison to actual urine samples. It is thus likely that eNose's performance in discriminating bacterial cultures was significantly better than it would have been using actual urine samples.

The comparison of limitations of studies II and III highlights the differences between the eNose and a traditional LC-MS analysis. Even though we achieved a wide array of polyamines in a single run, a LC-MS/MS faced problems with concentrations that were either too low or too high for detection, and also suffered from ME. The eNose fared well in these kinds of challenges and no similar problems occurred, apart from the accumulation of humidity that was resolved by improved instrumentation used in Study IV, no similar problems occurred. Since LC-MS/MS probably is more sensitive for specific molecules, a hybrid approach might provide a better performance than either method used alone. Since chromatography instrumentation would compromise device's mobility, a quantification of specified polyamines with lightweight solutions, such as antibody assays, should be examined.

This study lays a foundation for a number of new research directions. First, findings with eNose in the detection of PCa should be repeated with a larger sample. A special emphasis should be paid to the correlation of eNose signal with the Gleason score, in order to explore eNose's ability in discriminating between

aggressive, radically treatable cancers and those for which observation would suffice. An ideal study design would be one where patients referred for biopsies would be studied and eNose's to predict biopsy result would be the primary target parameter. This would allow evaluation of eNose in a completely real-world application. It would also be of interest to see how eNose signal changes in response to treatment, which in turn would allow the development of applications for the monitoring of cancer recurrence or favorable outcomes. eNose's capability in detecting other urologic malignancies - such as bladder and renal cancer - from urine, should also be tested.

12 SUMMARY AND CONCLUSIONS

The main finding of this thesis is that an eNose is able to discriminate PCa and BPH cells in vitro, as well as from the urine headspace of patients suffering from PCa or BPH. In vitro findings show that PCa cells seem to modify the smell of their medium, suggesting that the distinguishable smell of PCa is directly excreted into urine by cancer cells. eNose's performance regarding the detection of PCa from urine is comparable to those documented for existing PCa markers, namely PCA3 and PSA.

We have shown the ability of eNose to detect polyamines from the headspace of aqueous solutions. In order to study the mechanism behind the distinguishable smell of cancer, we developed a LC-MS/MS method for a simultaneous and accurate quantification of 14 polyamines from the urine of healthy individuals and patients suffering from PCa. A LC-MS/MS analysis revealed that polyamines exist in the urine of PCa patients in similar concentrations to those detected by from water by an eNose. This suggests that polyamines contribute to the smell of cancer. Although the small sample did not allow us to detect statistically significant differences between the two groups, we discovered two polyamines - DiAcPUT and DiAcCAD - whose existence in human urine has not been previously documented. DiAcPUT has been documented to have a role in immune reaction against cancer.

Lastly, we demonstrated the ability of the same eNose in discriminating the most common bacterial pathogens of UTI from cultures with high precision.

In conclusion, we have demonstrated eNose's ability to detect PCa and bacterial pathogens. We have developed a method for an accurate detection of polyamines from urine and demonstrated

eNose's ability to detect similar concentrations of polyamines in aqueous solutions. Therefore, we suggest that polyamines at least contribute to distinct smell of cancer.

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14 References

Aathithan, S. et al., 2001. Diagnosis of Bacteriuria by Detection of Volatile Organic Compounds in Urine Using an Automated Headspace Analyzer with Multiple Conducting Polymer Sensors. *Journal of Clinical Microbiology*, 39(7), pp.2590–2593.

Albertsen, P.C., Hanley, J.A. & Fine, J., 2005. 20-year outcomes following conservative management of clinically localized prostate cancer. *JAMA : the journal of the American Medical Association*, 293(17), pp.2095–101.

Alexeyev, O. et al., 2006. Association between the presence of bacterial 16S RNA in prostate specimens taken during transurethral resection of prostate and subsequent risk of prostate cancer (Sweden). *Cancer causes & control : CCC*, 17(9), pp.1127–33.

Altomare, D.F. et al., 2013. Exhaled volatile organic compounds identify patients with colorectal cancer. *British Journal of Surgery*, 100(1), pp.144–150.

Amundsen, T. et al., 2013. Can dogs smell lung cancer? First study using exhaled breath and urine screening in unselected patients with suspected lung cancer. *Acta oncologica*,54(3), pp.1–9.

Andriole, G.L. et al., 2009. Mortality results from a randomized prostate-cancer screening trial. *The New England journal of medicine*, 360(13), pp.1310–9.

Arshak, K. et al., 2004. A review of gas sensors employed in electronic nose applications. *Sensor Review*, 24(2), pp.181–198.

Asimpakopoulos, AD, et al. Prostate cancer diagnosis through electronic nose in the urine headspace: a pilot study. Prostate Cancer Prostatic Dis. 2014 Apr 1. doi: 10.1038/pcan.2014.11.

Bartolazzi, a. et al., 2010. A sensor array and GC study about VOCs and cancer cells. *Sensors and Actuators B: Chemical*, 146(2), pp.483–488.

Basu, H.S. et al., 2009. A small molecule polyamine oxidase inhibitor blocks androgen-induced oxidative stress and delays prostate cancer progression in the transgenic adenocarcinoma of the mouse prostate model. *Cancer research*, 69(19), pp.7689–95.

Bell, C.R. et al., 1998. Prostate specific antigen in urinary tract infection. *The British journal of general practice : the journal of the Royal College of General Practitioners*, 48(427), pp.1005–6.

Bello-Fernandez, C., Packham, G. & Cleveland, J.L., 1993. The ornithine decarboxylase gene is a transcriptional target of c-Myc. *Proceedings of the National Academy of Sciences of the United States of America*, 90(16), pp.7804–8.

Van den Berg, G.A. et al., 1986. Simultaneous gas-chromatographic determination of free and acetyl-conjugated polyamines in urine. *Clinical chemistry*, 32(10), pp.1930–7.

Bernabei, M. et al., 2008. A preliminary study on the possibility to diagnose urinary tract cancers by an electronic nose. *Sensors and Actuators B: Chemical*, 131(1), pp.1–4.

Bhandare, P., Pendbhaje, N. & Narang, A., 2013. Electronic Nose: A Review. *Research & Reviews: Journal of Engineering and Technology*, 2(4).

Bikov, A. et al., 2011. Exhaled breath volatile alterations in pregnancy assessed with electronic nose. *Biomarkers*, 16(6), pp.476–84.

Bird, I.M., 1989. High performance liquid chromatography: principles and clinical applications. *BMJ (Clinical research ed.)*, 299(6702), pp.783–7.

Bishop, C.M., 2006. *Pattern recognition and machine learning*, New York: Springer.

Bjartell, A.S., 2011. Dogs sniffing urine: a future diagnostic tool or a way to identify new prostate cancer markers? *European urology*, 59(2), pp.202–3.

Borsdorf, H. & Eiceman, G. a., 2006. Ion Mobility Spectrometry: Principles and Applications. *Applied Spectroscopy Reviews*, 41(4), pp.323–375.

Bos, L.D.J., Sterk, P.J. & Schultz, M.J., 2013. Volatile metabolites of pathogens: a systematic review. *PLoS pathogens*, 9(5), p.e1003311.

Bower, J.M., Gordon-Raagas, H.B. & Mulvey, M. a, 2009. Conditioning of uropathogenic Escherichia coli for enhanced colonization of host. *Infection and immunity*, 77(5), pp.2104–12.

Bower, J.M. & Mulvey, M.A., 2006. Polyamine-mediated resistance of uropathogenic Escherichia coli to nitrosative stress. *Journal of bacteriology*, 188(3), pp.928–33.

Bowlin, T.L. et al., 1990. Potentiation of natural killer cell activity and tumor immunity by diacetylputrescine. Cancer research, 50(17), pp.5460–3.

Bruins, M. et al., 2009. Device-independent, real-time identification of bacterial pathogens with a metal oxide-based olfactory sensor. *European journal of clinical microbiology & infectious diseases*, 28(7), pp.775–80.

Burdette, S.D. & Bernstein, J.M., 2007. Does the nose know? The odiferous diagnosis of Clostridium difficile-associated diarrhea. *Clinical infectious diseases,* 44(8), p.1142.

Byun, J.A. et al., 2008. Analysis of polyamines as carbamoyl derivatives in urine and serum by liquid chromatography-tandem mass spectrometry. Biomedical chromatography, 22(1), pp.73–80.

Byun, J.A. et al., 2009. Serum polyamines in pre- and post-operative patients with breast cancer corrected by menopausal status. *Cancer letters*, 273(2), pp.300–4.

Caras, R.J. & Sterbis, J.R., 2014. Prostate cancer nomograms: a review of their use in cancer detection and treatment. *Current urology reports*, 15(3), p.391.

Casero, R.A. & Pegg, A.E., 1993. Spermidine/spermine N1-acetyltransferase--the turning point in polyamine metabolism. *FASEB journal,* 7(8), pp.653–61.

Catalona, W.J. et al., 1991. Measurement of prostate-specific antigen in serum as a screening test for prostate cancer. *The New England journal of medicine*, 324(17), pp.1156–61.

Cecco, L. et al., 1992. Pattern and concentration of free and acetylated polyamines in urine of cirrhotic patients. *The International journal of biological markers*, 7(1), pp.52–8.

Chapman, E. a et al., 2012. A breath test for malignant mesothelioma using an electronic nose. The European respiratory journal, 40(2), pp.448–54.

Chattopadhyay, M.K., Tabor, C.W. & Tabor, H., 2003. Polyamines protect Escherichia coli cells from the toxic effect of oxygen. *Proceedings of the National Academy of Sciences of the United States of America*, 100(5), pp.2261–5.

Chen, M.E. et al., 2000. Detailed mapping of prostate carcinoma foci: biopsy strategy implications. *Cancer*, 89(8), pp.1800–9.

Chen, S., Wang, Y. & Choi, S., 2013. Applications and Technology of Electronic Nose for Clinical Diagnosis. *Open Journal of Applied Biosensor*, 02(02), pp.39–50.

Chen, X. et al., 2007. A study of the volatile organic compounds exhaled by lung cancer cells in vitro for breath diagnosis. *Cancer*, 110(4), pp.835–44.

Church, J. & Williams, H., 2001. Another sniffer dog for the clinic? *Lancet*, 358(9285), p.930.

Cipolla, B. et al., 1996. Pretherapeutic Erythrocyte Polyamine Spermine Levels Discriminate High Risk Relapsing Patients. *Cancer*, 78(5), pp.1055–1065.

Collins, D.C. & Lee, M.L., 2002. Developments in ion mobility spectrometry-mass spectrometry. *Analytical and bioanalytical chemistry*, 372(1), pp.66–73.

Cornu, J.-N. et al., 2011. Olfactory detection of prostate cancer by dogs sniffing urine: a step forward in early diagnosis. *European urology*, 59(2), pp.197–201.

Critz, F. a et al., 1996. Prostate-specific antigen nadir: the optimum level after irradiation for prostate cancer. *Journal of clinical oncology*, 14(11), pp.2893–900.

D'Amico, a et al., 2008. Identification of melanoma with a gas sensor array. *Skin research and technology*, 14(2), pp.226–36.

D'Amico, A. et al., 2010. An investigation on electronic nose diagnosis of lung cancer. *Lung cancer)*, 68(2), pp.170–6.

Darves-bornoz, A., Park, J. & Katz, A., 2013. Prostate Cancer Epidemiology. In P. Whelan, A. K. Tewari, & J. D. Graham, eds.

Prostate Cancer: Diagnosis and Clinical Management. John Wiley & Sons Inc.

Davies, J. & Davies, D., 2010. Origin and Evolution of Antibiotic Resistance. Microbiology and Molecular Biology Reviews, 74(3), pp.417-433.

Devillé, W. & Yzermans, J., 2004. The urine dipstick test useful to rule out infections. A meta-analysis of the accuracy. *BMC Urology*, 4, p.4.

DiCiccio, T. & Efron, B., 1996. Bootstrap confidence intervals. *Statistical Science*, 11(3), pp.189–228.

Dragonieri, S. et al., 2009. An electronic nose in the discrimination of patients with non-small cell lung cancer and COPD. Lung cancer, 64(2), pp.166–70.

Dragonieri, S. et al., 2012. An electronic nose distinguishes exhaled breath of patients with Malignant Pleural Mesothelioma from controls. Lung cancer, 75(3), pp.326–31.

Draisma, G. et al., 2003. Lead times and overdetection due to prostate-specific antigen screening: estimates from the European Randomized Study of Screening for Prostate Cancer. *Journal of the National Cancer Institute*, 95(12), pp.868–78.

Dunzendorfer, U. & Russell, D.H., 1978. Altered polyamine profiles in prostatic hyperplasia and in kidney tumors. *Cancer research*, 38(8), pp.2321–4.

Dutta, R. et al., 2005. Identification of Staphylococcus aureus infections in hospital environment: electronic nose based approach. Sensors and Actuators B: Chemical, 109(2), pp.355-362.

Ehmann, R. et al., 2012. Canine scent detection in the diagnosis of lung cancer: revisiting a puzzling phenomenon. *The European respiratory journal*, 39(3), pp.669–76.

El-Beqqali, A., Kussak, A. & Abdel-Rehim, M., 2007. Determination of dopamine and serotonin in human urine samples utilizing microextraction online with liquid chromatography/electrospray tandem mass spectrometry. *Journal of Separation Science*, 30(3), pp.421–424.

Epstein, J.I., 2010. An update of the Gleason grading system. *The Journal of urology*, 183(2), pp.433–40.

Erdman, S.H. et al., 1999. APC-dependent changes in expression of genes influencing polyamine metabolism, and consequences for gastrointestinal carcinogenesis, in the Min mouse. *Carcinogenesis*, 20(9), pp.1709–13.

FDA, 2001. Guidance for Industry Bioanalytical Method Validation Guidance for Industry Bioanalytical Method Validation.

Feldman, B.J. & Feldman, D., 2001. The development of androgen-independent prostate cancer. *Nature reviews. Cancer*, 1(1), pp.34–45.

Feliciano, J. et al., 2008. The incidence of fluoroquinolone resistant infections after prostate biopsy--are fluoroquinolones still effective prophylaxis? *The Journal of urology*, 179(3), pp.952–5.

Fonseca, R.C. et al., 2008. Prostate specific antigen levels following transurethral resection of the prostate. *BJU International*, 34(1), pp.41–8.

Fowler, F.J. et al., 2006. The impact of a suspicious prostate biopsy on patients' psychological, socio-behavioral, and medical care outcomes. *Journal of general internal medicine*, 21(7), pp.715–21.

Foxman, B., 2002. Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. *The American journal of medicine*, 113 Suppl , p.5S–13S.

Furumitsu, Y. et al., 1993. Levels of urinary polyamines in patients with rheumatoid arthritis. *The Journal of rheumatology*, 20(10), pp.1661–5.

Gendron, K.B. et al., 2007. In vitro discrimination of tumor cell lines with an electronic nose. *Otolaryngology--head and neck surgery*, 137(2), pp.269–73.

Gerner, E.W. & Meyskens, F.L., 2004. Polyamines and cancer: old molecules, new understanding. *Nature reviews. Cancer*, 4(10), pp.781–92.

Gibson, T.D. et al., 1997. Detection and simultaneous identification of microorganisms from headspace samples using an electronic nose. *Sensors and Actuators B: Chemical*, 44(1-3), pp.413–422.

Van Gils, M.P.M.Q. et al., 2007. The time-resolved fluorescence-based PCA3 test on urinary sediments after digital rectal examination; a Dutch multicenter validation of the diagnostic performance. Clinical cancer research, 13(3), pp.939–43.

Giskeødegård, G.F. et al., 2013. Spermine and citrate as metabolic biomarkers for assessing prostate cancer aggressiveness. PloS one, 8(4), p.e62375.

Gordon, R.T. et al., 2008. The use of canines in the detection of human cancers. *Journal of alternative and complementary medicine*, 14(1), pp.61–7.

Gordon, S.M. et al., 1985. Volatile organic compounds in exhaled air from patients with lung cancer. *Clinical chemistry*, 31(8), pp.1278–82.

Gosetti, F. et al., 2013. Simultaneous determination of sixteen underivatized biogenic amines in human urine by HPLC-MS/MS. *Analytical and bioanalytical chemistry*, 405(2-3), pp.907–16.

Van der Graaf, M. et al., 2000. Proton MR spectroscopy of prostatic tissue focused on the detection of spermine, a possible biomarker of malignant behavior in prostate cancer. *MAGMA*, 10(3), pp.153–9.

Grabe, M. et al., 2008. Guidelines on The Management of Urinary and Male Genital Tract Infections. *Uroweb*. Available at: http://www.uroweb.org/fileadmin/user_upload/Guidelines/The%20Management%20of%20Male%20Urinary%20and%20Genital%20Tract%20Infections.pdf

Greulich, T. et al., 2013. Detection of obstructive sleep apnoea by an electronic nose. *The European respiratory journal*, 42(1), pp.145–55.

Groskopf, J. et al., 2006. APTIMA PCA3 molecular urine test: development of a method to aid in the diagnosis of prostate cancer. *Clinical chemistry*, 52(6), pp.1089–95.

Haas, G.P. et al., 2008. The worldwide epidemiology of prostate cancer: perspectives from autopsy studies. *The Canadian journal of urology*, 15(1), pp.3866–71.

Haese, A. et al., 2008. Clinical utility of the PCA3 urine assay in European men scheduled for repeat biopsy. *European urology*, 54(5), pp.1081–8.

Hakim, M. et al., 2011. Diagnosis of head-and-neck cancer from exhaled breath. *British journal of cancer*, 104(10), pp.1649–55.

Hamaoki, M. & Nagata, A., 2006. Host macrophages produce diacetylspermine related with tumorigenesis. *Cancer letters*, 243(1), pp.128–34.

He, T.C. et al., 1998. Identification of c-MYC as a target of the APC pathway. *Science*, 281(5382), pp.1509–12.

Heidenreich, A. et al., 2011. EAU guidelines on prostate cancer. Part 1: screening, diagnosis, and treatment of clinically localised disease. *European urology*, 59(1), pp.61–71.

Henschke, C.I. et al., 2006. Survival of patients with stage I lung cancer detected on CT screening. *The New England journal of medicine*, 355(17), pp.1763–71.

Hiramatsu, K. et al., 1995. Determination of amounts of polyamines excreted in urine: demonstration of N1,N8-diacetylspermidine and N1,N12-diacetylspermine as components commonly occurring in normal human urine. *Journal of biochemistry*, 117(1), pp.107–12.

Hiramatsu, K. et al., 1997. Diagnostic and prognostic usefulness of N1,N8-diacetylspermidine and N1,N12-diacetylspermine in urine as novel markers of malignancy. *Journal of cancer research and clinical oncology*, 123(10), pp.539–45.

Hiramatsu, K. et al., 2005. N(1),N(12)-Diacetylspermine as a sensitive and specific novel marker for early- and late-stage colorectal and breast cancers. *Clinical cancer research,* 11(8), pp.2986–90.

Hoogendam, a, Buntinx, F. & de Vet, H.C., 1999. The diagnostic value of digital rectal examination in primary care screening for prostate cancer: a meta-analysis. *Family practice*, 16(6), pp.621–6.

Hooton, T.M. et al., 2000. A prospective study of asymptomatic bacteriuria in sexually active young women. *The New England journal of medicine*, 343(14), pp.992–7.

Horoszewicz, J.S. et al., 1983. LNCaP model of human prostatic carcinoma. *Cancer research*, 43(4), pp.1809–18.

Horton, R. & Tait, J.F., 1966. Androstenedione production and interconversion rates measured in peripheral blood and studies on the possible site of its conversion to testosterone. *The Journal of clinical investigation*, 45(3), pp.301–13.

Horvath, G. et al., 2008. Human ovarian carcinomas detected by specific odor. *Integrative cancer therapies*, 7(2), pp.76–80.

Horvath, G., Andersson, H. & Nemes, S., 2013. Cancer odor in the blood of ovarian cancer patients: a retrospective study of detection by dogs during treatment, 3 and 6 months afterward. *BMC cancer*, 13, p.396.

Horvath, G., Andersson, H. & Paulsson, G., 2010. Characteristic odour in the blood reveals ovarian carcinoma. *BMC cancer*, 10(1), p.643.

Horvath, G., Chilo, J. & Lindblad, T., 2010. Different volatile signals emitted by human ovarian carcinoma and healthy tissue. *Future oncology)*, 6(6), pp.1043–9.

Huggins, C., 1978. Endocrine-induced regression of cancers. *American journal of surgery*, 136(2), pp.233–8.

Ito, K. et al., 2003. Free/total PSA ratio is a powerful predictor of future prostate cancer morbidity in men with initial PSA levels of 4.1 to 10.0 ng/mL. *Urology*, 61(4), pp.760–764.

Johansen, A., 2002. Clostridium difficile associated diarrhoea: how good are nurses at identifying the disease? *Age and Ageing*, 31(6), p.487–a–488.

De Jong, W.H. a et al., 2010. Automated mass spectrometric analysis of urinary free catecholamines using on-line solid phase extraction. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, 878(19), pp.1506–12.

Juthani-Mehta, M., 2007. Asymptomatic bacteriuria and urinary tract infection in older adults. *Clinics in geriatric medicine*, 23(3), pp.585–94, vii.

Kabir, A. & Suresh Kumar, G., 2013. Binding of the biogenic polyamines to deoxyribonucleic acids of varying base composition: base specificity and the associated energetics of the interaction. *PloS one*, 8(7), p.e70510.

Kalač, P. & Krausová, P., 2005. A review of dietary polyamines: Formation, implications for growth and health and occurrence in foods. *Food Chemistry*, 90(1-2), pp.219–230.

Kang, J., 2012. Principles and Applications of LC-MS / MS for the Quantitative Bioanalysis of Analytes in Various Biological Samples. In J. Prsasain, ed. *Tandem Mass Spectrometry - Applications and Principles*.

Kano, Y. et al., 2007. Increased blood spermine levels decrease the cytotoxic activity of lymphokine-activated killer cells: a novel mechanism of cancer evasion. *Cancer immunology, immunotherapy*, 56(6), pp.771–81.

Karlovsky, M.E. & Pontari, M. a, 2002. Theories of prostatitis etiology. *Current urology reports*, 3(4), pp.307–12.

Kateb, B. et al., 2009. Sniffing out cancer using the JPL electronic nose: a pilot study of a novel approach to detection and differentiation of brain cancer. *NeuroImage*, 47 Suppl 2, pp.T5–9.

Kee, K. et al., 2004. Metabolic and antiproliferative consequences of activated polyamine catabolism in LNCaP prostate carcinoma cells. *The Journal of biological chemistry*, 279(26), pp.27050–8.

Kodogiannis, V., 2005. Intelligent classification of bacterial clinical isolates in vitro, using an array of gas sensors. *Journal of Intelligent and Fuzzy Systems*, 16, pp.1–14.

Kodogiannis, V. & Wadge, E., 2005. The use of gas-sensor arrays to diagnose urinary tract infections. *International journal of neural systems*, 15(5), pp.363–76.

Kogan, I. et al., 2006. hTERT-immortalized prostate epithelial and stromal-derived cells: an authentic in vitro model for differentiation and carcinogenesis. *Cancer research*, 66(7), pp.3531–40.

Krieger, J.N. et al., 2000. Bacterial DNA sequences in prostate tissue from patients with prostate cancer and chronic prostatitis. *The Journal of urology*, 164(4), pp.1221–8.

Kubota, S. et al., 1997. Ornithine decarboxylase overexpression in mouse 10T1/2 fibroblasts: cellular transformation and invasion. *Journal of the National Cancer Institute*, 89(8), pp.567–71.

Kushnir, M.M. et al., 2002. Analysis of catecholamines in urine by positive-ion electrospray tandem mass spectrometry. *Clinical chemistry*, 48(2), pp.323–31.

Lee, J.A. & Verleysen, M., 2007. *Nonlinear Dimensionality Reduction* C L Giles, S J Hanson, & J D Cowan, eds., Springer.

Lee, S.H. et al., 2003. Altered urinary profiles of polyamines and endogenous steroids in patients with benign cervical disease and cervical cancer. *Cancer Letters*, 201(2), pp.121–131.

Lewenhoeck, D.A., 1677. Observationes D. Anthonii Lewenhoeck, De Natis E Semine Genitali Animalculis. *Philosophical Transactions of the Royal Society of London*, 12(133-142), pp.1040–1046.

Lim, E. et al., 2010. Guidelines on the radical management of patients with lung cancer. *Thorax*, 65 Suppl 3(Suppl III), pp.iii1–27.

Lippi, G., 2011. Re: Jean-Nicolas Cornu,Géraldine Cancel-Tassin, Valérie Ondet, et Al. Olfactory detection of prostate cancer by dogs sniffing urine: a step forward in early diagnosis. Eur urol 2011; 59: 197-201. *European urology*, 60(4), p.e29; author reply e30.

Lykos, P. et al., 2001. Rapid detection of bacteria from blood culture by an electronic nose. *Journal of Microbiology- Seoul*, 39(3), pp.213–218.

Löser, C. et al., 1990. Polyamines in colorectal cancer. Evaluation of polyamine concentrations in the colon tissue, serum, and urine of 50 patients with colorectal cancer. *Cancer*, 65(4), pp.958–66.

Maaten, L. Van der, 2009. Dimensionality reduction: A comparative review. *Journal of Machine learning*, 10(January), pp.1–41.

Machado, R.F. et al., 2005. Detection of lung cancer by sensor array analyses of exhaled breath. *American journal of respiratory and critical care medicine*, 171(11), pp.1286–91.

Madersbacher, S. et al., 2004. EAU 2004 guidelines on assessment, therapy and follow-up of men with lower urinary tract symptoms suggestive of benign prostatic obstruction (BPH guidelines). *European urology*, 46(5), pp.547–54.

Marks, L.S. et al., 2007. PCA3 molecular urine assay for prostate cancer in men undergoing repeat biopsy. *Urology*, 69(3), pp.532–5.

McCulloch, M. et al., 2006. Diagnostic accuracy of canine scent detection in early- and late-stage lung and breast cancers. *Integrative cancer therapies*, 5(1), pp.30–9.

McGuire, B.B. et al., 2012. Outcomes in patients with Gleason score 8-10 prostate cancer: relation to preoperative PSA level. *BJU International*, 109(12), pp.1764–1769.

De Meij, T.G. et al., 2014. Electronic nose can discriminate colorectal carcinoma and advanced adenomas by fecal volatile biomarker analysis: proof of principle study. *International Journal of Cancer*, 134(5), pp.1132–1138.

Metz, C.E., 1978. Basic principles of ROC analysis. *Seminars in nuclear medicine*, VIII(4), pp.283–298.

Murray, P.R., 2012. What Is New in Clinical Microbiology—Microbial Identification by MALDI-TOF Mass Spectrometry. *The Journal of Molecular Diagnostics*, 14(5), pp.419–423.

Nagler, H.M. et al., 2005. Digital rectal examination is barrier to population-based prostate cancer screening. *Urology*, 65(6), pp.1137–40.

Di Natale, C. et al., 2003. Lung cancer identification by the analysis of breath by means of an array of non-selective gas sensors. *Biosensors and Bioelectronics*, 18(10), pp.1209–1218.

Park, M.H. & Igarashi, K., 2013. Polyamines and Their Metabolites as Diagnostic Markers of Human Diseases. *Biomolecules & therapeutics*, 21(1), pp.1–9.

Pavlou, A., Turner, A.P.F. & Magan, N., 2002. Recognition of anaerobic bacterial isolates in vitro using electronic nose technology. *Letters in applied microbiology*, 35(5), pp.366–9.

Pegg, A.E. et al., 1982. Polyamine biosynthesis and interconversion in rodent tissues. *Federation proceedings*, 41(14), pp.3065–72.

Peng, G. et al., 2010. Detection of lung, breast, colorectal, and prostate cancers from exhaled breath using a single array of nanosensors. *British journal of cancer*, 103(4), pp.542–51.

Pennazza, G. et al., 2011. Monitoring of melanoma released volatile compounds by a gas sensors array: From in vitro to in vivo experiments. *Sensors and Actuators B: Chemical*, 154(2), pp.288–294.

Penney, K.L. et al., 2013. Gleason grade progression is uncommon. *Cancer research*, 73(16), pp.5163–8.

Phillips, M. et al., 1999. Early report Volatile organic compounds in breath as markers of lung cancer : a cross-sectional study. *The Lancet*, 353, pp.1930–1934.

Pickel, D. et al., 2004. Evidence for canine olfactory detection of melanoma. *Applied Animal Behaviour Science*, 89(1-2), pp.107–116.

Pitt, J.J., 2009. Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry. *The Clinical biochemist*, 30(1), pp.19–34.

Popiolek, M. et al., 2013. Natural history of early, localized prostate cancer: a final report from three decades of follow-up. *European urology*, 63(3), pp.428–35.

Pöyhönen, M.J. et al., 1990. Urinary excretion of polyamines: importance of circadian rhythm, age, sex, menstrual cycle, weight, and creatinine excretion. *The American journal of clinical nutrition*, 52(4), pp.746–51.

Rao, K. et al., 2013. The nose knows not: poor predictive value of stool sample odor for detection of Clostridium difficile. *Clinical infectious diseases*, 56(4), pp.615–6.

Roberts, R.O. et al., 2002. Trends and risk factors for prostate biopsy complications in the pre-PSA and PSA eras, 1980 to 1997. *Urology*, 59(1), pp.79–84.

Ronald, A., 2003. The etiology of urinary tract infection: traditional and emerging pathogens. *Disease-a-month : DM*, 49(2), pp.71–82.

Rudman, D. et al., 1980. Abnormal polyamine metabolism in hereditary muscular dystrophies: effect of human growth hormone. *The Journal of clinical investigation*, 65(1), pp.95–102.

Russell, D.H. et al., 1979. Altered polyamine metabolism in cystic fibrosis. *Pediatric research*, 13(10), pp.1137–40.

Russell, D.H. et al., 1971. Urinary polyamines in cancer patients. *Cancer research*, 31(11), pp.1555–8.

Satink, H.P. et al., 1989. Microbial influences on urinary polyamine excretion. *Clinica chimica acta*, 179(3), pp.305–14.

Schiffman, S. et al., 2000. Effectiveness of an electronic nose for monitoring bacterial and fungal growth. *Electronic noses and olfaction 2000: Proceedings of the Seventh International Symposium on Olfaction and Electronic Noses*, pp.1–8.

Schipper, R.G. et al., 2003. Polyamines and prostatic cancer. *Biochemical Society transactions*, 31(2), pp.375–80.

Schröder, F.H. et al., 2012. Prostate-cancer mortality at 11 years of follow-up. *The New England journal of medicine*, 366(11), pp.981–90.

Seber, G.A.F., 1984. *Multivariate Observations*, Wiley.

Selnaes, K.M. et al., 2013. Spatially matched in vivo and ex vivo MR metabolic profiles of prostate cancer -- investigation of a correlation with Gleason score. *NMR in biomedicine*, 26(5), pp.600–6

Seng, P. et al., 2010. MALDI-TOF-mass spectrometry applications in clinical microbiology. *Future microbiology*, 5(11), pp.1733–54.

Sfoungaristos, S. & Perimenis, P., 2012. PSA density is superior than PSA and Gleason score for adverse pathologic features prediction in patients with clinically localized prostate cancer. *Canadian Urological Association*, 6(1), pp.46–50.

Shah, P. & Swiatlo, E., 2008. A multifaceted role for polyamines in bacterial pathogens. *Molecular microbiology*, 68(1), pp.4–16.

Simoneau, A.R. et al., 2008. The effect of difluoromethylornithine on decreasing prostate size and polyamines in men: results of a year-

long phase IIb randomized placebo-controlled chemoprevention trial. *Cancer epidemiology, biomarkers & prevention*, 17(2), pp.292–9.
Smith, R.C. et al., 1995. Identification of an endogenous inhibitor of prostatic carcinoma cell growth. *Nature medicine*, 1(10), pp.1040–5.
Sobel, R.E., Wang, Y. & Sadar, M.D., 2006. Molecular analysis and characterization of PrEC, commercially available prostate epithelial cells. *In vitro cellular & developmental biology. Animal*, 42(1-2), pp.33–9.
Sobin, L. & Wittekind, C. eds., 2002. *TNM classification of malignant tumours* 6th ed., New York: Wiley-Liss.
Soda, K., 2011. The mechanisms by which polyamines accelerate tumor spread. *Journal of experimental & clinical cancer research*, 30(1), p.95.
Sonoda, H. et al., 2011. Colorectal cancer screening with odour material by canine scent detection. *Gut*, 60(6), pp.814–9.
Sporn, M.B. & Hong, W.K., 2008. Concomitant DFMO and sulindac chemoprevention of colorectal adenomas: a major clinical advance. *Nature clinical practice. Oncology*, 5(11), pp.628–9.
Strauch, M. et al., 2014. More than apples and oranges - Detecting cancer with a fruit fly's antenna. *Scientific reports*, 4, p.3576.
Suh, J.W. et al., 1997. Urinary polyamine evaluation for effective diagnosis of various cancers. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, 688(2), pp.179–86.
Teti, D., Visalli, M. & McNair, H., 2002. Analysis of polyamines as markers of (patho)physiological conditions. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences* , 781(1-2), pp.107–49.
Thompson, I.M. et al., 2005. Operating characteristics of prostate-specific antigen in men with an initial PSA level of 3.0 ng/ml or lower. *JAMA : the journal of the American Medical Association*, 294(1), pp.66–70.
Tsujinaka, S. et al., 2011. Spermine accelerates hypoxia-initiated cancer cell migration. *International journal of oncology*, 38(2), pp.305–12.
Turner, A.P. & Magan, N., 2004. Electronic noses and disease diagnostics. *Nature reviews. Microbiology*, 2(2), pp.161–6.
Utriainen, M., Kärpänoja, E. & Paakkanen, H., 2003. Combining miniaturized ion mobility spectrometer and metal oxide gas sensor for the fast detection of toxic chemical vapors. *Sensors and Actuators B: Chemical*, 93(1-3), pp.17–24.
Uusitupa, M. et al., 1993. Increased urinary polyamine excretion after starting a very low calorie diet. *Scandinavian journal of clinical and laboratory investigation*, 53(8), pp.811–9.
Weber, C.M. et al., 2011. Evaluation of a gas sensor array and pattern recognition for the identification of bladder cancer from urine headspace. *The Analyst*, 136(2), pp.359–64.
Westhoff, M. et al., 2009. Ion mobility spectrometry for the detection of volatile organic compounds in exhaled breath of patients with lung cancer: results of a pilot study. Thorax, 64(9), pp.744–8

Vernat-Rossi, V., Garcia, C. & Talon, R., 1996. Rapid discrimination of meat products and bacterial strains using semiconductor gas sensors. *Sensors and Actuators B: Chemical*, 37, pp.43–48.

Vickers, A.J. & Brewster, S.F., 2012. PSA Velocity and Doubling Time in Diagnosis and Prognosis of Prostate Cancer. *British journal of medical & surgical urology*, 5(4), pp.162–168.

Wieling, J., 2002. LC-MS-MS experiences with internal standards. *Chromatographia*, 55(S1), pp.S107–S113.

Wilcox, M.H., 2007. Diagnosis of Clostridium difficile-associated diarrhea and odor. *Clinical infectious diseases*, 45(8), p.1110.

Williams, H. & Pembroke, A., 1989. Sniffer dogs in the melanoma clinic? *Lancet*, 1(8640), p.734.

Willis, C.M. et al., 2004. Olfactory detection of human bladder cancer by dogs: proof of principle study. *BMJ (Clinical research ed.)*, 329(7468), p.712.

Wilson, A.D. & Baietto, M., 2011. Advances in Electronic-Nose Technologies Developed for Biomedical Applications. *Sensors*, 11(1), pp.1105–1176.

Wilson, A.D. & Baietto, M., 2011. Advances in electronic-nose technologies developed for biomedical applications. *Sensors*11(1), pp.1105–76.

Wilson, A.D. & Baietto, M., 2009. Applications and advances in electronic-nose technologies. *Sensors*, 9(7), pp.5099–148.

Wolf, A.M.D. et al., 2010. American Cancer Society guideline for the early detection of prostate cancer: update 2010. *CA: a cancer journal for clinicians*, 60(2), pp.70–98.

Voss, A. et al., 2005. Smelling Renal Dysfunction via Electronic Nose. *Annals of Biomedical Engineering*, 33(5), pp.656–660.

Yeoman, C.J. et al., 2013. A multi-omic systems-based approach reveals metabolic markers of bacterial vaginosis and insight into the disease. *PloS one*, 8(2), p.e56111.

15. ORIGINAL COMMUNICATIONS

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Analysis of free, mono- and diacetylated polyamines from human urine by LC–MS/MS

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Cancer diagnostic markers
Prostate cancer

ABSTRACT

Polyamines are promising biochemical markers of cancer and many other pathophysiological conditions, and thus their concentrations in biological fluids are a matter of interest. However, since the concentrations of these compounds are low, their quantitation is typically based on methods requiring laborious sample preparation. Here we developed and validated an LC–MS/MS method to analyze simultaneously free (DAP, PUT, CAD, SPD, SPM) monoacetylated (AcPUT, AcCAD, N^1AcSPD, N^8AcSPD, N^1AcSPM) and diacetylated (DiAcPUT, DiAcCAD, DiAcSPD, DiAcSPM) polyamines from human urine without the need for derivatization. Deuterium labeled polyamines were the internal standards for each analyte. Diluted urine samples spiked with internal standards were filtered through a strong anion exchange resin prior to LC–MS/MS analysis. The chromatographic separation of 14 polyamines was achieved in 12 min on C18 column with 0.1% HFBA (v/v) as the ion-pairing agent and a water–acetonitrile gradient. Ionization was performed with positive electrospray ionization (ESI) and detection was with a triple quadrupole mass spectrometer with selected reaction monitoring. Calibration curves ranged from up to 5 to 10,000 nM. The accuracy and precision of the method were determined using urine based quality control samples, and matrix effects were examined by using standard addition methods. This novel method is suitable for elucidating differences in urinary polyamine excretion in cancer patients and healthy humans.

1. Introduction

Putrescine, spermidine and spermine are the most common polyamines in mammalian cells. These agents play important roles in cell growth, proliferation, and differentiation. Polyamines can exist as either free or conjugated forms in cells and physiological fluids, but in human urine, they are mainly excreted as their acetylated forms [1]. The transition of cells from a healthy state to malignancy is characterized by increased cellular proliferation. This is related to increased cellular polyamine concentrations, and thus polyamines can be considered as interesting biochemical markers of cancer. Their concentrations in human fluids become normalized in cancer patients receiving curative treatments and conversely these levels can further increase in patients who experience tumor relapses and metastasis [2]. Additionally, altered levels of different polyamines in biological fluids have been proposed as possible clinical biochemical markers for several other diseases [3,4].

The urine of human cancer patients was first reported to contain elevated levels of polyamines in the early 1970s [5]. Thereafter, numerous studies have been published describing polyamine

Abbreviations: DAP, propane-1,3-diamine dihydrochloride; PUT, butane-1,4-diamine dihydrochloride; SPD, N^1-(3-aminopropyl)butane-1,4-diamine trihydrochloride; SPM, *N*,*N*′-bis-(3-aminopropyl)butane-1,4-diamine tetrahydrochloride; N^1AcSPM, *N*-(3-((4-((3-aminopropyl)amino)butyl)amino)propyl)acetamide trihydrochloride; CAD, pentane-1,5-diamine dihydrochloride; AcPUT, *N*-(4-aminobutyl)acetamide hydrochloride; AcCAD, *N*-(5-aminopentyl)acetamide hydrochloride; DiAcPUT, *N*,*N*′-(butane-1,4-diyl)diacetamide; DiAcCAD, *N*,*N*′-(pentane-1,5-diyl)diacetamide; N^8AcSPD, *N*-(4-((3-aminopropyl)amino)butyl)acetamide dihydrochloride; N^1AcSPD, *N*-(3-((4-aminobutyl)amino)propyl)acetamide dihydrochloride; DiAcSPD, *N*-(3-((4-acetamidobutyl)amino)propyl)acetamide hydrochloride; DiAcSPM, *N*,*N*′-((butane-1,4-diylbis(azanediyl))bis(propane-3,1-diyl))diacetamide dihydrochloride.

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concentrations in urine in the hope of screening cancers or to monitor therapy response [2,3,6,7]. Since the results from total polyamine concentration measurements as tumor marker have been conflicting, more specific methods have been recommended to analyze several individual polyamine components separately in order to determine whether a specific pattern of polyamines exists in different pathophysiological conditions [4,8–11]. The diacetylated polyamines, such as N^1,N^{12}-diacetylspermine (DiAcSPM) and N^1,N^8-diacetylspermine (DiAcSPD), have also been shown to be potential cancer markers in several studies and should preferably be included in the measurements [12–16]. Nonetheless, in spite of extensive studies there is still no method which can analyze free, mono- and diacetylated diamines and -polyamines simultaneously.

Due to the poorly volatile, non-fluorescent and weakly UV absorbing nature of the polyamine backbone, most of the existing analytical methods have been based on the detection of different derivatives [3]. However, there are several possible drawbacks associated with derivatization, including prolonged analysis time, incomplete reactions, unstable reaction products, side reactions, and solubility problems [3,17,18]. In addition, neutral polyamine derivatives lacking a free amino group (e. g. diacetylated putrescine) are not derivatized, and thus remain undetectable by conventional methods.

Polyamines are present at nM–μM levels (or nmol per mg of creatinine) in human urine [9,10,19], and are thus minor components, requiring highly sensitive methods for their measurements. The most of the previous methods to analyze polyamines from food or other biological matrixes without any derivatization include separation of analytes with LC, IC, or CE. The detection systems of these applications mainly involve electrochemical detection like amperometric, integrated pulsed or mass spectrometric methods. Also methods without prior separation of analytes have been studied [20]. In the present study, we developed and validated an LC–ESI-MS/MS method for the simultaneous analysis of polyamines and their mono- and diacetylated forms in human urine. The method covers a total of 14 individual polyamines or their acetylated metabolites in a single analytical run, with better or comparable ease of sample preparation, LC running time and sensitivity to earlier published methods for different polyamine analysis from biological samples. In addition, to our knowledge, we also show for the first time the presence of diacetylated putrescine and diacetylated cadaverine in human urine. These two diacetylated diamines are predicted to have similar clinical relevance to pathological malignancies and neoplasms as other diacetylated polyamines might have [21] However, they have remained undetectable in all previous studies.

2. Materials and methods

2.1. Reagents

Ultra-gradient HPLC-grade acetonitrile (ACN) was from J.T.Baker, Avantor, Deventer, Netherlands. Heptafluorobutyric acid (HFBA, ≥99.5%), 2-propanol (LC–MS chromasolv grade, ≥99.9%), Trifluoroacetic acid (TFA, eluent additive for LC–MS, ≥99.0%), and methanol (LC–MS chromasolv grade, ≥99.9%) were from Fluka, Sigma–Aldrich, St. Louis, Mo. USA. Water was purified in a MilliQ Gradient A10 purification system (Millipore, Milford, MA, USA).

Standard compounds propane-1,3-diamine dihydrochloride (DAP, 98%), butane-1,4-diamine dihydrochloride (PUT, 98%), N^1-(3-aminopropyl)butane-1,4-diamine trihydrochloride (SPD, 98%), *N*,*N*′-bis-(3-aminopropyl)butane-1,4-diamine tetrahydrochloride (SPM, >95%), *N*-(3-((4-((3-aminopropyl)amino)butyl)amino)propyl)acetamide trihydrochloride (N^1AcSPM, ≥97.0%), were from Sigma–Aldrich. Pentane-1,5-diamine dihydrochloride (CAD) was from Calbiochem, Darmstadt, Germany. The publication describing the preparation methods for *N*-(4-aminobutyl)acetamide hydrochloride (AcPUT), *N*-(5-aminopentyl)acetamide hydrochloride (AcCAD), *N*,*N*′-(butane-1,4-diyl)diacetamide (DiAcPUT), *N*,*N*′-(pentane-1,5-diyl)diacetamide (DiAcCAD), *N*-(4-((3-aminopropyl)amino)butyl)acetamide dihydrochloride (N^8AcSPD), *N*-(3-((4-aminobutyl)amino)propyl)acetamide dihydrochloride (N^1AcSPD), *N*-(3-((4-acetamidobutyl)amino)propyl)acetamide hydrochloride (DiAcSPD), *N*,*N*′-((butane-1,4-diylbis(azanediyl))bis(propane-3,1-diyl))diacetamide dihydrochloride (DiAcSPM) (all >95% purity) is in preparation.

Deuterated internal standard compound 1,1,2,2,3,3,4,4-$^2H_8$-butane-1,4-diamine dihydrochloride (PUT-d_8) was prepared as previously described [22]. 1,1,5,5-$^2H_4$-pentane-1,5-diamine dihydrochloride (CAD-d_4) was prepared from 1,1,5,5,-$^2H_4$-pentane-1,5-diamine (98%, 98 atom % D) from QMX Laboratories, CDN Isotopes Inc., Canada. 1,1,2,2-$^2H_4$-propane-1,3-diamine dihydrochloride (DAP-d_4) was prepared starting from N^1-benzyl-3,3-$^2H_4$-propane-1,3-diamine dihydrochloride [23] using the previously described methods [23]. The publication describing the preparation methods for N^1-(3-amino-3,3-$^2H_2$-propyl)-1,1-$^2H_2$-butane-1,4-diamine trihydrochloride (SPD-d_4), N^3,$N^{3\prime}$-(butane-1,4-diyl)bis(1,1-$^2H_2$-propane-1,3-diamine) tetrahydrochloride (SPM-d_4), *N*-(4-aminobutyl)-2,2,2-$^2H_3$-acetamide (AcPUT-d_3), *N*-(5-aminopentyl)-2,2,2-$^2H_3$-acetamide, (AcCAD-d_3), *N*-(3-((4-aminobutyl)amino)-1,1-$^2H_2$-propyl)-2,2,2-$^2H_3$-acetamide (N^1AcSPD-d_5), *N*-(4-((3-aminopropyl)amino)butyl)-2,2,2-$^2H_3$-acetamide (N^8AcSPD-d_3), *N*-(3-((4-((3-aminopropyl)amino)butyl)amino)propyl)acetamide (N^1AcSPM-d_4), *N*,*N*′-(butane-1,4-diyl)bis(2,2,2-$^2H_3$-acetamide) (DiAcPUT-d_6), *N*,*N*′-(pentane-1,5-diyl)bis(2,2,2-$^2H_3$-acetamide) (DiAcCAD-d_6), *N*-(4-((3-acetamido-3,3-$^2H_2$-propyl)amino)-1,1-$^2H_2$-butyl)acetamide (DiAcSPD-d_4), *N*,*N*′-((butane-1,4-diylbis(azanediyl))bis(1,1-$^2H_2$-propane-3,1-diyl))diacetamide (DiAcSPM-d_4) is in preparation.

2.2. Urine samples

Study population was formed of patients that had been referred to a urologist for operative treatment in Tampere University Hospital (Tampere, Finland) and of normal healthy controls. Patients were divided into cases and controls: confirmed prostate cancer (PCa) patients (n = 22, men, aged 49–73) and healthy controls (n = 6, men, aged 22–38 years). The confirmed PCa group was formed of patients with biopsy-proven PCa scheduled for robot-assisted radical prostatectomy.

Subjects, who were consuming self-selected diets, provided a routine morning urine sample. Urine was collected without preservatives, aliquots were taken for separate analysis for creatinine, and within 2 h, the urine samples were stored at −70 to 80 °C until measurements were done. Before sample preparation, the urine specimens were thawed unassisted at room temperature and centrifuged for 5 min, 13,000 rpm, 20 °C.

Written, informed consent was acquired from all subjects. The study was approved by ethical committee of Tampere University Hospital (code: R10066).

2.3. Creatinine determination

The creatinine concentration in the urine samples was determined enzymatically using Cobas 6000, C 501 – module (Roche diagnostics GmbH, Mannhein, Germany) at Fimlab laboratory (Tampere, Finland).

2.4. LC–MS/MS instrumentation and analytical conditions

Validation was performed according to the FDA guideline on bioanalytical method validation [24] and taking into account the additional instructions for endogenous compound analysis [25–27].

The LC separation was performed using an Agilent 1200 Series Rapid Resolution LC System and the mass analysis was carried out with an Agilent 6410 Triple Quadrupole mass spectrometer equipped with an electrospray ionization source. Data acquisition and quantification were conducted using Agilent MassHunter Workstation software (Agilent Technologies, Palo Alto, CA, USA).

The reversed phase column used was Phenomenex Kinetex C18 (150 mm × 2.1 mm, 2.6 μm) protected with a Phenomenex SecurityGuard Ultra C18 guard cardridge for 2.1 mm ID columns (Phenomenex, Torrance, CA, USA). The eluent flow rate was 200 μL/min and the eluents were 0.1% HFBA (v/v) in water (eluent A) and 0.1% HFBA (v/v) in ACN (eluent B). A linear mobile phase gradient was used; eluent B was increased from 5% to 40% in 10 min, and held at 40% B for 1.5 min. The gradient was then decreased from 40% to 5% of eluent B in 0.1 min and the column was stabilized with 6.4 min flow of 5% eluent B (Total run-time 18 min). The column temperature was held at 35 °C, and the autosampler temperature at 4 °C. The injection volume was 10 μL. Injection was performed with 10 s needle wash with 0.1% TFA (v/v) and 50% 2-propanol in water.

A divert valve was used to direct the eluent flow into the mass spectrometer from 2.8 min to 12 min. The following ionization conditions were used: positive electrospray ionization (ESI), drying gas (nitrogen) temperature 300 °C, drying gas flow rate 8 L/min, nebulizer pressure 40 psi and capillary voltage 4000 V. Detection was performed using multiple reaction monitoring (MRM). Four time segments were used in the analyses, turning points being 2.8, 4.3, 6.9 and 9.5 min. Dwell times were 20 ms and mass resolution (peak width) for MS1 and MS2 quadrupoles were 0.7 FWHM for each transition. The collision induced dissociation (CID) was made by nitrogen. Deuterated internal standards were used for quantification. Fragmentor voltage value was set to 60 V for DAP, DAP-d_4, PUT, PUT-d_8, CAD and CAD-d_4, and to 90 V for rest of the analytes. Ion transitions, and collision energy values for all analytes are shown in Table 1.

2.5. Preparation of standard working solutions for calibration standards and IS

Stock solutions of each polyamine were prepared in water and stored in a freezer at −20 °C. Weighing of the analytes was done with a calibrated analytical balance (Ohaus GA 200D, England). Standard stock solutions were prepared by dissolving an appropriate amount of analyte in water to yield a concentration of 100 mM. All 100 mM solutions were analyzed by NMR spectrometer as previously described [28] to ensure the actual concentration of the solutions and the purity of the analytes. 100 mM solutions were further diluted to 400 μM stock solutions with water.

For the working standard solutions, 500 μL of each 400 μM stock solution were mixed and diluted up to 10 mL with water. This 20 μM standard solution was further diluted with water to give a series of eleven standard working solutions with polyamine concentrations of 5, 10, 30, 50, 100, 300, 500, 1000, 3000, 5000, 10,000 nM. The IS stock solutions were mixed and diluted with water to make a working solution with IS concentration of 1000 nM. Working solutions for calibration curve quality control (CC) samples were prepared from a separate 20 μM standard solution by diluting with water to give four CC standard working solutions with polyamine concentrations of 30, 300, 1000, and 5000 nM.

2.6. Sample preparation for polyamine LC–MS/MS determination

Samples were prepared by mixing 120 μL of urine with the 60 μL of the IS working solution and 420 μL of water. 550 μL of this IS spiked and water diluted (1:5) urine was then passed through solid-phase extraction (SPE) cartridges (Phenomenex Strata SAX cartridges, 100 mg/3 mL (Phenomenex, Torrance, CA, USA). The sorbent was conditioned with 1 mL methanol, and equilibrated with 1 mL of water before the sample handling. After the IS spiked urine had been eluted, 450 μL water was passed through the cartridge and combined to the sample. Samples for LC–MS/MS analysis were then prepared by mixing 100 μL of SPE treated sample and 25 μL of 10% HFBA, and centrifuged for 20 min, 13,000 rpm, 4 °C. Diluted urine samples were prepared by diluting the urine samples 1:10 with water before the sample preparation.

Calibration standards and CC samples were prepared like the urine samples starting from 120 μL of each standard working solution or CC working solution and mixing it with 60 μL of the IS working solution and 420 μL of water. In addition to eleven standard levels, the calibration curve included a blank sample (no standard or IS added) and a zero sample (only IS added).

2.7. Quality control samples

Quality controls were made in human urine. The endogenous concentration of polyamines from urine samples of six healthy men was measured, and urine from these 6 individuals were pooled by mixing equal amounts of urine from each subject and this pooled urine (QC matrix) was used in the preparation of quality controls (QC).

The 20 μM standard working solution was diluted 1:20 with QC matrix to prepare the high QC working solution (HQC = endogenous

Table 1
Ion transitions, and collision energy values for analytes and their internal standards (IS). QT = quantifier, QL = qualifier.

Analyte	M+H	QT	CID QT	QL 1	CID QL1	QL2	CID QL2	IS	M+H	QT	CID QT	QL	CID QL
DAP	75.1	58.1	7					DAP-d_4	79.1	62.1	7		
PUT	89.1	72.1	5					PUT-d_8	97.1	80.1	5		
CAD	103.1	86.1	5					CAD-d_4	107.1	90.1	5		
AcPUT	131.1	114.1	5	72.1	15			AcPUT-d_3	134.1	117.1	5	72.1	15
AcCAD	145.1	86.1	15	128.1	5			AcCAD-d_3	148.1	86.1	15	131.1	5
SPD	146.2	72.1	15	112.1	10			SPD-d_4	150.2	74.1	15		
DiAcPUT	173.1	131.1	10	72.1	20	114.1	15	DiAcPUT-d_6	179.1	135.1	10	117.1	10
DiAcCAD	187.1	145.1	10	128.1	15			DiAcCAD-d_6	193.1	149.1	10		
N^1AcSPD	188.2	100.1	15	72.1	15			N^1AcSPD-d_5	193.2	105.1	15		
N^8AcSPD	188.2	114.1	15	72.1	20			N^8AcSPD-d_3	191.2	117.1	15		
SPM	203.2	112.1	15	129.1	10			SPM-d_4	207.2	114.1	20		
DiAcSPD	230.2	100.1	20	72.1	30			DiAcSPD-d_4	234.2	102.1	15		
AcSPM	245.2	112.1	20	129.1	10			AcSPM-d_4	249.2	114.1	20		
DiAcSPM	287.2	100.1	20	171.1	10			DiAcSPM-d_4	291.2	102.1	25		

matrix polyamine concentration × 0.95 + 1000 nM). The HQC was then diluted 5-fold with QC matrix to prepare the middle QC working solution (MQC = endogenous matrix polyamine concentration × 0.995 + 200 nM). Unspiked QC matrix was used as the low QC working solution (LQC = endogenous matrix polyamine concentration). To prepare the lower limit QC working solution (LLQC), unspiked QC matrix was diluted 1:1 with water (LLQC = endogenous matrix polyamines × 0.5).

The final QC samples for LC–MS/MS were prepared similarly as samples and calibration standards described in Section 2.6. Pooled urine QC matrix without IS was used as a cross-talk check.

3. Results and discussion

3.1. Sample preparation

Polyamines are endogenous compounds which are also present in human urine, thus analyte free urine is not available. For this reason, standards and CC samples were prepared in water. However, QC samples were prepared in pooled urine matrix to be as closely related to the study samples as possible.

In the sample preparation, SPE cartridges were used to remove interfering compounds such as amino acids from the urine samples. This extraction step was shown to decrease markedly column contamination and column clogging. Usually SPE of polyamines from different matrixes has been performed using cation exchanging resins [29], however those are not suitable for extracting neutral polyamine derivatives, which do not contain free amino groups, like DiAcPUT and DiAcCAD. Strong anion exchanging sorbents retain organic acids and possibly also phenolic compounds and carbohydrates, but allows polyamines to elute in the void fraction [30–32], and are thus more suitable for neural polyamine derivatives.

3.2. LC–MS/MS method

LC separation of polyamines was based on HFBA as an ion-pairing reagent, and MS/MS analysis was performed utilizing selected reaction monitoring (SRM) [22,23,28,33,34]. It is known, that mass detection is not constrained by chromatographic resolution if the compounds differ in molecular weight. If the co-eluting compound has the same molecular weight, but its collision induced dissociation produces different fragment ions, the compounds can be separated using SRM. A common practice to increase the confidence of the analyte identification in SRM methods is to monitor a second, qualifier MS/MS transition in addition to that being used for quantification. A branching ratio of the precursor ion fragmentation to the qualifier and quantifier ions is used as an acceptance criterion for the identification. However, if the co-eluting compound has the same molecular weight, and it produces same fragments, which has the same ratio as the fragments originating from the analyte of interest, we cannot detect that there is something which interferes the analysis. To prevent this as much as possible, LC conditions were studied using several different columns, and the one used in the studies was selected as the best one to separate the analytes from each other and from the matrix compounds as well as possible. The Overlaid chromatograms of 14 polyamines are shown in Fig. 1. Also labeled internal standards for each analyte were used to compensate any co-eluting compounds which are not seen in MS, but could cause matrix effects.

During the setup of MRM transitions, it was found that deuterated DiAcCAD-d_6 and DiAcPUT-d_6 were fragmented probably through a four-center cleavage mechanism as described previously [35]. One of the deuterium atoms from the cleaved acetyl group was shifted to the remaining ammonium group. This was demonstrated by dissolving the compound to D_2O prior CID studies, after the deuterium shift from acetyl group did not increase m/z of the product ion. Fig. 2 illustrates the CID spectra of DiAcCAD-d_6 dissolved in water and in D_2O prior to the measurements.

For the DiAcPUT and DiAcCAD, the internal standard retention time slightly deviates from the analyte, deuterated analogs eluting earlier from the column (see Fig. 1 for compounds **1** and **3**). This means that not all of the matrix effect is compensated, and thus the method is only quasi-quantitative for these compounds. One of the reasons for the retention time difference might be the neutral character of these molecules, i.e. there can be no ion pairing interaction between them and the solid phase of the column.

3.3. Method validation

3.3.1. Calibration ranges and limit of detection

Calibration standards including a blank and a zero sample were analyzed before the samples within each analysis batch. Calibration curve quality control samples (CC) were used to verify the accuracy and precision of the calibration curves. Selection of the best curve fit

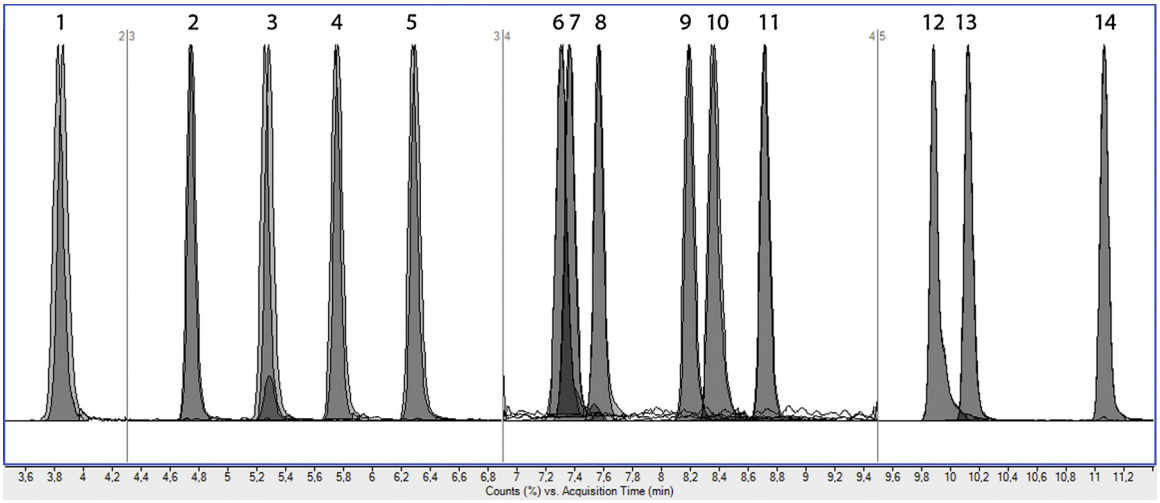

Fig. 1. Overlaid LC–MS/MS SRM chromatograms of 14 polyamines and their deuterated internal standards. All peaks are scaled to the same height. (1) DiAcPUT + DiAcPUT-d_6, (2) AcPUT + AcPUT-d_3, (3) DiAcCAD + DiAcCAD-d_6, (4) AcCAD + AcCAD-d_3, (5) DiAcSPD + DiAcSPD-d_4, (6) DAP + DAP-d_4, (7) PUT + PUT-d_8, (8) CAD + CAD-d_4, (9) N^1AcSPD + N^1AcSPD-d_5, (10) N^8AcSPD + N^8AcSPD-d_3, (11) DiAcSPM + DiAcSPM-d_4, (12) SPD + SPD-d_4, (13) AcSPM + AcSPM-d_4 (14) SPM + SPM-d_4. The third, smaller peak at t_R 5.3 is due to in-source fragmentation of DiAcCAD ion m/z 187 to m/z 145, which is the same as $[M+H]^+$ ion of AcCAD.

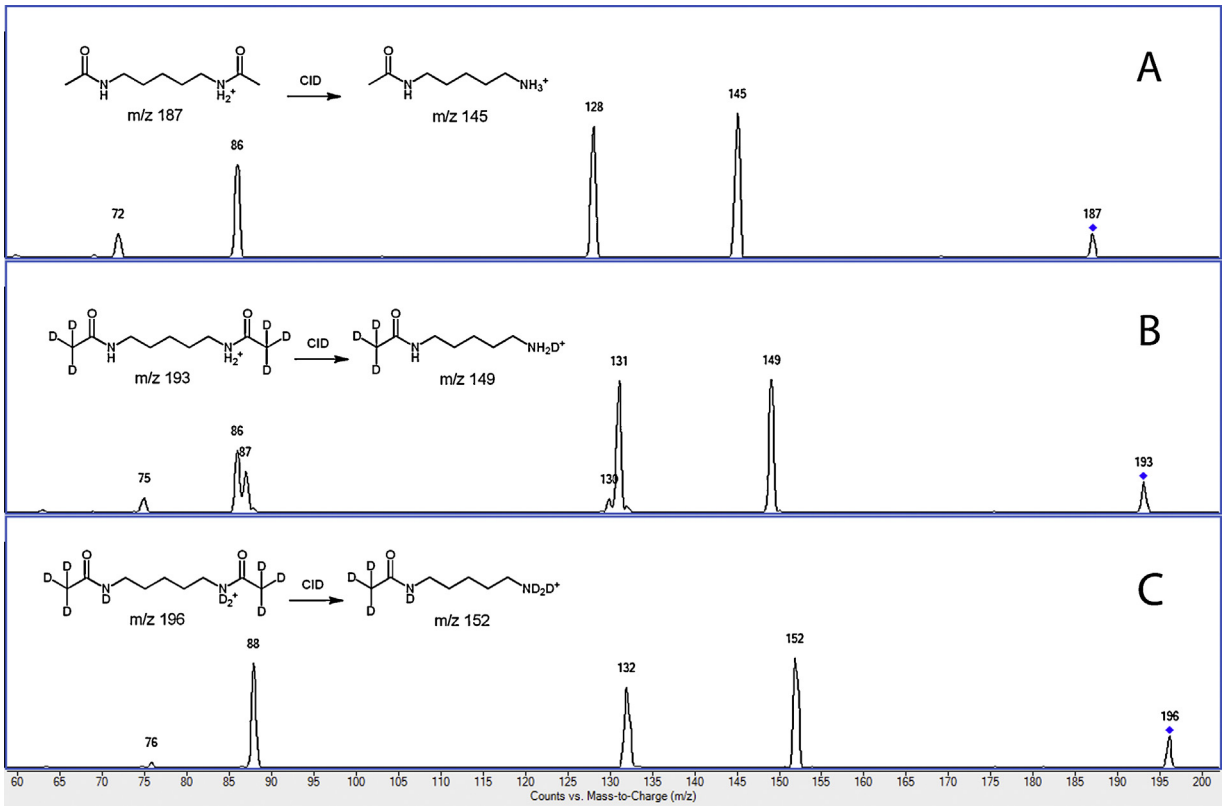

Fig. 2. DiAcCAD-d_6 is fragmented probably through a four-center cleavage mechanism. (A) DiAcCAD in H_2O m/z 187 ($M+H^+$), (B) DiAcCAD-d_6 in water m/z 193 ($M+H^+$) and (C) DiAcCAD-d_6 in D_2O m/z 196 ($M+D^+$).

(quadratic) was based on the comparison of the sum of deviations of standards from their nominal concentrations, and the weighing scheme that gave the smallest sum of deviations was used for the weighing ($1/x^2$). Correlation coefficients were also calculated. The acceptance value for intra and inter-run precision error (RSD%) and accuracy were <15% and 85–115% for all standards, respectively (except for the lowest calibration point the precision error limit was 20% and accuracy should be between 80 and 120%). Calibration ranges for each polyamine are shown in Table 2. Limit of detection for each polyamine standard in water after sample preparation was defined as the concentration which had signal to noise ≥3 (see Table 2, fmol/injection). However, it should be noted that in the real urine samples the LLOQ and LOD is dependent on the matrix, which can vary. Internal standard, if co-eluting with the analyte, should compensate the matrix effects. If there is co-eluting matrix compounds which suppresses the signal, the value for matrix factor

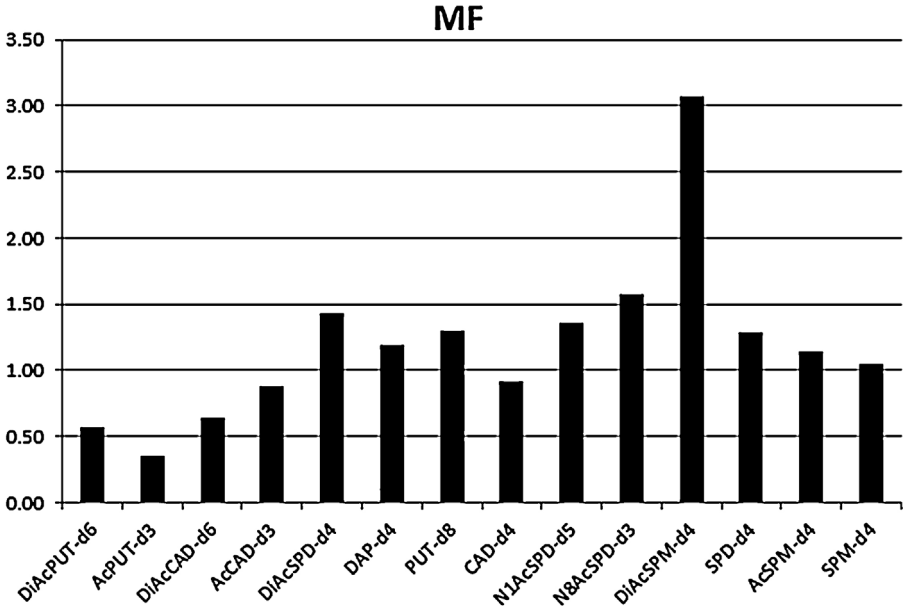

Fig. 3. Matrix factor (MF) was used to estimate the strength of matrix effect, and was calculated by taking the ratio of average peak area response of the IS spiked in QC samples and study samples (urine matrix, $n = 166$) and its average peak area response in the standards in water ($n = 135$). Ionization suppression MF < 1, Ionization enhancement MF > 1.

Table 2
Calibration ranges, limit of detection (LOD) and concentrations in pooled QC-urine for different polyamines. LOD and LLOQ (the lowest calibration point) are measured and validated using water based standards according to the FDA guidelines [24].

	Calibration range (nM)	LOD (S/N ≥3, fmol/injection)	Measured pooled, healthy QC-urine concentrations (nM), mean ± sd, $n = 20$
DAP	243–8114	71.4	219 ± 36*
PUT	103–10,250	45.1	485 ± 37
CAD	31–10,472	27.6	88 ± 12
AcPUT	25–8396	3.7	12,785 ± 488**
AcCAD	24–8136	7.2	1041 ± 67
SPD	30–10,145	4.5	208 ± 10
DiAcPUT	30–5000	8.8	228 ± 31
DiAcCAD	5–5000	2.6	25 ± 5
N^1AcSPD	27–9083	8.0	5828 ± 165
N^8AcSPD	30–10,145	4.5	4349 ± 150
SPM	32–10,552	9.3	1170 ± 39
DiAcSPD	6–11,587	1.0	361 ± 12
AcSPM	5–4881	0.9	≪5
DiAcSPM	4–8612	0.8	172 ± 5

* Below lloq.
** Calculated from 1:1 diluted sample.

would be smaller than 1 (see Fig. 3) Thus, for analytes with MF < 1 there might be an elevated risk to have higher real LLOQ values than what has been measured with the calibration curve quality control samples. However, false negative results are possible if ion suppression for any analyte in the individual sample is strong enough to diminish the analyte response below the detection limit. Thus, special attention should be paid to follow the analyte as well as the internal standard peak response.

3.3.2. Selectivity and matrix effect

Selectivity and matrix effect were assessed in several ways. Human urine samples from six independent lots, measured without IS added, were devoid of any interfering components at the retention time of interest in the SRM channel for the internal standard. For DiAcCAD, some of the low concentration urine samples did contain endogenous components that emitted a signal close to the retention time of the analyte, and thus some of the samples may not be able to be quantified reliably. In addition, while the SRM transition for DiAcPUT standard (m/z 173–114) provided the best sensitivity, the urine matrix had matrix-based components that interfered with the peak of interest and, therefore, this transition was only monitored for confirmatory purposes.

Carry over was investigated by analyses of the highest standard samples (10,000 nM) followed by analyses of blank samples. Percent carry over was determined by comparing the found peak areas in the blank samples versus the peak area of the standard solution. Mean %-carry over values ($n = 3$) of the first and second blank were 0.038% and 0.014% for SPM, 0.024% and 0.006% for AcSPM, 0.022% and 0.015% for SPD and 0.012% and 0.002% for DiAcSPM. For rest of the analytes, carry over was below 0.01%. To minimize any possible carry over, a blank sample was injected after the highest standards and urine samples with high concentrations.

A matrix factor (MF) was used to estimate the strength of matrix effect caused by the urine matrix as shown in Fig. 3. It can be seen that without internal standards to compensate for the matrix effect, the method might give average DiAcSPM concentrations three times too high whereas the concentrations for AcPUT would be less than half of the actual concentration.

Recovery was tested to evaluate the possible differences between the elution of analytes and their deuterated internal standards through the SAX column (Table 3). Both urine and water as a sample matrix were tested with two concentration levels. The elutions of both analyte and its deuterated analog in these two matrixes were similar, and all recovery values were between 80 and 104%.

STD addition to water, or to QC matrix, before the sample preparation was used to evaluate the bias of the endogenous concentration, calculated using the STD addition to urine, or to the water as shown in Fig. 4. Three samples were made at each concentration level and analyzed. Standard addition curves were drawn and slopes were calculated. If the slope for the two lines were equal, then internal standards could compensate for the matrix effect in the samples and water based standards could be used in the quantitation. For DiAcCAD, the slope differed in urine and water samples, thus the results for DiAcCAD are no more than quasi-quantitative.

Dilution linearity was measured to test if high concentration samples could be diluted with water prior to sample preparation, without affecting assay accuracy. Dilution linearity was tested in two ways. In method A, the QC-urine with a known endogenous concentration was spiked with 1000 nM polyamine standards and analyzed after 0, 2, 4, 10 or 20-fold dilution with water. In method B, unspiked QC-urine was analyzed after 0, 5, 10 or 20-fold dilution with water. The mean back-calculated concentrations, corrected for the dilution, were compared with the theoretical total concentration (measured endogenous plus spiked analyte). The results are shown in Table 4. There were no significant changes in the accuracies after up to 5-fold dilution for each analyte, and AcPUT, N^1AcSPD and N^8AcSPD, which are the most abundant polyamines in the urine samples, can be analyzed also from the 1:20 diluted samples, if necessary.

3.3.3. Stability studies

The stability of polyamine standard mixture in water was tested by analyzing working standard solutions (300 and 3000 nM) after being stored for 6 h at room temperature (short term stability), after storage at −20 °C two months (long term stability) and after going through three cycles of freezing at −20 °C before sample preparation. Standard mixtures were stable in all tested conditions.

Stability of QC samples (LQC, MQC, HQC) was examined after storage for 6 h at room temperature (short term stability), after being stored at −80 °C two months (long term stability) and after going through three cycles of freezing at −80 °C before sample preparation. QC samples were stable in all tested conditions.

3.3.4. Accuracy and precision

Quality controls were used to verify the accuracy and precision of the study samples. These were prepared from the pooled urine from healthy subjects. The endogenous concentration of polyamines from these six healthy men was measured separately. As the study samples from cancer patients were expected to yield higher polyamine levels, a 1:1 dilution of the QC matrix was expected to be sufficient to achieve the lower limit QC (LLQC). Intra- and inter-batch precision and accuracy were measured by the analysis of QCs over four separate analytical batches, each containing four levels of QCs at low (LLQC), basal (LQC), middle (MQC) and high (HQC) concentrations, each with five replicates. Back-calculated concentrations of the QCs were measured against the surrogate matrix calibration line using the relative peak area response of the analyte to IS versus the theoretical concentration of the calibration standards.

The assessments of precision and accuracy were performed by analyzing multiple aliquots of unspiked and spiked samples of the QC matrix against a surrogate calibration curve. The unspiked sample represented the mean endogenous background concentration. Accuracy was calculated by comparing the mean experimental concentrations of measured QC samples with their nominal values, and percentage values were used as the index. Relative standard deviation (RSD) of the concentrations was used as an index of precision, with acceptance criteria being 15%. Mean endogenous

Table 3
Recovery (%) calculated as the ratio of the mean peak area of an analyte and IS spiked to A) QC urine or B) water before SAX extraction to the mean peak area of an analyte and IS spiked post-extraction multiplied by 100 ($n = 3$).

A	+250 nM	+2500 nM		+250 nM	+2500 nM	B	+250 nM	+2500 nM		+250 nM	+2500 nM
DiAcPUT	96	98	DiAcPUT-d_6	93	101	DiAcPUT	93	97	DiAcPUT-d_6	91	100
AcPUT	99	98	AcPUT-d_3	92	93	AcPUT	93	100	AcPUT-d_3	91	102
DiAcCAD	94	95	DiAcCAD-d_6	94	96	DiAcCAD	92	94	DiAcCAD-d_6	91	96
AcCAD	99	99	AcCAD-d_3	102	102	AcCAD	95	101	AcCAD-d_3	94	97
DiAcSPD	99	98	DiAcSPD-d_4	100	100	DiAcSPD	96	97	DiAcSPD-d_4	96	98
DAP	104	92	DAP-d_4	99	101	DAP	108	102	DAP-d_4	101	98
PUT	104	98	PUT-d_8	104	93	PUT	100	100	PUT-d_8	93	96
CAD	106	99	CAD-d_4	103	102	CAD	94	95	CAD-d_4	92	100
N^1AcSPD	98	97	N^1AcSPD-d_5	95	100	N^1AcSPD	94	97	N^1AcSPD-d_5	95	98
N^8AcSPD	101	98	N^8AcSPD-d_3	99	97	N^8AcSPD	93	105	N^8AcSPD-d_3	96	102
DiAcSPM	98	96	DiAcSPM-d_4	97	98	DiAcSPM	95	97	DiAcSPM-d_4	97	97
SPD	91	80	SPD-d_4	90	82	SPD	96	90	SPD-d_4	97	89
AcSPM	97	88	AcSPM-d_4	96	90	AcSPM	94	91	AcSPM-d_4	96	90
SPM	100	92	SPM-d_4	96	90	SPM	94	90	SPM-d_4	94	88

concentrations in pooled QC-urine are shown in Table 2, and accuracy and precision values for QC and CC samples are shown in Tables A and B in the supplementary materials.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2013.10.009.

Accuracies of DiAcPUT and DiAcCAD in QC samples were acceptable in the first two batches (>300 injections), but no longer in the third and fourth batch with the same column (see Table A). DiAcPUT starts to display lower concentrations, and DiAcCAD starts to exhibit higher concentrations than in the first two batches (more than 15% bias in accuracy). The signal variation due to matrix effects cannot be totally compensated by using deuterated internal standards as there is a slight difference between the retention times of analyte and IS (see Fig. 1). In some conditions, the matrix contains a background compound that interferes with the DiAcPUT and

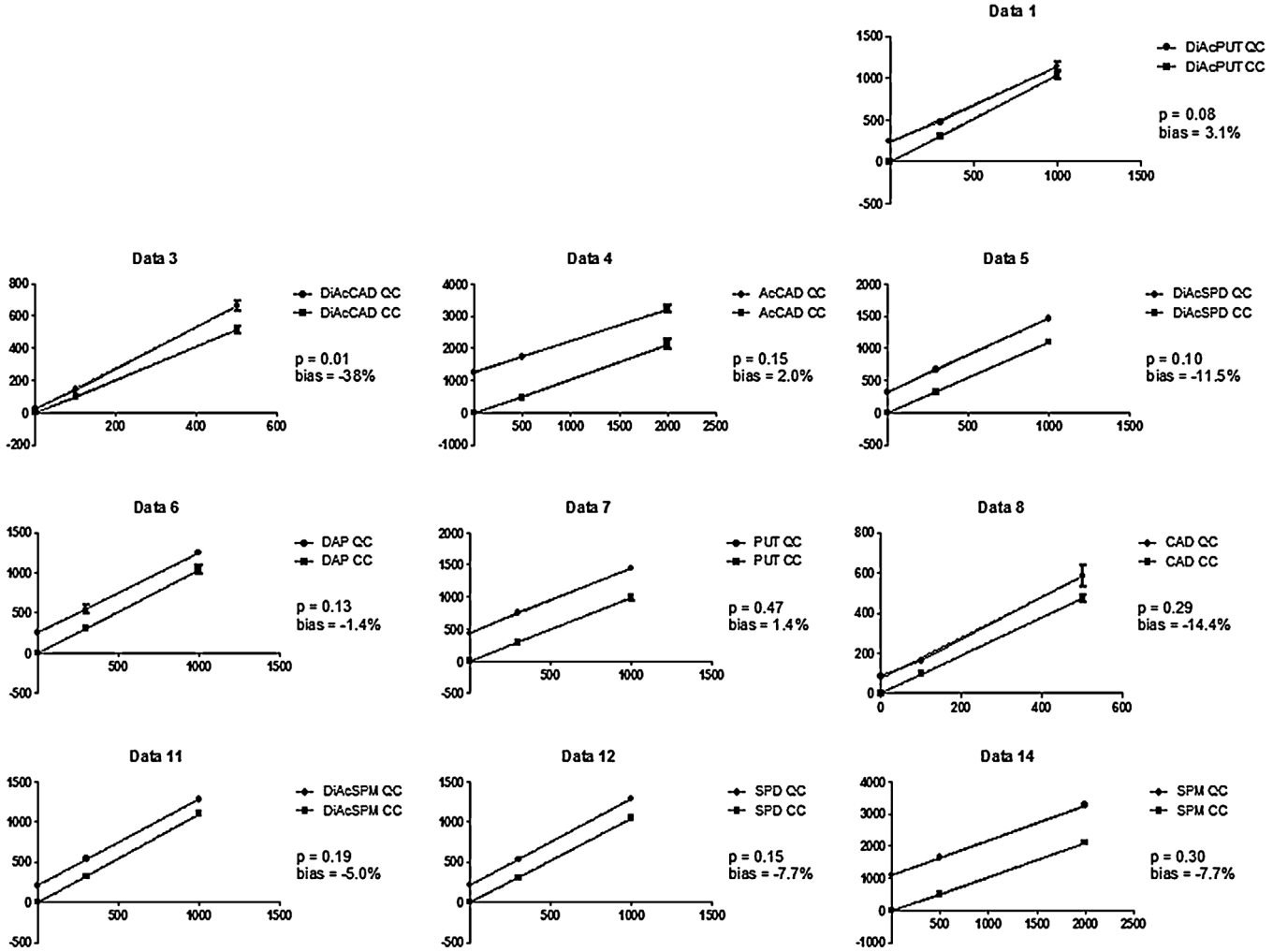

Fig. 4. STD addition to water, or to QC urine before the sample preparation. Attempts were made to keep the amount of the STD added between one and five times the amount of analyte in QC urine. Bias% is the relative error between the endogenous concentration calculated using the STD addition to urine or to the water. The endogenous concentrations of AcPUT, N^1AcSPD and N^8AcSPD were too high for STD addition, and the endogenous concentration of AcSPM was too far below the calibration range.

Table 4
Dilution linearity. (A) QC-urine was spiked with 1000 nM polyamine standards and the spiked sample was analyzed after 0, 2, 4, 10 or 20-fold dilution with water ($n \geq 3$). (B) Unspiked QC-urine was analyzed after 0, 5, 10 or 20-fold dilution with water ($n \geq 3$). The mean dilution corrected concentrations were then divided with the theoretical total concentration, and multiplied by 100% to obtain the % from the nominal concentration. NA = analyzed concentration was below lowest calibration point.

	DiAcPUT	AcPUT	DiAcCAD	AcCAD	DiAcSPD	DAP	PUT	CAD	N^1AcSPD	N^8AcSPD	DiAcSPM	SPD	AcSPM	SPM
(A) Dilution linearity (%) spiked sample														
DF 2	91	110	93	100	104	102	95	95	102	105	98	94	103	97
DF 4	91	117	95	103	101	94	97	98	107	105	95	92	100	98
DF 10	93	114	92	96	92	NA	101	99	101	96	94	94	93	118
DF 20	99	114	88	89	88	NA	NA	107	98	94	86	102	88	124
(B) Dilution linearity (%) unspiked sample														
PL 5	96	115	NA	93	80	NA	111	NA	102	96	92	115	NA	109
PL 10	NA	115	NA	97	77	NA	NA	NA	100	95	87	NA	NA	124
PL 20	NA	114	NA	83	77	NA	NA	NA	95	92	87	NA	NA	165

Table 5
Polyamines and metabolites (umol/g of Creatinine) and (nM in urine) in the urine samples of healthy men and in prostate cancer patients.

	Healthy men ($n = 6$)			Prostate cancer patients ($n = 22$)		
	Range μmol/g creatinine	Range nM in urine	Found in samples	Range μmol/g creatinine	Range nM in urine	Found in samples
DAP	NA–0.19	NA–349	3/6	NA–4.58	NA–3216	5/22
PUT	0.14–0.59	179–829	6/6	NA–6.27	NA–4400	21/22
CAD	NA–0.21	NA–290	5/6	NA–0.59	NA–411	8/22
AcPUT	5.35–9.20	7618–25239	6/6	6.01–49.24	2637–27213	22/22
AcCAD	0.14–1.52	284–4181	6/6	0.13–12.54	158–5608	22/22
SPD	0.09–0.15	139–364	6/6	0.09–1.35	77–892	22/22
DiAcPUT	0.05–0.45	71–881	6/6	NA–1.14	NA–552	21/22
DiAcCAD	0.002–0.026	6–70	6/6	NA–3.24	NA–922	19/22
N^1AcSPD	1.67–5.39	2180–13994	6/6	1.54–20.03	1069–11310	22/22
N^8AcSPD	1.40–2.67	2332–6648	6/6	1.79–9.11	1031–7203	22/22
SPM	0.33–1.04	457–2863	6/6	0.06–6.48	74–7013	22/22
DiAcSPD	0.14–0.24	189–581	6/6	0.12–0.94	60–735	22/22
AcSPM	NA	NA	0/6	NA–0.014	NA–12	3/22
DiAcSPM	0.06–0.17	68–454	6/6	0.05–0.39	32–582	22/22

NA = analyzed concentration was below the lowest calibration point.

DiAcCAD signals. In the future it would be interesting to determine whether C-13 or N-15 labeled internal standards would help to quantitate also of these two polyamine metabolites. In addition, DAP, CAD and AcSPM concentrations in some of the studied urine samples were too low when compared to the quantitation range of the method, thus these polyamine metabolites can be quantitated only if the concentration of the samples is sufficiently high. AcPUT and N^1AcSPD concentrations in urine samples were high and some samples had to be diluted 1:10 in order that they would fit into the calibration range.

To solve the matrix problems with DiAcPUT and DiAcCAD, as well as sensitivity problems with DAP, CAD and AcSPM, different LC columns and conditions, as well as different sample preparation methods were studied, but the best selectivity and sensitivity as a compromise for all analytes was achieved with the used conditions. However, the sensitivity of the QQQ instrument used for this study is not comparable with the current, more sensitive QQQ instruments available in the market. If more modern instruments would have been available for our studies, it might be possible to reach sufficient sensitivity also for DAP, CAD and AcSPM analysis. Also the samples could have been diluted more, without compromising the sensitivity, which could have decreased the matrix effects and thus help the analysis of DiAcPUT and DiAcCAD. In addition, with more modern instruments, narrower window for the selected ions might be used, which could enhance the selectivity and reduce the signals from possible interfering components to any analyte's MRM channels.

3.4. Polyamine concentration in human urine

Human urine is non-invasively accessible body fluid, and is thus patient-friendly method for sample collection. On the other hand, urine contains numerous compounds at different concentrations, and there may be large variation in the concentration because of individual variations in the urinary volume output [36,37]. For this reason, creatinine was used to correct urinary data for the effects of urine volume on total urinary concentration. Urine creatinine is a widely accepted way to perform normalization of urinary excretion data, despite the fact that its levels in urine are somewhat dependent on several endogenous and exogenous factors like age, gender, and lean body mass of the individual, sleep deprivation, drugs in use, etc. [37,38]. For this reason, in addition to the creatinine normalized polyamine concentration, also molar concentrations of urinary polyamines are presented in Table 5.

4. Conclusions

The LC–MS/MS method described for the analysis of urinary polyamines and their acetylated metabolites was proved to be suitable for the quantitation of at least nine polyamines (PUT, SPD, SPM, AcPUT, AcCAD, N^1AcSPD, N^8AcSPD, DiAcSPD, DiAcSPM) from human urine samples, and three polyamines (DAP, CAD, AcSPM) can be quantitated if the concentration in samples is sufficiently high. In addition, two neutral, acetylated diamines can be analyzed semiquantitatively. The simultaneous analysis of these 14 polyamines was performed without any derivatization using solid phase extraction columns in sample preparation and deuterium labeled internal standards for each analyte in the MS detection.

The method is suitable for the quantification of polyamine levels from human urine samples taken from prostate or urinary tract cancer patients and selected reference patients. The variation of polyamine concentrations in urine samples is high, and for this reason also diluted samples were measured in order to avoid detector suppression. In addition, measured polyamine amounts were normalized to urine creatinine concentration. This method will be used

in the analysis of urine from cancer patients to evaluate whether polyamine profiles can be used in the diagnostics of various types of cancers, in the estimation of cancer stage and treatment response.

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References

[1] F.A.J. Muskiet, B. Dorhout, G.A. van den Berg, J. Hessels, J. Chromatogr. B 667 (1995) 189–198.
[2] A. Önal, Crit. Rev. Anal. Chem. 40 (2010) 60–67.
[3] D. Teti, M. Visalli, H. McNair, J. Chromatogr. B 781 (2002) 107–149.
[4] M.-J. Paik, S. Lee, K.-H. Cho, K.-R. Kim, Anal. Chim. Acta 576 (2006) 55–60.
[5] D.H. Russel, Nat. New Biol. 233 (1971) 144–145.
[6] D.H. Russel, Crit. Rev. Clin. Lab. Sci. 18 (1983) 261–311.
[7] M.Y. Khuhawar, G.A. Qureshi, J. Chromatogr. B 764 (2001) 385–407.
[8] J.W. Suh, S.H. Lee, B.C. Chung, J. Park, J. Chromatogr. B 688 (1997) 179–186.
[9] K. Hiramatsu, M. Sugimoto, S. Kamei, M. Hoshino, K. Kinoshita, K. Iwasaki, M. Kawakita, J. Biochem. 117 (1995) 107–112.
[10] H. Inoue, K. Fukunaga, S. Munemura, Y. Tsuruta, Anal. Biochem. 339 (2005) 191–197.
[11] A.P. Stevens, K. Dettmer, G. Kirovski, K. Samejima, C. Hellerbrand, A.K. Bosserhoff, P.J. Oefner, J. Chromatogr. A 1217 (2010) 3282–3288.
[12] K. Shimpo, T. Chihara, C. Ida, K. Kawai, K. Wakamatsu, S. Ito, T. Matsui, M. Hirano, T. Watanabe, T. Nagatsu, H. Kuzuya, Biog. Amines 16 (2001) 211–223.
[13] S.H. Lee, Y.J. Yang, K.M. Kim, B.C. Chung, Cancer Lett. 201 (2003) 121–131.
[14] M. Kawakita, K. Hiramatsu, J. Biochem. 139 (2006) 315–322.
[15] Y. Umemori, Y. Ohe, K. Kuribayashi, N. Tsuji, T. Nishidate, H. Kameshima, K. Hirata, N. Watanabe, Clin. Chim. Acta 411 (2010) 1894–1899.
[16] K. Samejima, K. Hiramatsu, K. Takahashi, M. Kawakita, M. Kobayashi, H. Tsumoto, K. Kohda, Anal. Biochem. 401 (2010) 22–29.
[17] G.J. Feistner, Biol. Mass Spectrom. 23 (1994) 784–792.
[18] N. Seiler, J. Chromatogr. 379 (1986) 157–176.
[19] J.A. Byun, S.H. Lee, B.H. Jung, M.H. Choi, M.H. Moon, B.C. Chung, Biomed. Chromatogr. 22 (2008) 73–80.
[20] N.N. Al-Hadithi, B. Saad, Anal. Lett. 44 (2011) 2245–2264.
[21] S.H. Lee, J.W. Suh, B.C. Chung, S.O. Kim, Cancer Lett. 122 (1998) 1–8.
[22] M.R. Häkkinen, M.T. Hyvönen, S. Auriola, R.A. Casero Jr., J. Vepsäläinen, A.R. Khomutov, L. Alhonen, T.A. Keinänen, Amino Acids 38 (2010) 369–381.
[23] M.R. Häkkinen, T.A. Keinänen, A.R. Khomutov, S. Auriola, J. Weisell, L. Alhonen, J. Jänne, J. Vepsäläinen, Tetrahedron 65 (2009) 547–562.
[24] Guidance for industry, bioanalytical method validation, US department of health and human services food and drug administration, center for drug evaluation and research (CDER), 2001, available: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf
[25] N.C. van de Merbel, Trends Anal. Chem. 27 (2008) 924–933.
[26] R. Houghton, C.H. Pita, I. Ward, R. Macarthur, Bioanalysis 1 (2009) 1365–1374.
[27] C.T. Viswanathan, S. Bansal, B. Booth, A.J. DeStefano, M.J. Rose, J. Sailstad, V.P. Shah, J.P. Skelly, P.G. Swann, R. Weiner, Pharm. Res. 24 (2007) 1962–1973.
[28] M.R. Häkkinen, T.A. Keinänen, J. Vepsäläinen, A.R. Khomutov, L. Alhonen, J. Jänne, S. Auriola, J. Pharm. Biomed. Anal. 48 (2008) 414–421.
[29] C. Molins-Legua, P. Campins-Falcó, Anal. Chim. Acta 546 (2005) 206–220.
[30] J.B. Wehr, J. Chromatogr. A 709 (1995) 241–247.
[31] O. Busto, J. Guasch, F. Borrull, J. Chromatogr. A 718 (1995) 309–317.
[32] O. Busto, M. Miracle, J. Guasch, F. Borrull, J. Chromatogr. A 757 (1997) 311–318.
[33] M.R. Häkkinen, T.A. Keinänen, J. Vepsäläinen, A.R. Khomutov, L. Alhonen, J. Jänne, S. Auriola, J. Pharm. Biomed. Anal. 45 (2007) 625–634.
[34] M.R. Häkkinen, Methods Mol. Biol. 720 (2011) 505–518.
[35] W. Hu, E. Reder, M. Hesse, Helv. Chim. Acta 79 (1996) 2137–2151.
[36] D.F. Putman, Composition and concentrative properties of human urine. NASA contractor report, Washington D.C., 1971.
[37] E.J. Saude, D. Adamko, B.H. Rowe, T. Marrie, B.D. Sykes, Metabolomics 3 (2007) 439–451.
[38] D. Ryan, K. Robards, P.D. Prenzler, M. Kendall, Anal. Chim. Acta 684 (2011) 17–29.

Detection of Prostate Cancer by an Electronic Nose: A Proof of Principle Study

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Purpose: We evaluate the ability of an electronic nose to discriminate prostate cancer from benign prostatic hyperplasia using urine headspace, potentially offering a clinically applicable noninvasive and rapid diagnostic method.

Materials and Methods: The ChemPro® 100-eNose was used to discriminate prostate cancer from benign prostatic hyperplasia using urine sample headspace. Its performance was tested with 50 patients with confirmed prostate cancer and 24 samples from 15 patients with benign prostatic hyperplasia (15 patients provided urine preoperatively and 9 patients provided samples 3 months postoperatively) scheduled to undergo robotic assisted laparoscopic radical prostatectomy or transurethral resection of prostate, respectively. The patients provided urine sample preoperatively and those with benign prostatic hyperplasia also provided samples 3 months postoperatively to be used as a pooled control sample population. A discrimination classifier was identified for eNose and subsequently, sensitivity and specificity values were determined. Leave-one-out cross-validation was performed.

Results: Using leave-one-out cross-validation the eNose reached a sensitivity of 78%, a specificity of 67% and AUC 0.77.

Conclusions: The electronic nose is capable of rapidly and noninvasively discriminating prostate cancer and benign prostatic hyperplasia using urine headspace in patients undergoing surgery.

Key Words: prostatic hyperplasia, electronic nose, prostatic neoplasms

Abbreviations and Acronyms

BPH = benign prostatic hyperplasia
DRE = digital rectal examination
eNose = electronic nose
G/LC-MS = gas/liquid chromatography-mass spectrometry
LOOCV = leave-one-out cross-validation
NMR = nuclear magnetic resonance spectroscopy
PCa = prostate cancer
PSA = prostate specific antigen
TURP = transurethral resection of prostate

PROSTATE cancer is the second most common cancer in males and one of the leading causes of cancer mortality.[1] The heterogeneity of PCa makes it difficult to diagnose and to predict tumor progression. Current cornerstones of the diagnosis, ie DRE and plasma PSA,[2] have limited sensitivity and specificity, although free PSA adds to the performance.[3] Histological examination of transrectal ultrasound guided biopsies leads to definitive diagnosis, but is associated with considerable costs, discomfort and risk of infectious complications.[4] In addition, a significant proportion of diagnosed cancers is low grade and will not cause symptoms or disease

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specific mortality. Therefore, aggressive treatment can lead to decreased quality of life without affecting the longevity of the patient.[5] Thus, there is a need for novel diagnostic tools.

The first report on the olfactory detection of cancer was a case study published in *The Lancet*.[6] Since then, experimental studies of the use of trained dogs in the detection of cancer have confirmed the preliminary findings.[7–9] Previous results of PCa detection were discouraging[10] until Cornu et al showed that trained dogs are able to detect PCa using urine with high sensitivity and specificity.[11] This finding sparked considerable attention.[12,13]

The concern with canine olfaction is the heterogeneity of the performance of the dogs between and within studies.[11,14] The eNose is a device that consists of a cluster of nonspecific sensors. When the device is exposed to the sample, it produces a profile or a smell print. eNoses are best suited for qualitative analysis of complex gaseous mixtures of molecules,[15] and are routinely used in food and agricultural quality control and in military applications.[16] eNoses have been studied in various medical applications, including the early detection of cancer,[15] especially from exhaled air.[17,18]

Exhaled air is a problematic sample material since it requires good cooperation and technique from the patient as well as immediate analysis, while urine is simple to attain and store and, therefore, more feasible in clinical practice. Urine has also been used in several metabolomics studies using G/LC-MS or NMR.[19] Preliminary data suggest that the detection of urological malignancies using urine headspace is possible.[20]

Recently we showed that the discrimination of PCa cells and cells from BPH is possible.[21] These results encouraged us to launch a prospective clinical study recruiting patients undergoing robotic assisted laparoscopic radical prostatectomy and symptomatic patients with BPH undergoing TURP. In this study we test the hypothesis that the eNose system is capable of discriminating patients with PCa from those with BPH using urine headspace. We also evaluate potential sources of diagnostic error such as prostate volume and tumor size.

MATERIALS AND METHODS

Patients and Samples

The study population was prospectively formed of consecutive patients referred to a urologist for operative treatment of PCa or BPH at Tampere University Hospital (Tampere, Finland) or Hatanpää City Hospital (Tampere, Finland) between March 2011 and November 2012. Inclusion criteria were operative treatment of PCa or BPH. Exclusion criteria were patient refusal, material insufficient for histopathology, known malignancy other than PCa, persistent urinary infection and urinary catheter in place. Patients were divided into cases and controls, as in patients with biopsy proven PCa scheduled to undergo robotic assisted laparoscopic radical prostatectomy (50) and PCa-free patients (15). The PCa-free group comprised patients with BPH scheduled to undergo TURP, having later confirmed benign histology. Of the patients with BPH 3 had previously had negative prostate biopsies.

The control group consisted of 15 patients who gave a urine sample preoperatively, and of these patients 9 provided another sample 3 months postoperatively, resulting in a total of 24 samples. As the measurement of postoperative prostate volume was not included in the study protocol, we reduced prostate volume 40% from the postoperative results. Subjects provided a standard morning urine sample before the operation and 3 months after the operation. No standardization of diet, hydration status or bladder time was conducted. The samples were stored at −70C until eNose analysis.

Written informed consent was acquired from all subjects. The study was approved by the ethical committee of Tampere University Hospital (code: R10066). The baseline clinical characteristics of the patients are provided in the table. [T1]

eNose and Measurement Chamber

The eNose used in this study is a commercially available model (ChemPro® 100, Environics Inc., Mikkeli, Finland) based on the ion mobility spectrometry principle. The device contains an ion mobility cell that consists of 8 electrode strips producing 2-channel output and a metal oxide based semiconductor cell. Together these sensors produce 18-channel measurement data. The sensors do not specify molecules but produce a characteristic smell print of the sample. Ambient air is used as carrier gas. The device is described in detail by Utriainen et al.[22] The device was connected to a Windows® based portable

Baseline clinical and pathological characteristics of study subjects

	Confirmed PCa Group		Control Group	
Age:				
Mean	62		66	
Median (range)	63.5	(49—73)	67	(53—72)
Total PSA:				
Mean	7		3	
Median (range)	6.3	(2—18.2)	1.75	(0.2—9)
Prostate vol:				
Mean	36.7		48.6	
Median (range)	33	(15—75)	40.3	(18.2—100)
PSA density (PSA/prostate vol):				
Mean	0.22		0.06	
Median (range)	0.18	(0.04—0.69)	0.05	(0.02—0.2)
No. postop Gleason score:				
6	9		Not applicable	
7	34		Not applicable	
8	1		Not applicable	
9	6		Not applicable	
No. pT status (%):				
pT2	27	(50)	Not applicable	
pT3	23	(50)	Not applicable	
No. presence of Gleason 4 or greater (%)	41	(50)	Not applicable	

computer with software (Environics Inc.) for monitoring and logging the data.

The measurement chamber was similar to that described in our previous work.[21] It consists of a polystyrene cell culture plate and a cover in which 3 holes are drilled. Two holes are for replacement air and the third hole is inserted with a modified 16G intravenous cannula. The injection port of the cannula is removed and a Teflon® pipe is connected to it to provide sample air for eNose. The cover is secured using parafilm. The covers were recycled during the study and washed with sterile water between measurements. Culture plates were used only once. Sample material (5 ml) was defrosted and pipetted to the plate, resulting in a thin layer of fluid, maximizing the area for evaporation. The sample plate was heated and maintained at 37C by a water poultice and kept in a laminar flow cabinet for the duration of the measurement. Each measurement lasted approximately 15 minutes and a recovery period of 10 minutes was used to avoid carryover. These intervals were previously determined to be sufficient.[21] The maximum absolute values during the measurement interval were extracted for further analysis. Of 18 channels 2 had no response in the measurements and were excluded, effectively resulting in 16 channels for the analysis. The samples were analyzed in random order and the persons performing the measurements were blinded to the status of the patient.

Statistical Analysis

To emphasize the smell print profile produced by the eNose each sample was scaled by dividing each element (16 channels) with the L2-norm of the vector. Thus, the L2-norm of each scaled sample equaled one. Linear discriminant analysis was used to assign each sample into predefined classes of PCa and BPH. LOOCV was used to avoid overfitting the classifiers.[23]

Pointwise confidence intervals for the ROC curves were computed as bias corrected and accelerated percentiles from 500 bootstrap samples.[24] The optimal thresholds were identified as the upper leftmost contact points between a straight line that has a slope S and the ROC curve. Assuming equal costs for false-positive and false-negative classes, the slope S reduces to the ratio N/P, the number of negative samples divided by the number of positive samples.[25]

Finally, the correlations between the eNose data and prostate volume in the BPH group as well as tumor volume in the PCa group were examined by a multilinear regression model, predicting prostate size using the eNose data. A stepwise regression was performed using p values of 0.05 and 0.1 for including and removing variables from the model correspondingly.[25] The same procedure was applied to examine the correlation between the eNose and tumor size.

RESULTS

Performance of eNose

The ability of eNose to differentiate the patients with operable PCa from those with symptomatic BPH requiring surgery was evaluated. The classifier comprised all the variables in the 16-dimensional data set without performing dimension reduction. A sensitivity of 82%, a specificity of 88% and AUC 0.92 were achieved using linear discriminant analysis. This represents the potential maximum performance of the methodology, but it is prone to show overly positive results in small sample cohorts due to overfitting. LOOCV was performed to minimize the effect of overfitting and to model an actual diagnostic implementation more accurately. In LOOCV analysis, a sensitivity and specificity of 78% and 67%, respectively, and AUC 0.77 were achieved. ROC curve is shown in figure 1. The [F1] stepwise regression model for predicting prostate size using eNose measurements resulted in the final model including only one eNose channel, number 9. Its correlation coefficient with prostate size was 0.34 (p value 0.02 probability of zero correlation). The R^2 was 0.11 and, thus, the model captured 11% of the total variance. No significant model for predicting tumor size was found. The average profile of urine and characteristics of average smell prints of BPH and PCA are presented in figure 2. [F2]

DISCUSSION

For the first time to our knowledge we have shown that the eNose is capable of differentiating patients with PCa from subjects with symptomatic BPH by rapid analysis of urine headspace. Our results are in line with the preliminary findings of Bernabei et al.[20] An important distinction is that while demonstrating the capability of the eNose to differentiate various urological malignancies (ie bladder and prostate cancer) from each other, they did not attempt to distinguish BPH from PCa. Cornu et al

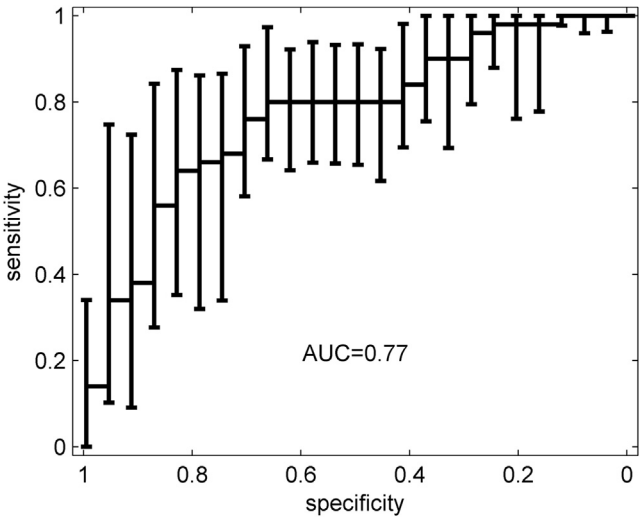

Figure 1. ROC curve for eNose with 95% CIs

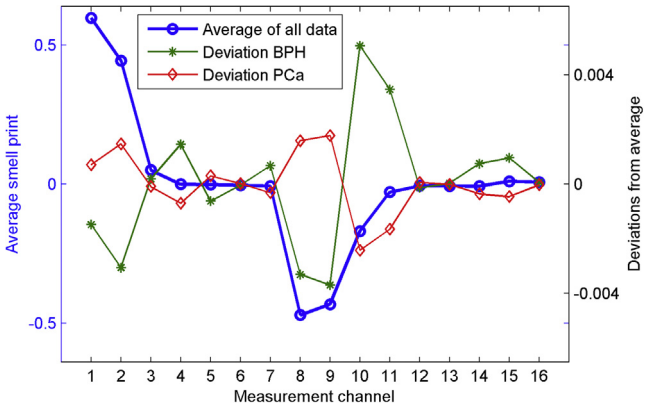

Figure 2. Total average of all data represents typical smell print of urine. Characteristics of average smell prints of BPH and PCa groups are depicted by deviation from total average.

discriminated between BPH and PCa with excellent results by using trained dogs.[11] The performance of the dog described in the study was superior to that of the eNose, potentially indicating that the dog's olfactory apparatus is superior to the eNose or that during the training phase the dog learned to ignore distracting factors such as variable kidney function, diet or medication, which may alter the smell of urine. Cornu et al collected urine samples after attentive DRE, which might partially explain the higher sensitivity and specificity. However, DRE makes the testing more invasive.

NMR and G/LC-MS offer an approach analogous to eNose when used in a holistic way. Most studies focus on plasma or tissue as sample material and, instead of a holistic approach, aim to find new biomarkers for cancer. Sarcosine was found using this methodology but proved disappointing in further studies.[19] In a recently published study Zhang et al attempted to distinguish patients with PCa from healthy controls using holistic LC-MS.[26] Their biomarker panel reached a high AUC of 0.896. It should be noted that PSA reached an AUC of 0.94, a result that could be expected when comparing patients with metastasized PCa to healthy individuals. It should be noted that NMR and G/LC-MS are considerably bulkier and more expensive than eNose. NMR requires little sample preparation but has limited sensitivity, whereas mass spectrometry offers significantly higher sensitivity but requires costly and time-consuming sample preparation. With these limitations in mind, eNose, NMR and G/LC-MS are complementary, rather than competing methods.

PSA is known to correlate positively with prostate volume,[27] which is a potential source of diagnostic error when comparing PCa with BPH. According to our current analysis prostate volume did not affect the eNose results, potentially indicating the high specificity of our sensor array to cancer. We also studied whether eNose signal correlates with tumor size and no such correlation was found.

To simulate real-life performance we conducted LOOCV in which the sample subject to discrimination is excluded from the data used to identify the classifier. Due to the limited sample number, splitting the sample into training and test populations was not feasible. LOOCV reduced the performance of eNose to a level similar to that reported by Thompson et al for PSA (AUC 0.678).[28]

It should be noted that in our study the control patients with symptomatic BPH were confirmed cancer-free by histological examination of the material resected in TURP. This is noteworthy since the majority of cancers develop in the peripheral zone of the prostate.[29] Thus, some patients in the control group may actually have subclinical cancer and this may negatively affect the discriminatory capability of the eNose. The false-positive rate of 21% is in line with the reported rate of 23% of biopsy negative PCa.[30]

Another factor to be considered is that our BPH sample consisted entirely of patients sufficiently symptomatic to require surgical treatment. We hypothesize that discrimination efficacy could be enhanced in a population consisting of patients with BPH with mild symptoms and smaller prostates. An additional confounding factor is that patients with BPH may have more lower urinary tract symptoms such as increased voiding frequency and incomplete voiding, which may affect the time urine is exposed to the prostate.

While our study confirms our hypothesis of eNose technology use in the detection of PCa, further studies are needed to evaluate the effect of comorbid conditions, hydration, diet and medications. As Cornu et al pointed out another prospect is to pinpoint the specific molecules responsible for the different smell prints.[11] In our previous work we showed that malignant prostatic cells modify their culture medium.[21] One possibility is that the distinct smell of PCa is indeed caused directly by molecules secreted to the urinary tract by cancerous cells. One future prospect is to conduct prostate massage and to study whether it enhances diagnostic performance by stimulating prostatic excretion. Due to the small sample size and that most of the cancers were Gleason score 7 it was not possible to investigate reliably whether a positive eNose finding was associated with histological PCa aggressiveness. This would be especially valuable in PCa screening to differentiate the clinically significant cancers from those which would not need further investigations and treatment. A simple,

rapid urine test with sensitivity and specificity matching that of PSA as compared to the previous published literature would be worthy of further evaluation in PCa screening.

CONCLUSIONS

eNose is able to discriminate PCa from BPH using urine headspace. The performance matches that of PSA in the literature, and results are achieved rapidly and in a completely noninvasive manner. Future studies to enhance current technology and to identify the molecules behind the distinct odors are warranted.

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REFERENCES

1. Jemal A, Bray F, Center MM et al: Global cancer statistics. CA Cancer J Clin 2011; **61:** 69.
2. Heidenreich A, Bellmunt J, Bolla M et al: EAU guidelines on prostate cancer. Part 1: screening, diagnosis, and treatment of clinically localised disease. Eur Urol 2011; **59:** 61.
3. Holmström B, Johansson M, Bergh A et al: Prostate specific antigen for early detection of prostate cancer: longitudinal study. BMJ 2009; **339:** b3537.
4. Feliciano J, Teper E, Ferrandino M et al: The incidence of fluoroquinolone resistant infections after prostate biopsy—are fluoroquinolones still effective prophylaxis? J Urol 2008; **179:** 952.
5. Roobol MJ and Carlsson SV: Risk stratification in prostate cancer screening. Nat Rev Urol 2012; **10:** 38.
6. Williams H and Pembroke A: Sniffer dogs in the melanoma clinic? Lancet 1989; **1:** 734.
7. Willis CM, Church SM, Guest CM et al: Olfactory detection of human bladder cancer by dogs: proof of principle study. BMJ 2004; **329:** 712.
8. McCulloch M, Jezierski T, Broffman M et al: Diagnostic accuracy of canine scent detection in early- and late-stage lung and breast cancers. Integr Cancer Ther 2006; **5:** 30.
9. Sonoda H, Kohnoe S, Yamazato T et al: Colorectal cancer screening with odour material by canine scent detection. Gut 2011; **60:** 814.
10. Gordon RT, Schatz CB, Myers LJ et al: The use of canines in the detection of human cancers. J Altern Complement Med 2008; **14:** 61.
11. Cornu JN, Cancel-Tassin G, Ondet V et al: Olfactory detection of prostate cancer by dogs sniffing urine: a step forward in early diagnosis. Eur Urol 2011; **59:** 197.
12. Lippi G: Re: Jean-Nicolas Cornu, Géraldine Cancel-Tassin, Valérie Ondet et al. Olfactory detection of prostate cancer by dogs sniffing urine: a step forward in early diagnosis. Eur Urol 2011; **59:** 197—201. Eur Urol 2011; **60:** e29.
13. Bjartell AS: Dogs sniffing urine: a future diagnostic tool or a way to identify new prostate cancer markers? Eur Urol 2011; **59:** 202.
14. Lippi G and Cervellin G: Canine olfactory detection of cancer versus laboratory testing: myth or opportunity? Clin Chem Lab Med 2011; **50:** 435.
15. Wilson AD and Baietto M: Advances in electronic-nose technologies developed for biomedical applications. Sensors (Basel) 2011; **11:** 1105.
16. Wilson AD and Baietto M: Applications and advances in electronic-nose technologies. Sensors 2009; **9:** 5099.
17. D'Amico A, Pennazza G, Santonico M et al: An investigation on electronic nose diagnosis of lung cancer. Lung Cancer 2010; **68:** 170.
18. Peng G, Hakim M, Broza YY et al: Detection of lung, breast, colorectal, and prostate cancers from exhaled breath using a single array of nanosensors. Br J Cancer 2010; **103:** 542.
19. Trock BJ: Application of metabolomics to prostate cancer. Urol Oncol 2011; **29:** 572.
20. Bernabei M, Pennazza G, Santonico M et al: A preliminary study on the possibility to diagnose urinary tract cancers by an electronic nose. Sens Actuators B Chem 2008; **131:** 1.
21. Roine A, Tolvanen M, Sipiläinen M et al: Detection of smell print differences between nonmalignant and malignant prostate cells with an electronic nose. Future Oncol 2012; **8:** 1157.
22. Utriainen M, Kärpänoja E and Paakkanen H: Combining miniaturized ion mobility spectrometer and metal oxide gas sensor for the fast detection of toxic chemical vapors. Sens Actuators B Chem 2003; **93:** 17.
23. Bishop CM: Pattern Recognition and Machine Learning. New York: Springer 2006; pp. 1—66.
24. DiCiccio T and Efron B: Bootstrap confidence intervals. Stat Sci 1996; **11:** 189.
25. Metz CE: Basic principles of ROC analysis. Semin Nucl Med 1978; **8:** 283.
26. Zhang T, Watson DG, Wang L et al: Application of holistic liquid chromatography-high resolution mass spectrometry based urinary metabolomics for prostate cancer detection and biomarker discovery. PLoS One 2013; **8:** e65880.
27. Oesterling JE, Jacobsen SJ, Chute CG et al: Serum prostate-specific antigen in a community-based population of healthy men. Establishment of age-specific reference ranges. JAMA 1993; **270:** 860.
28. Thompson IM, Ankerst DP, Chi C et al: Operating characteristics of prostate-specific antigen in men with an initial PSA level of 3.0 ng/ml or lower. JAMA 2005; **294:** 66.
29. Chen ME, Johnston DA, Tang K et al: Detailed mapping of prostate carcinoma foci: biopsy strategy implications. Cancer 2000; **89:** 1800.
30. Roehl KA, Antenor JA and Catalona WJ: Serial biopsy results in prostate cancer screening study. J Urol 2002; **167:** 2435.

EDITORIAL COMMENT

Should you follow your eNose? Roine et al present some exciting new technology whereby an electronic nose might be able to sniff urine and discern prostate cancer from benign prostatic hyperplasia.

Rapid and accurate detection of urinary pathogens by mobile IMS-based electronic nose: a proof-of-principle study

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Abstract

Urinary tract infection (UTI) is a common disease with significant morbidity and economic burden, accounting for a significant part of the workload in clinical microbiology laboratories. Current clinical chemisty point-of-care diagnostics rely on imperfect dipstick analysis which only provides indirect and insensitive evidence of urinary bacterial pathogens. An electronic nose (eNose) is a handheld device mimicking mammalian olfaction that potentially offers affordable and rapid analysis of samples without preparation at athmospheric pressure. In this study we demonstrate the applicability of ion mobility spectrometry (IMS) –based eNose to discriminate the most common UTI pathogens from gaseous headspace of culture plates rapidly and without sample preparation. We gathered a total of 101 culture samples containing four most common UTI bacteries: *E. coli*, *S. saprophyticus*, *E. faecalis*, *Klebsiella spp* and sterile culture plates. The samples were analyzed using ChemPro® 100i device, consisting of IMS cell and six semiconductor sensors. Data analysis was conducted by linear discriminant analysis (LDA) and linear regression (LR). The results were validated by leave-one-out cross validation analysis. In discrimination of sterile and bacterial samples sensitivity of 95% and specificity of 97% were achieved. The bacteria were identified with sensitivity of 95% and specificity of 96% using eNose as compared to urine bacterial cultures. In conclusion: These findings strongly demonstrate the ability of our eNose to discriminate bacterial cultures and provides a proof of principle to use this method in urinanalysis of UTI.

Introduction

Urinary tract infection (UTI) is a condition where pathogen enters urinary system either by ascending via urethra or via hematological route and causes an infection that can range from mild cystitis to life-threatening pyelonephritis [1]. UTI is one of the most common infections in humans. It is associated with considerable economical costs [2].

80% of all uncomplicated UTIs are caused by gastrointestinal bacteria *Escherichia coli*, approximately 15% are caused by *Staphylococcus saprophyticus*, a bacterium commonly present in female genital tract [3]. The remaining 5-10% are caused by *Klebsiella* species or *Enterococcus faecalis*, both originating from the gastrointestinal tract [3]. A complicated UTI occurs in a patient who has a predisposing factor such as diabetes or an abnormality in the urinary tract that lowers natural resistance and enables opportunistic infections. *E. coli* is the most common pathogen in this population as well, but the composition of other pathogens depends on the predisposing factor of the patient [3].

Physicians must distinguish UTI from other diseases that have a similar clinical presentation, some UTIs are asymptomatic or present with atypical signs and symptoms. The diagnosis of UTI relies on urinalysis and bacterial culture. Urinalysis is achieved either by on-site dipstick analysis, or by flow cytometry in a laboratory-setting [4]. Dipstick analysis detects urinary leucocytes, nitrate and blood, whose clinical implications are interpreted by a physician. Urinalysis offers more accurate analysis of aforementioned parameters and an estimation of bacterial content of the urine [4]. Dipstick analysis is effective in ruling out infection but positive results need confirmation by evaluation of pre-test probability or by culture [5]. Bacterial culture is the only method that offers definite knowledge of antibiotic susceptibility. The disadvantage of culture is that it typically takes a minimum of 24 hours to complete. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) is the current state-of-the-art method that offers inexpensive identification of micro-organisms. It is able to identify the bacterial species in about 90% of the cases [6]. Its disadvantage is that high fixed costs make it only viable in high volume laboratories where its high throughput can be fully utilized.

An electronic nose (eNose) is a device that mimics the working principle of mammalian olfaction. It consists of an array of nonselective sensors, preprocessing electronics and a computer that interprets sensor signals by pattern detection [7]. It is designed for qualitative, not quantitative analysis. This enables eNose to analyze complex mixtures that

would be unanalyzable with more sensitive methods such as gas chromatography mass spectrometry without extensive preparation [8]. These characteristics, handheld size, and relative affordability make eNose a tempting tool for detection of UTI.

The ability of eNose to discriminate cultured bacteria has been demonstrated in [9–15]. Preliminary studies of detection of UTI from cultured urine samples have also been published [16–18]. Studies have largely employed a single device that is bulky and not suitable for point-of-care use. Only a single study has employed a clearly handheld device in classification of eye infection pathogens [19].

To date, no studies employing a hand-held IMS-based eNose device have been published. We have recently demonstrated the ability of such device to discriminate malignant prostate cells from benign ones [20], as well as shown that this prostatic cancer can be differentiated from benign hyperplasia in clinical setting with urine gaseous headspace as sample material [21]. UTI is a significant confounding factor for diagnostic methods employing urine as sample material. UTI can also be comorbid or diagnostic option for urological malignancies or benign prostatic hyperplasia. In this study we demonstrate the ability of IMS-based eNose to discriminate cultured urinary pathogens with high sensitivity and specificity.

Materials and methods

Bacterial cultures

Four most common UTI pathogens, *Escherichia col*i, *Staphylococcus saprophyticus*, *Klebsiella species* and *Enterococcus faecalis*, were included in the study. Pure culture samples were made to 92 mm x 16 mm polystyrene Petri dishes (No. 82.1472, Sarstedt AG & Co., Nümbrecht, Germany) containing cysteine lactose electrolyte deficient (CLED) medium (LAB041, Lab M Limited, Lancashire, UK). Urine samples for pure cultures were acquired anonymously from patients in Tampere University Hospital collected and processed by Fimlab laboratories, the provider of laboratory services for Pirkanmaa Hospital District.

The sample contained a total of 101 samples including all four pathogens and reference samples containing only the CLED agar. Detailed description of samples presented in table 1. All samples containing pathogens were marked with an individual identification number and were stored in the refrigerator between the measurements (2 - 8 ºC).

Device and measurement

A commercially available eNose (ChemPro® 100i, Environics Inc., Mikkeli, Finland) was employed in this study. It is based on ion mobility spectrometry principle. The device consists of an ion mobility cell (IMCell) which features eight electrode strips that produce 16-channeled data. It contains a radiation source of Am-234 5.92 MHq and a heater element that maintains the cell at a constant temperature. In addition to IMCell, the device has six metal oxide-semiconductor sensors. Ambient air is utilized as carrier gas. The device is described in more detail by Utriainen et al. [22] Flow was set at 1.30l/min and sensor temperature was on average 31.5 °C. In order to reduce background noise, the air entering measurement chamber was filtered through activated carbon and was then fed to the sensor.

Every sample was soaked into water bath (36ºC) and measured for approximately 15 minutes. Between the samples ambient air was measured for approximately 3 minutes and the empty Petri dish for approximately 4 minutes to avoid carryover from the previous sample. These time intervals were found sufficient in pre-study experiments. The switching

time was registered to the spreadsheet program and compared afterwards to the log file made by the Windows-based software provided with the eNose device. The log file was then submitted for statistical analysis.

Statistical analysis

Sample analysis was designed to mirror the clinical decision making. In the first phase, we created a classified that discriminates the samples either to sterile, or infected group. For the infected group we performed additional classification that aimed to identify the bacterial species in question.

Linear discriminant analysis (LDA) and logistic regression (LR) were employed to identify the classifier for discrimination of sterile and bacterial samples. Only LDA was employed in identification of bacterial species since LR is only suitable for discrimination into two classes. The generalization ability of the classifiers was tested by leave-one-out cross validation (LOOCV) [23].

Results

In discrimination of sterile and infected samples, eNose achieved sensitivity of 95% and specificity of 97% using LR. Sensitivity of 90% and specificity of 96% were achieved with LDA. These results were validated with LOOCV. Classification is presented in table 2.

In classification of four different bacteria and sterile culture plate, LOOCV-validated sensitivity of 95% and specificity of 96% were achieved using LDA. 2-dimensional Sammon projection is presented in figure 1 to demonstrate clustering of bacterial samples. Sterile and *Klebsiella spp* samples were all classified correctly. Three *E. coli* samples were classified as sterile. Discrimination of *S. saprophyticus* and *E. faecalis* was more challenging with six *S.* saprophyticus samples classified as *E. faecalis* and five *E. faecalis* as *S. saprophyticus* samples. One *S. sapropyticus* and *E. faecalis* were misclassified as *Klebisiella spp*. This is in contrast with high discrimination of sterile, *E. coli* and *Klebsiella spp*. Classifaction results are presented in table 3.

Discussion

In this study we demonstrated the ability of affordable IMS-based handheld eNose to rapidly discriminate bacterial cultures with high accuracy at atmospheric pressure without sample preparation.

Previous works have focused on bacteria and yeasts in food industry [24], common bacterial and fungal human pathogens [10,11,13,14], blood culture simulations [12]. The fact that every published study employed a different set of bacteria, makes direct comparison difficult. No studies focusing on sole discrimination of the most common urinary pathogen cultures have been published. Some studies have employed protocols of rapid incubation of urine, followed by analysis by eNose [17,18,25].

In all studies that focused solely on discrimination of bacterial cultures, complex sampling methods were used [9–11]; Bacterial cultures were sealed in a plastic bag where equilibrium of VOCs was allowed to form. The content of this bag was then fed to the sensor. In contrast, we employed a considerably simpler strategy where culture plate was simply connected to a plastic cover and measured immediately thus entirely omitting sample preparation. In the present study we used a relatively long measurement period of 15 minutes, but the fact that high discrimination can be achieved at 5 minutes already, suggests that the duration of measurement period can be significantly reduced. Based on our pilot experiments a minimum of four minutes of flushing with ambient air is required to avoid carryover from previous sample. In future we aim to improve the measurement cycle so that the duration can be reduced. This would directly translate to a higher throughput.

In point-of-care applications the mobility, rapidity and minimal cost of the device and analyses play a great role. A handheld device can be carried to patient's home for online analysis or used in medical wards without need to carry samples to centralized laboratory. A rugged, handheld device with minimal maintenance need would be suitable for use in developing countries. Commonly used conducting polymer sensor device Bloodhound BH114 is not suitable for mobile applications in its current form. Dutta et al used a handheld, battery powered Cyranose 320 device to discriminate pathogens responsible for eye infections [19]. The device relies solely on 32 conducting polymer sensors which are similar to six MOS-sensors in our device. They also achieved high overall discrimination of 96% with use of a combination of three simultaneous data clustering algorithms. By comparison, they only achieved a discrimination of 75% by PCA, while we achieved high

sensitivity and specificity by using only LR. This shows that IMS is well capable of discriminating cultured bacteria responsible for UTI and may be even be better suited for the task than more traditional conducting polymer based eNose. Our device also has the advantage of long runtime and military-grade ruggedness which enable its use in difficult conditions.

Vernat-Rossi et al focused on discrimination of bacterium that are responsible for spoilage of dry meat products, with S. saprophytius as the only overlapping bacteria in our study [9]. They employed measurement chamber design similar to ours. By using LDA and LOOCV they achieved discrimination of 86% which is inferior compared to our present results. This may be due to different bacterial species but more likely explanation is relatively primitive eNose consisting of only six semiconductor sensors. Gibson achieved comparable discrimination but used a complex neural network approach [10]. Our sampling technique is powerful enough to produce good discrimination with relatively simple LDA and LR approaches in relatively small sample. It should be noted that current sample consists of highly standardized cultured samples with virtually no confounding factors. Schiffman et al. focused more on fungal growth and employed only a single culture of 10 different bacteria that were measured four times. Although they reached clear clustering of samples in LDA, LOOCV reduced performance significantly, indicating that discrimination model overfitted the small sample.

High classification achieved with *E. coli*, *Klebisella* spp and sterile culture plates was shadowed by poorer discrimination of *S. saprophyticus* and *E. faecalis*. This suggests that the two bacteria have similar smell prints. The two species were separated by other samples that could be classified with near-perfect performance, speaking against systematic confounder in some part of the measurements. No studies investigating volatile compounds released by *S. saprophyticus* exist, although *S. aureus* and *E. faecalis* have been extensively studied. They have fewer common volatile compounds than *Klebisella* spp and *E. coli* which were completely discriminated from each other [26]. *S. saprophyticus* seems to form two clusters, one overlapping with *E. faecalis* and another clearly separated from other bacteria. *S. saprophyticus* has two known subspecies: *saprophyticus* and *bowis*. An important distinction is that unlike *saprophyticus* subsp, *bowis* features nitrate reductase [27]. The enzyme catalyzes the production of nitrogen dioxide, a volatile compound that could potentially alter the smell print. *E. coli*, *Klebisella spp* and *E. faecalis* all have nitrate reductase, meaning that *S. saprophyticus* sp.

saprophyticus is the only nitrate reductase –negative bacterium in this study, potentially explaining distinct clustering.

Limitations of this study are that bacteria were randomized within species but every species was carried out on separate batch. Complete randomization would rule out potential systemic factor that causes confusion of *E. faecalis* and *S. saprophyticus*. Another limitation is that standard culture in unable to discriminate subspecies of *S. saprophyticus*, which would clarify clustering of the bacteria.

With these promising results, the next step is to attempt discrimination of bacteria in urine samples. Aathithan et al decribed an eNose device that could discriminate incubated urine samples as infected or uninfected after overnight incubation with sensitivity of 72.3% and specificity of 89.4% by use of PCA. No cross-validation was reported implicating that discrimination algorithm may suffer from overfitting the study sample and the performance may be significantly poorer in new sample set. The device used there was designed for laboratory setting and received FDA approval. To authors' knowledge, the device is not currently available for commercial use. Kodogiannis et al used previously described Bloodhound device and by use of neural networks, managed to discriminate bacterial pathogens with a classification rate of 100%. Notably, this discrimination was achieved only after 5 hours of incubation. We aim to achieve similar discrimination with handheld device. The greatest challenge is that unlike cultured samples, urine samples are associated with high biological variance caused by kidney function, medications, age, sex and comorbid conditions.

Conclusions

A handheld, mobile eNose is able to rapidly, without sample preparation and at low cost to discriminate common urinary bacterial pathogens from culture plates. High discrimination is achieved both between sterile and bacterial plates as well as between different bacterial species. Our results warrant future trial attempting discrimination of urine samples.

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References

1. Grabe M, M.C. B, Bjerklund-Johansen TE, Botto H, Cek M, et al. (2008) Guidelines on The Management of Urinary and Male Genital Tract Infections. Uroweb. Available: http://www.uroweb.org/fileadmin/user_upload/Guidelines/The Management of Male Urinary and Genital Tract Infections.pdf. Accessed 4 February 2014.

2. Foxman B (2002) Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. Am J Med 113 Suppl : 5S–13S.

3. Ronald A (2003) The etiology of urinary tract infection: traditional and emerging pathogens. Dis Mon 49: 71–82.

4. Mach KE, Wong PK, Liao JC (2011) Biosensor diagnosis of urinary tract infections: a path to better treatment? Trends Pharmacol Sci 32: 330–336.

5. Devillé W, Yzermans J (2004) The urine dipstick test useful to rule out infections. A meta-analysis of the accuracy. BMC Urol 4: 4.

6. Murray PR (2012) What Is New in Clinical Microbiology—Microbial Identification by MALDI-TOF Mass Spectrometry. J Mol Diagnostics 14: 419–423.

7. Turner AP, Magan N (2004) Electronic noses and disease diagnostics. Nat Rev Microbiol 2: 161–166.

8. Wilson AD, Baietto M (2011) Advances in electronic-nose technologies developed for biomedical applications. Sensors (Basel) 11: 1105–1176.

9. Vernat-Rossi V, Garcia C, Talon R (1996) Rapid discrimination of meat products and bacterial strains using semiconductor gas sensors. Sensors Actuators B: Chem 37: 43–48.

10. Gibson TD, Prosser O, Hulbert JN, Marshall RW, Corcoran P, et al. (1997) Detection and simultaneous identification of microorganisms from headspace samples using an electronic nose. Sensors Actuators B Chem 44: 413–422.

11. Schiffman S, Wyrick DW, Gutierrez-Osuna R, Nagle HT (2000) Effectiveness of an electronic nose for monitoring bacterial and fungal growth. Electron noses olfaction 2000 Proc Seventh Int Symp Olfaction Electron Noses: 1–8.

12. Lykos P, Patel P, Morong C, Joseph A (2001) Rapid detection of bacteria from blood culture by an electronic nose. J Microbiol 39: 213–218.

13. Pavlou A, Turner APF, Magan N (2002) Recognition of anaerobic bacterial isolates in vitro using electronic nose technology. Lett Appl Microbiol 35: 366–369.

14. Kodogiannis V (2005) Intelligent classification of bacterial clinical isolates in vitro, using an array of gas sensors. J Intell Fuzzy Syst 16: 1–14.

15. Bruins M, Bos a, Petit PLC, Eadie K, Rog a, et al. (2009) Device-independent, real-time identification of bacterial pathogens with a metal oxide-based olfactory sensor. Eur J Clin Microbiol Infect Dis 28: 775–780.

16. Aathithan S, Plant JC, Chaudry AN, French GL (2001) Diagnosis of Bacteriuria by Detection of Volatile Organic Compounds in Urine Using an Automated Headspace Analyzer with Multiple Conducting Polymer Sensors. J Clin Microbiol 39: 2590–2593.

17. Pavlou AK, Magan N, McNulty C, Jones J, Sharp D, et al. (2002) Use of an electronic nose system for diagnoses of urinary tract infections. Biosens Bioelectron 17: 893–899.

18. Kodogiannis V, Wadge E (2005) The use of gas-sensor arrays to diagnose urinary tract infections. Int J Neural Syst 15: 363–376.

19. Dutta R, Hines EL, Gardner JW, Boilot P (2002) Bacteria classification using Cyranose 320 electronic nose. Biomed Eng Online 1: 4.

20. Roine A, Tolvanen M, Sipiläinen M, Kumpulainen P, Helenius MA, et al. (2012) Detection of smell print differences between nonmalignant and malignant prostate cells with an electronic nose. Future Oncol 8: 1157–1165.

21. Roine A, Veskimäe E, Tuokko A, Kumpulainen P, Koskimäki J, et al. (2014) Detection of prostate cancer by an electronic nose: a proof-of-principle study. J Urol. doi:10.1016/j.juro.2014.01.113.

22. Utriainen M, Kärpänoja E, Paakkanen H (2003) Combining miniaturized ion mobility spectrometer and metal oxide gas sensor for the fast detection of toxic chemical vapors. Sensors Actuators B Chem 93: 17–24.

23. Bishop CM (2006) Pattern recognition and machine learning. New York: Springer.

24. Vernat-Rossi V, Garcia C, Talon R (1996) Rapid discrimination of meat products and bacterial strains using semiconductor gas sensors. Sensors Actuators B: Chem 37: 43–48.

25. Aathithan S, Plant JC, Chaudry AN, French GL (2001) Diagnosis of Bacteriuria by Detection of Volatile Organic Compounds in Urine Using an Automated Headspace Analyzer with Multiple Conducting Polymer Sensors. J Clin Microbiol 39: 2590–2593.

26. Bos LDJ, Sterk PJ, Schultz MJ (2013) Volatile metabolites of pathogens: a systematic review. PLoS Pathog 9: e1003311.

27. Hájek V, Meugnier H, Bes M, Brun Y, Fiedler F, et al. (1996) Staphylococcus saprophyticus subsp. bovis subsp. nov., isolated from bovine nostrils. Int J Syst Bacteriol 46: 792–796.

Figure 1: Sammon map –projection of the dataset used in this study. Different species cluster in their own areas with some overlapping in 2-dimensional projections.

Table 1: Description of bacterial culture samples. The samples were analyzed in presented order.

Pathogen	**Number of samples**
Escherichia coli	20
Staphylococcus saprophyticus	19
Klebsiella species	20
Enterococcus Faecalis	21
CLED agar	21

Abbreviations: cysteine lactose electrolyte deficient

Table 2: Classification results of bacteria vs sterile samples with LDA and LR. Left columns shows the true class of the sample. Top rows identify used prediction model and how it classifies samples. Both methods achieve near perfect discrimination, LR demonstrating marginally better performance.

		Predicted LDA		Predicted LR	
		Sterile	Bacteria	Sterile	Bacteria
True	Sterile	19	2	20	1
	Bacteria	3	77	2	78

Table 3: Identification of bacterial species and sterile samples. Left-hand colums identify true classification of the samples. Top row shows the discrimination by LDA. S. saprophyticus is most commonly misclassified and is often confused with E. faecalis. Overall discrimination is very high.

		Predicted LDA				
		Sterile	S.Saprophyticus	E.Coli	Klebsiella spp	E. faecalis
True	Sterile	20	0	1	0	0
	S.Saprophyticus	0	12	0	1	6
	E.Coli	3	0	17	0	0
	Klebsiella spp	0	0	0	20	0
	E. faecalis	0	5	0	1	15

Figure 1
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