

THEMATIC REVIEW

Perspectives for metabolomics in testosterone replacement therapy

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Abstract

Testosterone is the major circulating androgen in men but exhibits an age-related decline in the ageing male. Late-onset hypogonadism or androgen deficiency syndrome (ADS) is a 'syndromic' disorder including both a persistent low testosterone serum concentration and major clinical symptoms, including erectile dysfunction, low libido, decreased muscle mass and strength, increased body fat, decreased vitality or depressed mood. Given its unspecific symptoms, treatment goals and monitoring parameters, this review will outline the various uncertainties concerning the diagnosis, therapy and monitoring of ADS to date. Literature was identified primarily through searches for specific investigators

in the PubMed database. No date or language limits were applied in the literature search for the present review. The current state of research, showing that metabolomics is starting to have an impact not only on disease diagnosis and prognosis but also on drug treatment efficacy and safety monitoring, will be presented, and the application of metabolomics to improve the clinical management of ADS will be discussed. Finally, the scientific opportunities presented by metabolomics and other -omics as novel and promising tools for biomarker discovery and individualised testosterone replacement therapy in men will be explored.

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Testosterone serum concentrations in men: physiology and epidemiology

Testosterone is the major circulating androgen in men and is essential for the development and maintenance of specific reproductive tissues, such as the testis, and other characteristic male properties, including increased muscle strength, bone mass and hair growth (Mooradian *et al.* 1987). In serum, most of the circulating testosterone (50–60%) is bound to sex hormone-binding globulin (SHBG), while a smaller fraction (40–50%) is loosely bound to albumin, leaving only 1–3% to circulate unbound as 'free' testosterone (Kaufman & Vermeulen 2005). To maintain testosterone at appropriate concentrations, a dynamic network of different interacting factors involved in the excretion and metabolic clearance of testosterone must be in balance. Briefly, the testosterone action in target cells depends on the amount of steroid that can penetrate into the cells, the extent of metabolic conversions within the cells, its interactions with the receptor proteins and finally the action of the androgen receptors at the genomic level (Rommerts 2004).

Based on the findings from various prospective epidemiological studies (Morley *et al.* 1997, Zmuda *et al.* 1997, Harman *et al.* 2001, Feldman *et al.* 2002, Haring *et al.* 2010), it is now well established that total testosterone (TT) concentration shows an age-related decline, with mean serum TT concentration at the age of 75 years being approximately two-thirds of that at age 25 years (Vermeulen *et al.* 1996). Over and above this established age-related decline at the population level, a considerably larger inter-individual variability of TT concentrations can be observed at any age. Dependent on the genetic background, accompanying comorbidities, medications or adverse lifestyle behaviours, individual TT concentrations could be either well preserved against this physiological decline or decrease even more progressively (Haring *et al.* 2010). Although the physiological basis and the extent of the suggested cofactors underlying the large inter-individual variability in TT concentrations are not yet fully elucidated, disturbances in the biosynthesis and actions of testosterone caused by acute illness or chronic diseases are well known (Kaufman & Vermeulen 2005).

Conversely, prospective epidemiological cohort studies have accumulated evidence suggesting that low TT concentration is an independent predictor of various cardiovascular risk factors, including obesity (Svartberg *et al.* 2004), subclinical inflammation (Haring *et al.* 2011a), dyslipidaemia

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(Haring *et al.* 2011b), hypertension (Torkler *et al.* 2011), metabolic syndrome (Laaksonen *et al.* 2004, Kupelian *et al.* 2006, Haring *et al.* 2009) and type 2 diabetes (Vikan *et al.* 2010, Schipf *et al.* 2011). Prospective population studies have also repeatedly observed that low TT is independently associated with an increased mortality risk (Araujo *et al.* 2011). However, although numerous prospective studies have shown that low TT precedes the onset of various cardiovascular risk factors, others have found reduced TT in men with type 2 diabetes (Barrett–Connor *et al.* 1990), metabolic syndrome (Laaksonen *et al.* 2003), obesity (Kaplan *et al.* 2006) and comorbidity (Wu *et al.* 2008).

Given the bidirectional nature of the observed low TT–cardiovascular risk factor associations, it is still unclear whether low TT is a risk factor (causal) or a risk marker (secondary) of mechanisms that ultimately lead to a higher cardiovascular risk factor burden; therefore, reverse causality remains a possibility (Box 1). In addition, neither case–control studies nor prospective cohort studies have observed an independent association between low TT and incident fatal or nonfatal cardiovascular disease (CVD) events (Ruige *et al.* 2010), providing further evidence for the role of low TT as a risk marker. In conclusion, recent epidemiological data suggest that serum TT concentration might be considered as a biomarker of good health and overall well-being in men (McLachlan 2010). Circulating TT shows a physiological decline in conjunction with ageing, cardiovascular comorbidity, obesity, medications and depression. Therefore, testosterone assessment may play a role as a personalised risk marker rather than an independent causal risk factor (Maggio & Basaria 2009).

Androgen deficiency syndrome in men: current practice

The primary clinical use of testosterone replacement therapy (TRT) is to diagnose primary or secondary hypogonadism caused by ‘classical’ disorders, such as Klinefelter syndrome, Kallmann syndrome or pituitary insufficiency (Nieschlag & Behre 2004). There is no doubt that these patients should receive TRT (Isidori & Lenzi 2007). However, in the

majority of patients, the diagnosis of low TT parallels with advanced age, accompanying acute or chronic diseases, medication use or adverse health-related lifestyle (Travison *et al.* 2007, Snyder 2008). Thus, testosterone deficiency is a frequent diagnosis in ageing men, with a prevalence of 10–20%, depending on the applied cut-off and studied population (Araujo *et al.* 2004, Haring *et al.* 2010). Androgen deficiency syndrome (ADS) is a ‘syndromic’ disorder including both clinical symptoms and persistently low TT. Thus, TT assessment is a crucial diagnostic criterion requiring proper evaluation and interpretation.

Laboratory diagnosis of testosterone deficiency

Current guidelines unequivocally highlight the measurement of morning serum TT by a reliable assay as the initial diagnostic test to assess the male androgen status (Wang *et al.* 2009a, Bhasin *et al.* 2010, McLachlan 2010). The presently used, extensively automated procedures for the analyses of TT concentration in routine diagnostics are commercial platform-based immunoassays. However, given a lack of specificity (Wang *et al.* 2004) and substantial inter-assay as well as inter-laboratory differences in measured absolute TT concentrations (Dorgan *et al.* 2002, Wang *et al.* 2004, Hsing *et al.* 2007), immunological procedures are considered insufficient for the low concentration range of TT in elderly comorbid men (Taieb *et al.* 2003). Hence, the more precise mass spectroscopic procedures, demonstrating considerably lower intra- and inter-laboratory variability (Vesper *et al.* 2009), are increasingly perceived as the gold standard for the measurement of TT (Wang *et al.* 2004, 2009a, Thienpont *et al.* 2008, Bhasin *et al.* 2010, Stanczyk & Clarke 2010). Beyond these analytical factors, several pre-analytical and physiological cofactors also need to be considered for the proper interpretation of TT measurements (Wheeler & Barnes 2008, Haring *et al.* 2011d). Taken together, TT measurements must be evaluated cautiously not only because of the insufficient diagnostic and analytic quality of immunological measurements in the low concentration range but also for the reasons of pre-analytical and physiological influencing factors.

Box 1 Outstanding questions

- Does a male andropause exist, or are testosterone concentrations well preserved in metabolically healthy ageing men over time?
- Which symptoms are specific to low testosterone concentrations?
- Which treatment goal meets an individual’s metabolic needs, or what is a ‘normal’ testosterone concentration at different ages?
- How can TRT take into consideration the large inter-individual variability in testosterone concentrations?
- Is there an optimal dose or duration of TRT to induce clinically relevant benefits with regard to cardiovascular risk, frailty or quality of life?
- Is it possible to predict which individuals will most likely benefit from TRT?
- Which parameters should be monitored to optimise the effectiveness and safety of TRT?

Clinical diagnosis of ADS

Even more uncertainty exists with regard to the clinical symptoms and signs presumably related to low serum TT. Conditions such as erectile dysfunction, low libido, decreased muscle mass and strength, increased body fat, decreased bone density, decreased vitality and depressed mood are suggested to be related to testosterone (Morales *et al.* 2010), but none of these symptoms are specific to low TT concentrations in men (Wang *et al.* 2009a). Therefore, one or more of these symptoms must be corroborated with repeatedly measured low morning TT to constitute the diagnosis of ADS and consider the initiation of TRT (Fig. 1). Similarly, an observational study of 3369 men aged 40–79 years showed that among the various symptoms, only sexual symptoms had a syndromic association with low TT (Wu *et al.* 2010). In contrast, a meta-analysis of 17 randomised placebo-controlled trials showed that TRT only moderately improved the number of sexual symptoms and had no effect on erectile function (Isidori *et al.* 2005). In conclusion, the clinical conditions related to low TT are of a non-specific and only suggestive nature, not diagnostic of ADS (Box 1). This absence of definite clinical correlates of ADS contributes to the uncertainties surrounding TRT in men (Sadovsky *et al.* 2007) and is reflected by the difficulties guideline expert panellists face in issuing firm recommendations and criteria for the initiation of TRT (Wang *et al.* 2009a, Bhasin *et al.* 2010).

Current therapy for ADS

Once a treatment decision has been made, improvements in signs and symptoms of ADS together with serum TT concentrations in the middle to lower range of young adult males should be sought (Wang *et al.* 2009a, Bhasin *et al.* 2010). To achieve these therapeutic goals, injectable, oral and transdermal preparations of natural testosterone are currently available. Due to inadequate data, much discussion exists regarding the critical threshold to determine a definite cut-off for the optimal serum TT concentration in terms of efficacy and safety, as well as a risk–benefit equation for intervention. A review and meta-analysis of 51 treatment studies concluded that the safety of TRT and its adverse cardiovascular effects are still unknown (Fernandez-Balsells *et al.* 2010). The observed increases in haemoglobin or haematocrit and small decreases in HDL cholesterol were of unknown clinical significance. The results from a randomised, double-blinded, placebo-controlled trial in 274 frail elderly men aged 65–90 years showed that the effects of 6-month TRT on muscle strength, lean mass and quality of life were not maintained 6 months post-treatment (O’Connell *et al.* 2011). Furthermore, the time course of the effects induced by TRT from their first manifestation to the maximum effects shows considerable variation (Saad *et al.* 2011), raising the question of optimal treatment duration. The potential risks of over-supplementation during TRT were exemplified by a discontinued trial among elderly men (mean age 74 years)

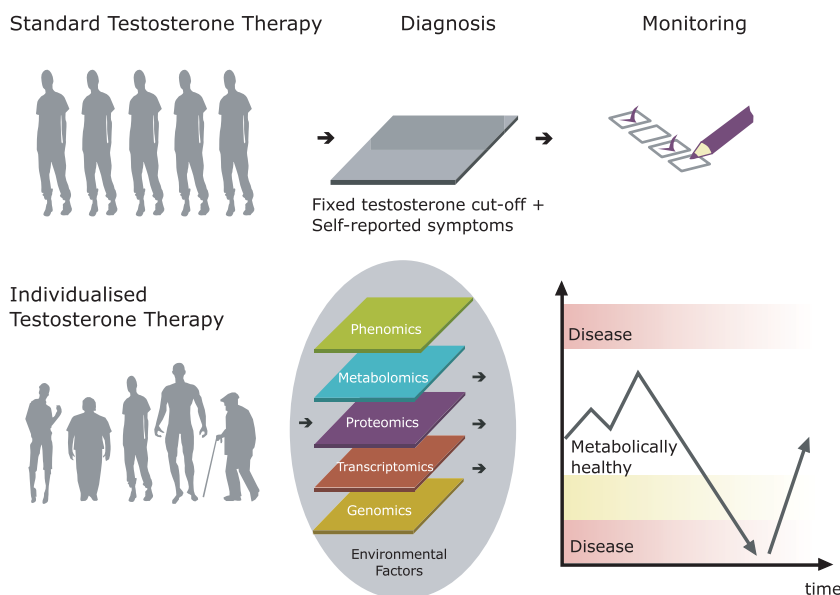


Figure 1 Standard vs individualised testosterone therapy in men. Instead of relying solely on a single biomarker (fixed testosterone cut-off) and self-reported symptoms for the diagnosis of ADS and monitoring of testosterone therapy, novel diagnostic techniques (multi-omics) promise to advance our understanding of the pathophysiology of ADS (leading to a metabolically healthy state) and thereby enable individualised treatment concepts for the improved diagnosis, therapy and monitoring of ADS in men.

with mobility limitations after testosterone replacement was associated with an increased risk of adverse cardiovascular events in the intervention group (Basaria *et al.* 2010). The starting doses applied in this trial were higher than those recommended by the manufacturer, and the treatment goal in these patients (34.7 nmol/l) was considerably higher than that recommended by the Endocrine Society (13.9–17.4 nmol/l; Bhasin *et al.* 2010).

Taken together, the current evidence about the safety of TRT in men with regard to important patient outcomes is of low quality and limited by short follow-up periods (Fernandez-Balsells *et al.* 2010). Thus, TRT should only be initiated in the presence of TT concentrations clearly below the lower normal limit for younger men, together with unequivocal signs and symptoms of TT deficiency, in the absence of other reversible causes of decreased TT and after screening for contraindications (Nieschlag & Behre 2004). Once initiated, TRT should induce and maintain secondary sex characteristics and improve sexual function, sense of well-being, muscle mass and strength and bone mineral density (Bhasin *et al.* 2010). Accordingly, the response to TRT should be assessed and monitored by patients' well-being, sexual activity and occasional measurement of serum TT, haemoglobin and haematocrit, bone density and prostate parameters (Nieschlag & Behre 2004, Wang *et al.* 2009a).

As stated earlier, the presumed syndromic nature of testosterone deficiency is often difficult to disentangle because symptoms and signs suggestive of ADS are readily accounted for by comorbidities and because borderline TT deficiency is a frequent biochemical accompaniment of systemic disease, which are the reasons why 'pure' ADS is quite uncommon. Furthermore, we do not yet have an operational definition for 'normal' testosterone concentrations at different ages, nor have we identified specific signs and symptoms to accurately discriminate between those who need treatment and those who do not (Isidori & Lenzi 2007). Thus, the conjunction between low TT and several non-specific symptoms, constituting the diagnosis of ADS or late-onset hypogonadism, remains a controversial concept with several outstanding questions (Box 1).

Metabolomics for the improved diagnosis, therapy and monitoring of ADS

The principle techniques of metabolomics

Against the background of the various uncertainties surrounding the diagnosis, therapy and monitoring of ADS described earlier, the application of metabolomics offers a variety of scientific opportunities to improve the clinical management of testosterone deficiency in men. Metabolomics or metabonomics addresses the comprehensive measurement of the total low-molecular weight or metabolite content of a cell, tissue or body fluid. Because small molecules are the end result of all regulatory and metabolic processes at

the cellular level within tissues in all organisms, metabolomics gives a view into whole-system biochemistry (Nicholson & Wilson 2003). With the global analysis of all or nearly all these cellular metabolites, metabolomics offers molecular information that is closest to the phenotype in that metabolites are an ultimate product of gene, mRNA and protein activity (Nordstrom & Lewensohn 2010).

There are two major complementary approaches in metabolomics: targeted analysis is used to measure the concentrations of a limited number of precisely known metabolites (Shulaev 2006), and the complementary non-targeted approach of metabolic profiling measures a large set of metabolites in a global semi-quantitative manner (Lindon & Nicholson 2008). Although a wide range of techniques can be used for metabolomics, the two principal analytical platforms used to analyse metabolites in body fluids, such as urine and plasma, are nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography coupled to mass spectrometry (LC-MS; Lindon & Nicholson 2008). MS-based metabolomics is currently the most developed technique to quantitatively analyse specific metabolites or a defined set of metabolites with a high sensitivity and throughput. Nevertheless, a recent inter-laboratory study showed that ultra-performance LC-MS of human urine is a novel technique for global metabolic profiling, yielding metabolite measurements that are highly reproducible both within and between different laboratories (Benton *et al.* 2012). Hence, the rapid technical progress increasingly enables an analytical flexibility that makes MS-based metabolomics amenable to targeted as well as untargeted metabolomics approaches.

Addressing the basic idea that specific molecules or metabolites for a certain disease or drug intervention are detectable and quantifiable in body fluids, NMR and MS-based metabolomics have been used for biomarker screening, pathway discovery and monitoring of metabolic changes in response to physiological and therapeutic perturbations or environmental stimuli (Clayton *et al.* 2006, Holmes *et al.* 2008a, O'Sullivan *et al.* 2011). Below, I will present the current state of research of these different metabolomics applications with a special focus on their potential to improve diagnosis, therapy and monitoring of ADS in men.

Metabolomics for biomarker discovery

The main application of metabolomics, by far, lies in the discovery of biomarkers in the clinical and pharmaceutical research setting. A biomarker is a biological characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention (Biomarkers Definitions Working Group 2001). An ideal biomarker is quantifiable, reproducible and analytically simple; it is also inexpensive to measure, its concentration or level does not vary widely, it is specific to the condition

of interest and it is not affected by comorbid factors (Vasan 2006).

One of the first applications of metabolomics for biomarker discovery was the diagnosis of inborn errors of metabolism (Moolenaar *et al.* 2003). The most prominent metabolomics biomarker discovery studies are presently conducted in the field of cancer research. Metabolomic analysis of plasma, tissue and urine from prostate cancer patients has identified and validated sarcosine as a potential candidate for early disease detection and aggressivity prediction in prostate cancer (Sreekumar *et al.* 2009). Further exploratory metabolomics studies have been conducted to identify biomarkers in serum and urine from various patient groups and cancer types, including lung, bladder, kidney, colon, oral, breast, ovarian, leukaemia and cervical cancer (Mamas *et al.* 2011).

CVD patients have been investigated to identify potential metabolic biomarkers for improved disease prediction, risk stratification and individualised diagnosis (Nordstrom & Lewensohn 2010). MS-based metabolomics on 36 patients (18 cases showing inducible ischaemia and 18 controls) revealed significant discordant regulation of multiple metabolites after exercise stress testing in cases but not in controls (Sabatine *et al.* 2005). Another serum metabolomics study revealed branched-chain amino acid metabolites and urea cycle metabolites associated with the presence of coronary artery disease (CAD), whilst dicarboxylic acylcarnitines predicted death/myocardial infarction outcomes (Shah *et al.* 2010). Wang *et al.* (2009b) demonstrated that metabolites of arginine methylation provide independent risk prediction for both obstructive CAD and incident major adverse cardiac events in stable patients undergoing cardiac evaluation. Serum metabolomics has correctly diagnosed not only the presence but also the severity of CAD (Brindle *et al.* 2002). Taken together, NMR- and MS-based metabolomics have shown much potential for the identification of novel biomarkers for a range of pathological processes affecting the heart (Barderas *et al.* 2011) and have been proposed as a novel tool for cardiac research (Griffin *et al.* 2011). Metabolomics-based biomarker discovery has also been applied to clinical conditions such as osteoarthritis (Lamers *et al.* 2005), diabetes mellitus (Bain *et al.* 2009) and neurological diseases (Madsen *et al.* 2010). Furthermore, recent metabolomics studies on human urine and serum samples identified numerous metabolites associated with cardiovascular risk factors (Barderas *et al.* 2011), including blood pressure (Holmes *et al.* 2008a), insulin resistance (Wang *et al.* 2011b) and atrial fibrillation (Mayr *et al.* 2008). Furthermore, metabolomics studies demonstrated the phenotypic heterogeneity of CVD and the limitations of single diagnostic biomarkers (Makinen *et al.* 2008).

Most of the metabolomics studies described earlier have been performed in small patient samples and have not yet been validated in larger external samples. Thus, large-scale validation is required to reassess the discovered biomarkers in prospective studies with well-phenotyped clinical outcomes. For this goal, metabolome-wide association studies (MWAS)

have been proposed as a powerful new approach not only to replicate discovered biomarkers but also to identify novel biomarkers of disease risk (Nicholson *et al.* 2008). Enabled by rapid technological advances, high-throughput metabolic profiling used in large-scale molecular epidemiology has proved its potential to detect associations between metabolic phenotypes and disease risk. Holmes *et al.* (2008a) demonstrated this population-level approach for the identification of discriminatory metabolic biomarkers for high blood pressure. Another MWAS identified urinary metabolites that discriminate between southern and northern Chinese study samples to explain the observed regional differences in CVD risk (Yap *et al.* 2010). Although a number of novel biomarkers have been discovered, external validation through MWAS is currently under investigation, and methodological challenges will be addressed (Dunn *et al.* 2011), none of the discovered biomarkers have currently made the transition to routine use in clinical practice (Mamas *et al.* 2011).

However, the described findings fuel confidence that metabolomics may address some of the outstanding questions related to TRT in men (Box 1). To date, the key area of controversy surrounding the diagnosis of ADS relates to 1) the biochemical determination of low TT and 2) the unspecific symptoms of ADS (Traish *et al.* 2011). The diagnostic information obtained from hypothesis-free metabolic profiling complemented with a targeted approach is likely to yield a biomarker set of multiple metabolites that could provide comprehensive insights into pathophysiological metabolic processes specific to the onset and progression of ADS (Fig. 1) that were previously not assessable with traditional single biomarkers such as TT alone (Wopereis *et al.* 2009). Thus, metabolic profiling may help to overcome the single-biomarker conservatism by analysing several biomarkers or biomarker combinations (Kenny *et al.* 2010).

Furthermore, metabolic signatures have the unique potential to disclose linkages between physiological, behavioural and environmental characteristics and could therefore offer a promising approach to account for the unspecific nature of the symptoms related to ADS and the existing doubts about the postulated syndromic nature of low TT (Gould *et al.* 2000, Morley & Perry 2003, Seidman 2006, Perheentupa & Huhtaniemi 2007). Thus, the discovery of metabolic disorders specific to low TT would considerably advance the diagnostic reliability of ADS. Because there is no specific cut-off value for serum testosterone concentration that clearly distinguishes between men with and without testosterone deficiency, one could envision a small case-control metabolomics study to reveal a potential metabolic biomarker that provides suitable discrimination. Furthermore, the identified biomarker could offer potential links to symptoms specifically related to low TT. Surely, metabolites identified from these early studies will need to be externally validated in larger, prospective clinical cohorts and MWAS for their future use as biomarkers.

Metabolomics for the definition of 'normal'

Before the application of metabolomics to study disease onset and progression or the effects of any drug intervention, it is first necessary to define the metabolic range covered by normal physiological variation. Thus, various metabolomics studies have been conducted to investigate the inherent metabolic variability based on intrinsic (gender, age, comorbidity and genetics) and extrinsic (diet, sleep, stress and temperature) factors (Aardema & MacGregor 2002, Lindon *et al.* 2003). Furthermore, each biological tissue or fluid has its own unique metabolic signature (see Fig. 1; Holmes & Nicholson 2007). Because changes in these factors could potentially mask molecular changes caused by the disease or intervention, defining a physiologically normal metabolic pattern is crucial to the identification of robust and specific biomarkers. In contrast to animal studies that have controlled for confounding factors, such as age, gender, diet or stress levels, human populations show a comparably greater variability. Thus, it is clearly necessary to demonstrate that it would be possible to detect the effects of disease progression or drug therapy above that inherent inter-individual variability.

A recent study investigating gender-specific differences in serum metabolite concentrations and their underlying genetic determination revealed significant differences in metabolite profiles between males and females (Mittelstrass *et al.* 2011). Among more than 3000 participants of a population-based epidemiological study, significant differences were shown for 101 of the 131 investigated metabolites. Based on these results, gender-specific therapies that would assign men and women to different categories and predictive biomarkers depending on gender were postulated (Mittelstrass *et al.* 2011). NMR-based metabolomics of urine and plasma profiles from 150 healthy humans showed similar gender-specific metabolic differences (Kochhar *et al.* 2006). Further urine metabolomics studies showed that inter-individual metabolic differences were related to a significant extent to gender and to a lesser extent to age (Psihogios *et al.* 2008) and uncovered additional population-wide metabolic differences related to gender, diurnal variation and age (Slupsky *et al.* 2007). The issue of the comparative effects of different influencing factors was addressed in a unique study that prospectively collected urine and plasma samples from 154 post-menopausal female twins to analyse NMR spectra and statistically decompose metabolic variability into familial (genetic and common environmental), individual environmental and longitudinally unstable components. The main result was the identification and quantification of a substantive proportion of stable biological variation (sum of genetic and environmental variation) in the plasma (60%) and urine metabolomes (47%) (Nicholson *et al.* 2011).

Because metabolic profiles are subject to a large inter-individual variability based on the above-described confounding factors, the importance of a standardised protocol for the application of metabolomics to clinical

research should not be underestimated. Analysing NMR-based metabolomics data of urine and plasma samples drawn from 12 healthy men on two separate occasions 14 days apart, the results of one study emphasise the possibility of collecting consistent metabolomics data in clinical studies (Lenz *et al.* 2003). In summary, metabolomics has been used in a number of studies to define a 'normal' biochemical profile and has proved capable of significantly differentiating effects related to age, gender, diet, comorbidities and drugs. These results offer valuable insights into the sources of human metabolic variability and will have implications for the effective design of biomarker discovery studies.

As stated earlier, there are still considerable uncertainties with regard to the definition of normal TT concentrations at different ages. Our understanding of individual set points for circulating TT concentrations (below which one, but not another, individual may develop metabolic changes indicative of testosterone deficiency) or the concept of reserve capacity (the possibility that men with TT concentrations below the fixed cut-off still may have adequate concentrations to meet their metabolic needs) is very limited. The two current guidelines (Wang *et al.* 2009a, Bhasin *et al.* 2010) provide little direction and arbitrarily defined fixed TT cut-offs respectively. Thus, there is currently no consensus about the definition of normal TT concentrations at different ages, further diluting any efforts to transparently define treatment goals for men under TRT.

Here, metabolomics offers the potential to measure testosterone metabolites and related molecules in urine and serum to provide a broader metabolic picture for the evaluation of the single, absolute serum TT measurement. In particular, the urinary steroid profile, which is mainly based on testosterone, reflects the metabolic pathways of androgenic compounds and is essential for the diagnosis of diseases related to steroid secretion (Mareck *et al.* 2008). Recently, MS-based metabolomics was applied to directly analyse and quantify major urinary metabolites as markers of exogenous steroid administration in routine doping controls (Badoud *et al.* 2011). This method was subsequently validated and applied to a clinical TRT trial comparing a group of healthy male volunteers with a placebo group (Badoud *et al.* 2011). Similar studies replicated this method, establishing a fast and sensitive analytical procedure for the simultaneous separation, determination and quantification of testosterone derivatives in human urine (Bean & Henion 1997, Strahm *et al.* 2008).

By incorporating detailed information on an individual's metabolic status, metabolomics offers the potential to obtain a multi-metabolite characterisation of the metabolic state of the patient for the diagnosis of ADS and monitoring of TRT in men (Fig. 1). Hence, assessment of the individual baseline metabolic profile allows changes in response to TRT to be monitored and treatment goals to be defined in accordance with an individual's metabolic needs. Furthermore, deviations from that 'normal' metabolic profile may elucidate the mechanisms by which TRT alters the metabolic profile of

an individual so that we can better understand and maintain a healthy metabolic profile (Fig. 1). Based on the metabolic profile of a pre-dose baseline urine sample, Clayton *et al.* (2009) demonstrated the feasibility of predicting acetaminophen metabolism and excretion as well as acetaminophen-induced hepatotoxicity in humans. These proof-of-principle studies show that defining a metabolic starting point or baseline is key to predicting the outcome of any drug or lifestyle intervention.

Metabolomics in the investigation of physiological variation and rhythms

Based on a large body of evidence from experimental research, it is now clear that metabolomics has the requisite reproducibility and sensitivity to characterise inter-individual variability in animals as well as humans and to distinguish between various normal physiological states and their changes due to environmental or drug-related influences (Bollard *et al.* 2005). The acquired metabolic spectra of a body fluid, such as urine, serum or saliva, reflects the metabolic status of the organism, which changes in response to stressors to maintain a homeostatic balance. The potential value of metabolomics in the investigation of physiological variation and rhythms important in TRT became apparent with the description of a hormonal cycle based on urine metabolomics (Bollard *et al.* 2001). In that particular study, Bollard *et al.* used NMR spectra of female rat urine to examine the influence of hormonal fluctuations over a 10-day period. Furthermore, hormonal variation was taken into account by analysing metabolite fluctuations and metabolic trajectories. Before that, an insightful approach was used to elucidate time-related effects of toxins on endogenous rat urine profiles (Holmes *et al.* 1992, Beckwith-Hall *et al.* 1998). Metabolomics was also demonstrated to be a non-invasive, sensitive and relatively fast method for assessing the individual changes in metabolic profiles of urine from rats following chronic exposure to pesticides (Wang *et al.* 2011a). Metabolomic phenotyping also revealed novel urinary metabolic markers in response to surgical trauma in Wistar rats (Kinross *et al.* 2011). However, to date, metabolomics has mainly been applied to experimental animal studies conducted in laboratory models of disease or toxicity under controlled genetic and environmental conditions (Gavaghan *et al.* 2000, 2001, 2002, Bollard *et al.* 2001). To investigate the translatability of the experimental data to humans, an NMR-based system-wide characterisation of the urine, serum, liver and kidney metabolomes of the pig showed that the metabolites observed in each of these biological compartments were qualitatively comparable to the metabolic signature of the same biological matrices in humans and rodents (Merrifield *et al.* 2011).

Hence, it is now appropriate to extend those methods to investigate human metabolism and its responses to controlled interventions (Holmes & Nicholson 2007). However, understanding the individual metabolic baseline profile and its inter-individual variability is only the first step, although it

already holds numerous scientific opportunities for the improved diagnosis, treatment and monitoring of ADS in men (Box 1). The aforementioned studies suggest that metabolomics can be an efficient tool for the individualised diagnosis of ADS in men. Metabolomics could help to explain the observed large inter-individual variability in TT concentrations by not only defining an individual metabolic baseline profile but also by uncovering homeostatic mechanisms in response to nutritional or environmental effects. However, ultimately, the potential of metabolomics lies in the ability to assess the response to a medical therapy or drug intervention, including TRT.

Metabolomics for drug response characterisation in time

As described in the previous sections, metabolomics encompasses the comprehensive and systematic profiling of metabolite levels as well as temporal changes in response to stimuli related to lifestyle, environment or drugs. Another major application of metabolomics will be to predict an individual's response to pharmaceutical treatment, perform drug safety studies and obtain drug effect endpoints – a concept termed pharmacometabolomics (Lindon *et al.* 2006). Pharmacometabolomics is envisioned to provide real-time metabolic profiles as dynamic markers reflecting the individual treatment response and to reveal indicators of treatment efficacy. Thus, sequentially collected urine or serum samples could be used to assess changes in the metabolic profile and investigate drug-related effects or disease processes over time (Holmes *et al.* 1998, Nicholson *et al.* 1999). To employ pharmacometabolomics to detect effects specifically related to a drug intervention such as TRT, we must first distinguish alterations occurring in response to a drug from those resulting from other sources of variability, as discussed earlier.

Because perturbations of the metabolic state of an individual generally manifest as particular patterns of metabolites, these metabolic signatures could be used to monitor individuals under TRT. To control for the large inter-individual variability in drug response and for potential confounders, serial sampling can be performed so that each patient serves as his own control. As a proof of principle, studies on patients undergoing controlled interventions, such as the exercise stress test (Sabatine *et al.* 2005) or oral glucose challenge (Shaham *et al.* 2008, Wopereis *et al.* 2009), showed that most metabolites displayed concordant changes in cases and controls, while the metabolites with significant discordant changes in cases remained unchanged in controls. In prospective biomarker studies, metabolomics has identified, categorised and profiled kinetic patterns of early metabolic biomarkers of planned and spontaneous myocardial infarction (Lewis *et al.* 2008b, Baumgartner *et al.* 2010). Based on the plasma samples from 36 patients undergoing a planned myocardial infarction, MS-based metabolomics identified different metabolic profiles in the early phase of myocardial injury (Lewis *et al.* 2008b). In animal studies, urine profiles

were used to predict individual drug metabolism and susceptibility to side effects (Nicholson & Lindon 2008).

A similar approach and another area of intense research is nutrimetabolomics for the investigation of the effects of dietary interventions on metabolic phenotypes (Rezzi *et al.* 2007). Combining untargeted metabolic profiling of urine specimens with cross-validation in large-scale epidemiological data, Heinzmann *et al.* (2010) presented a novel strategy for food biomarker discovery to objectively evaluate the effect of diet on health. In the first step, a standardised diet was administered to eight individuals to reveal putative urinary biomarkers of fruit consumption. Secondly, candidate biomarkers were validated using urinary NMR spectra from 499 UK participants of the International Collaborative Study of Macronutrients, Micronutrients, and Blood Pressure (INTERMAP). This approach identified urinary excretion of proline betaine as a specific and sensitive biomarker of citrus fruit intake (Heinzmann *et al.* 2010). Another short-term diet-controlled study investigated the contribution of metabolic baseline differences in healthy individuals to metabolomic outcomes under various dietary modulations and showed that over and above inter-individual metabolic differences, clear biochemical effects of single-type dietary interventions, animal protein and fruit and wine intake are detectable (Heinzmann *et al.* 2012).

Applying this strategy of untargeted metabolic profiling in a small clinical TRT trial and the subsequent validation of candidate biomarkers using large-scale epidemiological data promises valuable insights for TRT in men. First, investigating relative differences in biological processes rather than absolute treatment targets supersedes fixed TT cut-offs to define treatment goals in men under TRT. Secondly, specific metabolic biomarkers of treatment response may highlight the dynamic metabolic status of an organism in response to drug stimuli (Bollard *et al.* 2005) and could be applied to assess the efficacy of TRT. Finally, metabolomics provides excellent analytical and biological reproducibility with low costs per sample and analyte, allowing frequent monitoring to address time-dependent fluctuations of metabolites that occur in response to TRT. Overall, metabolomics offers a huge potential to accurately assess drug-induced changes and to individually initiate as well as monitor TRT in men.

Metabolomics for measuring drug effectiveness and safety

Pharmacometabolomics is also being embraced to take into account important environmental influences on drug absorption, distribution, metabolism and excretion and to thereby achieve maximal efficacy and avoid adverse drug reactions. The proof of principle of the pharmacometabolomics concept for the prediction of drug toxicity was shown in the rat (Coen *et al.* 2003, Lenz *et al.* 2005) and for drug metabolism in humans (Clayton *et al.* 2006). In a groundbreaking study by Clayton *et al.* (2006), the prediction of the metabolism and toxicity of a dosed substance was performed based solely on the analysis and modelling of

a pre-dose metabolic profile, offering an alternative approach to understand the inter-individual variability in drug response. After administration of paracetamol (acetaminophen) to rats, urinary drug metabolite profiles showed an association between pre-dose urinary composition and the extent of liver damage sustained after paracetamol administration (Clayton *et al.* 2006). This approach was subsequently tested in a clinical study on healthy adults receiving 4 g/day acetaminophen for 7 days, which identified predictive urine metabolite profiles that could distinguish responders from non-responders for liver injury (Winnike *et al.* 2010). Using NMR spectra of pre- and post-dose urine samples after a standard dose of acetaminophen, a human-gut microbiome cometabolite was identified whose high pre-dose urinary level was associated with a low post-dose urinary ratio of acetaminophen sulphate to acetaminophen glucuronide (Clayton *et al.* 2009). This finding adds to the rapidly growing recognition of the multiple metabolic interactions between humans and their gut microbiome (Arumugam *et al.* 2011, Kau *et al.* 2011) and of the potential significance of the latter with regard to disease, drug efficacy and adverse drug reactions (Clayton *et al.* 2009).

Another study used an untargeted MS-based metabolic profiling approach in prostate cancer cell lines to investigate androgen-induced metabolomic alterations in prostate cancer cells. Its findings indicate that androgen exposure results in elevated amino acid metabolism and altered methylation potential in prostate cancer cells. Further, metabolic phenotyping studies confirm higher flux through pathways associated with amino acid metabolism in prostate cancer cells treated with androgens. These findings provide valuable insights into the potential biochemical processes regulated by androgen signalling in prostate cancer (Putluri *et al.* 2011), especially given the hypothesised influence of serum sex hormones on prostate cancer risk (Roddam *et al.* 2008).

For these reasons, pharmacometabolomics is now recognised as an independent and widely used technique to evaluate the inter-individual variability of the beneficial and adverse effects of a drug intervention. Taking into account the genetic and modifying environmental influences that determine the individual metabolic fingerprint, together with drug-induced metabolomics changes, the pharmacometabolomics approach promises various potential applications for the improved treatment and monitoring of ADS in men.

Metabolomics and other omics for individualised TRT in men

The benefits of attaining knowledge about the metabolic state of an individual and the potential of this knowledge to help improve TRT in men have been discussed in the present review. In addition, general advances in molecular profiling (omics) methods, including genomics, transcriptomics, proteomics and metabolomics, coupled with statistical data integration, allows us to analyse different omic levels, their

interactions and finally a global model of the system characteristics. This integrative research strategy, named systems biology (Aderem 2005), is designed to address the complexity of biological systems at all levels of organisation, from molecules, cells and organs to organisms and ecosystems, using multi-omics data sets from high-throughput experimental and computational technologies (Auffray *et al.* 2009). The described advances in 'omics' technology also allow for the incorporation of novel biomarkers at multiple omic levels into integrative personal omics profiles (Chen *et al.* 2012) as well as into large-scale observational studies, which is a concept we previously referred to as systems epidemiology (Haring & Wallaschofski 2012).

This integration of multiple 'omics' data has been initially performed in small-scale animal studies integrating metabolic profiles with quantitative trait locus data in a diabetic rat model (Dumas *et al.* 2007) and in combined metabolic and proteomic data of a mouse model of prostate cancer (Rantalainen *et al.* 2006). With the availability of increasingly powerful high-throughput technologies, computational tools and integrated knowledge bases, multiple 'omics' integration is now being applied to large-scale human clinical and population studies. For example, an integrated analysis of metabolic and genome-wide data revealed new functional relationships between disease-associated variants (Suhre *et al.* 2011). Chen *et al.* (2012) presented an integrated personal omics profile that combined longitudinal genomic, transcriptomic, proteomic, metabolomic and autoantibody data from a single individual over a 14-month period, revealing several dynamic risk markers for various conditions, including type 2 diabetes. Another study analysed the genetic variance of human metabolism and identified 31 genetic variants associated with levels of circulating metabolites (Kettunen *et al.* 2012). Brockmoller *et al.* (2012) integrated the analysis of the lipidome and metabolome with protein and gene expression data from a cohort of breast cancer patients to underline the general relevance of metabolic changes in cancer pathogenesis and tumour progression. Altogether, there is an increasing consensus that systems biology principles will play important roles in tuberculosis (Comas & Gagneux 2011), cancer (Lund & Dumeaux 2008), type 2 diabetes mellitus (Hu 2011) and CVD research (MacLellan *et al.* 2012).

The application of metabolomics together with complementary omics promises to have far-reaching implications for the development of an individualised treatment concept for TRT in men (Fig. 1). For example, a recent genome-wide association study on 14 429 men investigated the genetic determinants of serum TT concentration in men and found that genetic variants in the *SHBG* locus and on the X-chromosome are associated with a substantial variation in TT concentration and increased risk of low TT (Ohlsson *et al.* 2011). Longitudinal epidemiological data from 1859 men aged 20–79 years also suggested genomic effects of the CAG repeat length on overall TT concentration and testosterone-related cardiometabolic effects (Haring *et al.* 2011c). These findings

proved helpful in elucidating the genetic basis of the observed inter-individual variability of TT concentrations in men. A MWAS of TT concentrations in men is currently underway and promises to provide novel insights into the aetiology, biological mechanisms and pathways of testosterone metabolism in men. The broader applications of metabolomics in mammalian systems biology for the study of the health–disease continuum, drug efficacy and drug toxicity were recently reviewed (Dunn *et al.* 2011).

Thus, systems approaches can be used to describe the changes in metabolism in different body compartments affected by exposure to, for example, TRT. Such multi-omic profiles are themselves characteristic of particular types and mechanisms of pathology and can be used to provide a more complete description of the biochemical consequences than can be obtained from one omic-level analysis alone. For example, the evaluation of transcriptomics as well as metabolic changes after the administration of bromobenzene provides a more sensitive approach for detecting the effects of the toxin (Heijne *et al.* 2005). However, although omics-based biomarkers and signatures identified from population-based studies can potentially be used to identify high-risk groups for targeted prevention and treatment, the translation of this knowledge into interventions has proved more difficult than anticipated (Lenfant 2003). It still needs to be determined whether the identified molecular biomarkers (genetic variant, protein and metabolite) confer effect sizes above and beyond traditional risk factors, such as obesity, smoking or family history, and thereby add to the clinical prediction. However, these high-throughput technologies are subject to rapid technological development, and their application to large-scale cohort studies has just begun. Thus, future technological advances in high-throughput methods with improved analytical sensitivity and reproducibility, enhanced bioinformatics and reduced costs will promote the widespread use of these techniques in medical research (Hu 2011), which is expected to foster notable successes in clinical practice (O'Donnell & Nabel 2011). Compared with other molecular profiling techniques, metabolomics represents the omic level closest to the phenotype because it not only provides a direct and dynamic snapshot of the current physiological status of an individual but also molecular information about metabolites that are the ultimate products of gene, mRNA and protein activity. Therefore, metabolomics is considered the omic level most capable of reflecting the non-linear impact of environmental and lifestyle factors on disease risk.

The present review highlights the application of metabolomics and other omics to open exciting avenues for biomarker discovery and individualised TRT in men. This review is based on literature search for specific investigators in the PubMed database without applying any date or language limits for the searches. Identifying markers that can be measured before treatment to individualise the type of therapy, relevant drug dose and treatment scheme plays a crucial role in the framework of personalised medicine.

Metabolomics is starting to have an impact not only on disease diagnosis and prognosis but also on drug treatment efficacy and safety monitoring. With the projected future growth of metabolomics and other omics techniques, systems biology principles are anticipated to contribute significantly to the advent of personalised medicine (Holmes *et al.* 2008b, Lewis *et al.* 2008a, Nicholson & Lindon 2008). Studies published to date have illustrated the potential for applying metabolomics to the field of andrology, and it becomes increasingly clear that systems approaches including multiple omic levels will be essential to address the outlined uncertainties in the current diagnosis and therapy of ADS (Box 1). Thus, the application of the described omics approaches in large, long-term, randomised, placebo-controlled trials of TRT in symptomatic middle-aged and elderly men with well-documented testosterone deficiency would be of great value to improve the diagnosis, therapy and monitoring of ADS in men and the clinical management of TRT.

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