Evaluation of molecular profiling platforms in clinical pharmacology

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SECTION 5

Summary, conclusions and general discussion

CHAPTER 10

Summary and conclusions

Section 1

The first section of this thesis is divided into two chapters. Chapter 1 is an introductory chapter in which the outline of the thesis and its context are described. This is followed by Chapter 2, which provides some more background on the '-omics' technologies (cDNA microarray-based transcriptomics and ^{1}H NMR-based metabolomics) evaluated in the studies comprising this thesis.

Section 2

The second section describes the results of the analyses performed to evaluate and demonstrate the treatment response of the two 'prototype' peroxisome proliferator activated receptor (PPAR) agonists rosiglitazone (PPARγ-agonist), and ciprofibrate (PPARα-agonist) by assessing both 'traditional' and a number of 'non-traditional' (candidate) plasma markers. In both studies, the experimental populations consisted of 16 type 2 Diabetes Mellitus (T2DM) patients and 16 healthy volunteers (HVs), balanced for gender (i.e. 8 males and 8 females).

In the first study (Chapter 3) we sought to demonstrate efficacy of the insulin sensitizing drug rosiglitazone *vs.* placebo, by studying its effects on 'traditional' parameters of gluco-regulation (i.e. fasting blood glucose, insulin and C-peptide) in a 6-week treatment period. In parallel, we investigated whether a selection of inflammation markers and pro-inflammatory cytokines could serve as early responding 'non-traditional' biomarkers for the pharmacological effects of rosiglitazone in T2DM patients and HVs. In addition, we explored the differences in baseline concentrations of these markers between the T2DM patients and HVs groups, in order to investigate and confirm some of the previously reported T2DM- associated biochemical changes.

By measuring the response of these candidate 'non-traditional' effect parameters, which we hypothesized to be more upstream in the cascade of the events leading to insulin sensitization, we aimed to identify a comprehensive new set of early responding biomarkers for PPARγ action that could potentially precede or predict the response of the 'traditional' parameters in T2DM patients. In addition, we sought to investigate if these candidate biomarkers would also respond in more easily studied and recruited HVs. If found, these new biomarkers could be used to support and expedite early clinical development 'proof of concept' studies with novel PPARγ-agonist-class compounds.

In this study we demonstrated efficacy of rosiglitazone in T2DM patients by showing significant decreases in parameters of gluco-regulation as well as a significant decrease in some of the 'non-traditional' markers (i.e. Interleukin-6 (IL-6), and white blood cell count (WBC)). However, we could not identify new markers that had an earlier response to treatment with rosiglitazone than the 'traditional' parameters of glucoregulation in this group. Also, we did not observe significant treatment effects in HVs, apart from a slight reduction in peak glucose and peak insulin following an oral glucose load. Comparing T2DM patients and HVs at baseline revealed elevated concentrations of IL-6 and HS-CRP in the T2DM group, which is consistent with previous observations and supports the concept that T2DM reflects a state of chronic inflammation.

We concluded that although IL-6 did not have a quicker response than the 'traditional' biomarkers, it might still have a place as complementary biomarker in the early clinical evaluation of novel thiazolidinediones in T2DM patients. Finally, the marginal response of the 'traditional' biomarkers, and entirely absent response of the 'non-traditional' biomarkers in the HVs group in this study, indicated that T2DM patients cannot easily be substituted with HVs in early clinical 'proof of concept' experiments evaluating novel thiazolidinediones.

In the subsequent chapter (Chapter 4) a study is described in which we sought to evaluate and validate the efficacy of the lipid-lowering drug ciprofibrate in T2DM patients and HVs by demonstrating its effects on 'traditional' parameters of lipid metabolism, including several (apo-)lipoproteins in a relatively short-term (3-week) treatment period. In parallel and for comparison with the effects on 'traditional' markers, we investigated the effects on two 'non-traditional' pro-inflammatory candidate markers (i.e. tumour necrosis factor α (TNFα), and monocyte chemoattractant protein 1 (MCP-1)).

Since recent evidence suggested that the previously observed (anti-inflammatory) effects of PPARα-agonists on TNFα and MCP-1 may be important factors related to the beneficial effects on human atherogenesis and cardiovascular disease outcome measures, we hypothesized that these non-traditional variables could serve as important 'mechanism-based' biomarkers for the early clinical evaluation of novel PPARα-agonist class compounds in T2DM patients. In addition, and in analogy to the study with rosiglitazone, we sought to investigate if these candidate markers would also respond in HVs.

In addition, to gain more insight in the mechanism and site of ciprofibrate action, we investigated whether the anticipated *in vivo* drug effects on TNFα and MCP-1 plasma levels were (partly) attributable to reduced

spontaneous as well as *ex vivo* lipopolysacharide (LPS) or C-reactive protein (CRP) stimulated whole-blood MCP-1 and TNFα secretion.

We demonstrated efficacy of ciprofibrate in both T2DM patients and HVs by showing significant decreases in most of the 'traditional' lipid and (apo)lipoprotein parameters after 3 weeks treatment with ciprofibrate *vs.* placebo. There was virtually no change in mean MCP-1 and TNFα concentrations in either study group. However, ciprofibrate treatment did significantly decrease the *ex vivo* whole blood unstimulated, LPS and CRP stimulated MCP-1 release in HVs. We concluded that MCP-1 and TNFα are unsuitable as 'mechanism-based' biomarkers in small clinical 'proof on concept' studies with novel PPARα agonist-class drug candidates. In addition, the fact that ciprofibrate significantly decreased the unstimulated, LPS and CRP stimulated MCP-1 release in whole blood in HVs suggests a possible role in the modulation of atherosclerosis and inflammation by PPARα agonists in general.

Section 3

The third section contains a description of the pilot study (Chapter 5) in which we assessed the feasibility of minimally invasive human skeletal muscle and adipose tissue biopsy procedures that were to be applied in our subsequent drug intervention microarray studies. In addition, this chapter portrays the evaluation of the RNA extraction techniques and methods for cDNA microarray hybridization, data normalisation procedures as well as methods to assess data reproducibility and perform outlier detection and removal. Furthermore, this section contains a description of the studies in which we evaluated the usefulness of transcriptional profiling using spotted cDNA microarray technology for the assessment of the pharmacological effects of the PPAR γ and α agonists rosiglitazone (Chapter 6) and ciprofibrate (Chapter 7), respectively, in their relevant human target tissues.

Since these drug-induced transcriptional profiles or 'treatment fingerprints' are hypothetically capable of capturing the pleiotropic 'upstream' actions of these types of drugs, they were proposed as potentially superior, rapidly responding 'mechanism-based' biomarkers, which would allow better, knowledge-based ('go / no go') decision-making in the clinical development of novel PPAR-class candidate drugs. In addition, in both these studies we sought to explore the baseline differences in global tissue gene expression profiles between the T2DM

patients and HVs (i.e. identify a molecular disease 'fingerprint') and subsequently compare these profiles between our two studies to see if we could identify putative disease related, so-called 'enriched' T2DM-associated genes, biological functions and pathways.

The results from our pilot study (Chapter 5) demonstrated that the muscle and adipose tissue biopsy procedures were well tolerated by the study subjects. The muscle biopsy procedure yielded sufficient amounts of high quality RNA, evidenced by gel electrophoresis as well as subsequent dynamic range, concordance correlation and cluster analyses. The adipose tissue biopsy procedure however, yielded insufficient amounts of RNA in some samples and did therefore not allow duplicate or triplicate hybridizations for all samples. This resulted in two putative outliers in a series of 12 samples. The blood samples, harbouring peripheral blood leukocytes, yielded sufficient RNA in most cases. However, it was concluded that future blood sampling should be performed in duplicate to prevent needless missing data points. Taken together, we concluded that the biopsy and RNA extraction procedures in this pilot study were suitable for use in subsequent clinical microarray intervention studies, provided that the adipose tissue biopsy weight was minimally 400 mg and the skeletal muscle biopsy weight minimally 100 mg. Furthermore, the tested methodology for microarray data normalization and elimination of outlier data points and subsequent clustering analysis proved to be an appropriate approach, which was expected to be fully applicable to the identification of (PPAR) treatment induced changes in global tissue gene expression profiles in a clinical setting.

The results from the subsequent intervention study with rosiglitazone (Chapter 6) indicated that none of the genes in adipose tissue, muscle tissue or PBLs of T2DM or HVs displayed a significant treatment response *vs.* placebo at Bonferroni adjusted values and α=0.05. It appeared that in this study design the signal-to-noise ratio was probably too low to adequately identify drug-induced effects on tissue gene expression levels using spotted cDNA microarray technology.

On the other hand, using identical statistical criteria, we identified several genes that were significantly differentially expressed between T2DM patients and HVs in skeletal muscle and adipose tissue at baseline. Exploratory pathway analysis of these baseline data identified several (previously reported) putative T2DM- associated genes that were implicated in carbohydrate and lipid metabolism, as well as a number of T2DM-associated canonical pathways (i.e. the 'Chemokine', 'Insulin receptor' and 'PPAR' signalling pathways) in adipose tissue. Analysis

of the skeletal muscle data set identified several canonical pathways implicated in lipid metabolism and inflammation (i.e. the 'IL-6' and 'Nuclear factor κB' signalling pathways).

From these results we concluded that gene expression profiling using spotted cDNA microarray technology (in the contemporary tested study design and wet lab design settings) was unable to provide a broad array of molecular biomarkers for drug response to be used in early clinical 'proof of concept studies' with novel thiazolidinediones in humans. Nonetheless, we demonstrated that microarray technology coupled with sophisticated bioinformatic pathway analyses appeared suited for metabolic disease exploration. Finally, we stated that the capability of cDNA microarray technology to detect PPAR agonist-induced changes in global tissue gene expression profiles should be evaluated further using a modified study design (i.e. studying patients on stable doses of oral hypoglycaemic medication and accounting for potential print batch issues), which might result in an improved signal-to-noise ratio.

The observations made in the subsequent study with ciprofibrate (Chapter 7) were similar to our previous findings in the study with the PPARγ agonist rosiglitazone, and showed that none of the genes in adipose tissue, skeletal muscle tissue or PBLs of T2DM and HVs displayed a significant response to ciprofibrate *vs.* placebo at Bonferroni adjusted values and α=0.05.

Also similar to the results from the study with rosiglitazone, the baseline comparisons revealed a large number of differentially expressed genes in adipose and skeletal muscle tissues between T2DM patients and HVs. Comparison of these exploratory baseline findings (using the Ingenuity Pathway Analysis application) between the two studies revealed a number of biological 'themes' in adipose tissue, of which 'uptake of monosaccharide' and 'PPAR signalling' were identified as most significantly 'enriched' biological function and canonical pathway, respectively. In the skeletal muscle data sets, although based on a very small number of focus genes, 'Release of eicosanoid' and 'IL6- signalling' were identified as most significantly enriched biological function and pathway, respectively. In contrast, there was very little overlap between the two studies at the individual gene level, with only one individually enriched gene that exhibited the same direction of differential expression (up- or downregulation) in both studies.

In summary, we concluded that the results from this study confirm the findings from our previous microarray study with PPARγ agonist rosiglitazone, and indicate that global tissue gene expression profiling using spotted cDNA microarray technology is currently unable to provide

a broad array of 'combinatorial biomarkers' for the action of PPAR agonists in relevant human tissues that could be used to support the early clinical development of novel PPAR agonist drug candidates. Nonetheless, as demonstrated by the identification of several enriched biological functions and pathways, the technology may still have important utility in metabolic disease exploration, and could yield important new starting points for future hypothesis driven investigations.

Section 4

Section four describes the studies in which we evaluated the usefulness of ^{1}H NMR spectroscopy-based metabolomics as a method to assess the pharmacological effects of PPAR α and γ agonists on global endogenous metabolite profiles in human biofluids of T2DM patients and HVs. Since most of the 'pleiotropic' actions of a drug would likely remain unnoticed when studying a limited number of pre-defined conventional biochemical plasma markers, we hypothesized that global endogenous metabolite profiles could serve as a broad, more complete array of biomarkers (i.e. 'treatment fingerprint') for the 'downstream' pharmacological effects of PPAR agonists in easily collected human biofluids like urine and blood plasma.

If found to respond in small groups of T2DM subjects, and preferably in more easily studied and recruited HVs, these global metabolite profiles could be useful effect parameters to be applied in the early clinical evaluation of novel PPAR-agonist drug candidates. In addition, the design of these studies provided the opportunity to explore the baseline differences in urine and plasma metabolite profiles between T2DM patients and HVs in order to attain a putative metabolic T2DM 'disease fingerprint'.

Chapter 8 describes the pharmacological effects of PPARγ agonist rosiglitazone on global endogenous metabolite profiles measured in plasma and urine samples of T2DM patients and HVs using ^{1}H NMR spectroscopy coupled with multivariate statistical analysis (Principal Component Discriminant Analysis; PC-DA). In addition, we sought to explore the baseline differences in plasma and urine metabolite profiles between the T2DM patients and HVs groups.
Treatment with rosiglitazone *vs.* placebo led to clear separation of the different treatment Visits in the PC-DA plots in the urine and to a lesser extent plasma of the T2DM group, but not in the HVs group. Interpretation of the peaks that could be identified in the proprietary

TNO database indicated that the treatment-induced separation was primarily related to a relative decrease in urinary hippurate and aromatic amino acids coinciding with an increase in plasma branched chain amino acids as well as alanine, glutamine and glutamate. Since the maximum effect on these metabolites was observed after 2 weeks of rosiglitazone treatment, we hypothesized that these observations could be related to the early improvement in hepatic insulin sensitivity reported to occur after short-term thiazolidinedione use. Furthermore, the baseline comparisons between T2DM patients and HVs showed that urine and plasma metabolite profiles convincingly discriminated between disease state and gender. Subsequent identification of metabolites in these profiles showed that T2DM patients exhibited a relative increase in urinary levels of several amino acids, citrate, phospho(enol)pyruvate and hippurate, whereas putative T2DM-associated changes in plasma appeared largely attributable to increased levels of lipids and lactate coinciding with decreased levels of several amino acids. From these results we concluded that NMR-based metabolomics of urine and blood plasma coupled with advanced bioinformatics can yield a comprehensive array of non-glucose biomarkers for the action of thiazolidinediones in small groups of T2DM patients but not in HVs. In addition, NMR-based metabolomics appeared capable of providing distinct endogenous metabolite profiles for T2DM patients and HVs (so-called 'disease fingerprints') in plasma and urine samples at baseline.

Analogous to the previous chapter, Chapter 9 describes the pharmacological effects of the PPARα agonist ciprofibrate as well as the baseline differences in global endogenous metabolite profiles measured in urine samples between T2DM patients and HVs using ^{1}H NMR spectroscopy.

In this study we demonstrated that supervised multivariate statistical analysis (partial least squares discriminant analysis; PLS-DA) of human urine samples was able to separate the various study groups – ciprofibrate *vs.* placebo treated – when the male and female subjects were analyzed separately. The contribution plots showed those regions of the urine spectra that were responsible for the differences between the various treatment groups. In addition, the T2DM patients and HVs were easily separated, but the variation in the urine profiles limited the number of metabolites observed that distinguished one group from another using this type of study design and method of analysis. We concluded that NMR-based metabonomics of easily collected urine samples could discern HVs from T2DM patients as well as the ciprofibrate-treated and placebo-treated categories.

CHAPTER II

General discussion

In this thesis a series of studies is described, in which we evaluated the capability of two distinct molecular profiling platforms – cDNA microarray-based transcriptomics and ^{1}H NMR-based metabolomics – to identify a broad array of (rapidly responding) molecular biomarkers for the pharmacological effects of the peroxisome proliferator activated receptor (PPAR)α agonist ciprofibrate and PPARγ agonist rosiglitazone (i.e. 'treatment fingerprint') in relevant target tissues and biofluids of type 2 Diabetes Mellitus (T2DM) patients. In addition, we assessed if these 'combinatorial biomarkers' [1] could also be identified in more easily studied and recruited healthy volunteers (HVs). If found, these 'prototype' molecular biomarker profiles could eventually be used to support and expedite early clinical development 'proof of concept' studies with novel PPAR agonist-class drug-candidates.

In addition, the design of these studies provided the opportunity to evaluate if these molecular profiling platforms were capable of identifying putative disease-related biomarker profiles (i.e. a 'disease fingerprint') by comparing the gene expression and metabolite profiles between the T2DM patients and HVs at baseline.

In the first intervention study with the PPARγ agonist rosiglitazone we 'validated' our study design by first demonstrating efficacy of rosiglitazone in T2DM patients using a 'traditional' set of biochemical plasma markers.

In parallel, we investigated the effects on global tissue gene expression profiles, but failed to detect significant ('upstream') rosiglitazone-induced changes in two relevant PPARγ target tissues (adipose tissue and skeletal muscle) and peripheral blood leukocytes (PBLs). In contrast, we did identify clear ('downstream') changes in urine and plasma metabolite profiles using ^{1}H NMR spectroscopy-based metabolomics. Moreover, a similar discordance between 'upstream' and 'downstream' treatment effects was observed in the consecutive study with the PPARα agonist ciprofibrate.

The lack of 'upstream' treatment effects on the transcriptome could be related to a number of issues related to study design, but likely also to some issues inherent to the sensitivity and limitations of microarray-based gene expression profiling and mechanisms of PPAR agonist action per se. To start with, our studies used a longitudinal design (e.g. comparison of active drug *vs.* placebo in time) that is typically employed in clinical pharmacology studies. This design is clearly more complicated than the commonly used cross-sectional design (i.e. tumour *vs.* healthy tissue) for microarray studies. A number of temporal factors including

intra- and inter-individual variability in gene expression, possible confounding effects of intercurrent illness, concomitant medications, diet and menstrual cycle (for pre-menopausal women) may all represent additional complicating factors that might introduce variability in the analysis [2]. Also, variability in gene expression profile related to age, gender, or ethnic background can become a potentially important issue when comparing subjects in a treatment cohort [2]. Besides these general issues related to the longitudinal study design, other more specific issues, although partially accounted for in the statistical analyses, may have increased variability.

In the first microarray study (with rosiglitazone), there were some concerns with regard to the glucose-lowering potential of rosiglitazone as add-on therapy and thus the demonstration of efficacy using 'traditional' markers. Therefore, it was decided to withdraw the patients' oral hypoglycaemic maintenance therapy and washout all subjects for two weeks before randomizing them to either rosiglitazone or placebo. Unfortunately, this led to a higher than anticipated number of dropouts (largely due to hyperglycaemia in the placebo group) and corresponding missing data points which had to be substituted with modelled data. In addition, metabolic derangement, after withdrawal of oral maintenance therapy in the remaining (non-dropout) patients, may have generated additional variability. However, considering the fact that we also failed to detect significant changes in gene expression profiles of (relatively stable) HVs, makes it less likely that these factors played a major role.

With regard to spotted cDNA microarray technology itself, we concluded that, in addition to other well-known technical pitfalls [3;4], differences in microarray print batch, as unavoidable in larger studies with multiple samples, appeared to be an important variability introducing factor, which if it had remained undetected and uncorrected, would have led to considerable misinterpretation of the data. In the second study (with ciprofibrate), the study design allowed studying patients that remained on steady doses of oral hypoglycaemic therapy instead of patients washed-out from oral hypoglycaemic therapy. This design thus avoided the confounding effects of metabolic derangement and resulted in considerably fewer subjects dropping out and hence less missing data in the patients' cohort. In addition, to avoid technical interpretation issues with regard to differences in print batch, we assured that the samples were well balanced. Hence, no major statistical correction was needed during the analysis. However, also with this improved study design, and using identical methods and statistical criteria, we were unable to demonstrate significant PPARα-

agonist induced effects on global tissue gene expression profiles in T2DM patients or HVs.

When taking into consideration that changes in gene expression profile induced by pharmacological interventions may be much smaller than differences commonly observed in (cross-sectional) disease *vs.* control comparisons (i.e. in cancer where many genes are differentially expressed due to multiple DNA mutations), it is conceivable that this relatively subtle treatment signal could be obscured by the large amount of 'noise' associated with a surfeit of uncontrolled factors in a more complicated longitudinal design. However, this view appears to be in contrast with the observed treatment effects on 'downstream' effect parameters, i.e. biochemical markers and metabolite profiles, which appeared less affected by this 'noise' and remained readily detectable in both studies. A possible explanation for this could be that after the activation of the PPAR receptor, many small and interlinked changes in (temporal) gene expression induce relatively large, more consistent changes in 'downstream' processes, which are therefore more easily measured in a 'noisy' clinical environment. In addition, especially the traditional sets of biochemical markers were measured using more targeted and robust methods. In other words, the discrepancy between 'upstream' and 'downstream' findings is likely to bear down to differences in signal-to-noise ratio, which, in this study design and use of methods, appears to be a considerably better ratio for the 'downstream' processes.

In addition to a low signal-to-noise ratio, the lack of observed treatment-induced transcriptome changes could be partially related to suboptimal timing and quantity of serial gene expression measurements performed in these studies. It has been shown in previous experiments with LPS infusions in healthy men that gene expression changes in peripheral blood mononuclear cells (PBMCs) can follow many different patterns of (sub)acute response during a 24 hour period after infusion [5]. The most commonly observed pattern was that genes do not change in expression at all, which is quite surprising considering the fact that LPS represents a very strong stimulus. However, some of the changes included a quick up or down regulation of several genes, which rapidly returned to baseline within 24 hours [5]. Only a few patterns included a slow rise in expression that was still continuing after 24 hours [5]. These observations show that changes in gene expression can follow many different patterns in time and illustrate that frequent sampling is eminent to provide a good picture of the transcriptome changes in time following an intervention.

However, in a clinical setting is it unfeasible to collect large series of tissue samples. For this reason, we also obtained more easily collected blood samples (harbouring PBLs) at more frequent time points during the study. However, although a subpopulation of PBLs, i.e. mononuclear cells, expresses PPARα and γ receptors [6], we failed to detect a significant acute (i.e within hours) or delayed (i.e after 2, 4 and 6 weeks) treatment response on their transcriptome. This could be related to the fact that white blood cells are by far the most transcriptionally active cells in the human body and thus exhibit even larger intra- and inter-individual variability than adipose and skeletal muscle tissue [7]. In addition, as is true for the biopsy samples, white blood cells represent a highly heterogeneous 'tissue', which is comprised of many different subtypes (i.e. lymphocytes (30%), monocytes (5%), and granulocytes (65%)) [8]. Furthermore, the proportions of these subtypes often vary from time to time, adding to the variability in relative gene expression [8]. Recent evidence suggests that better results can be obtained when studying the peripheral mononuclear cells (PBMCs; i.e. monocytes and lymphocytes) in isolation [7].

The suggestion that the additional 'noise' introduced in a longitudinal study design likely obscures the relatively weak treatment signal, appears to receive further support by the observation that in both studies (using virtually identical statistical criteria and methods) we were able to identify a large number of putative disease related genes when performing a typical cross-sectional comparison (i.e. T2DM *vs.* HVs at baseline). In other words, the supposedly larger difference in gene expression profile (i.e. 'signal') related to the disease state was more easily detected when making use of a simpler and thus less noisy cross-sectional comparison. Although the adipose tissue and skeletal muscle 'disease fingerprints' showed little overlap on the individual gene level across both studies, the analysis was still able to identify a number of enriched putative T2DM-associated biological functions and pathways that appear plausible findings in view of the pertaining literature on T2DM. For instance, the gene encoding PPARγ, which is the renowned target for thiazolidinediones (including rosiglitazone) in i.e. adipose tissue [9], and its corresponding signalling pathway were found to be significantly 'enriched' using the Ingenuity Pathway Analysis application, but remained unidentified using conventional p-value cut-offs and correction for multiple comparisons. Similar findings were reported by Bammler et al. who compared gene expression profiling results (from the same experiment) across different laboratories and across platforms [10]. Although they observed large inconsistencies

on a gene-by-gene basis, the biological themes (using Gene Ontology annotations) that emerged from the data from the different platforms and laboratories were overall the same. These observations underline the true potential of cDNA microarray technology, which in combination with sophisticated pathway-level analyses and in experienced hands can be an important tool for disease exploration by facilitating the identification of disease pathways and potentially novel 'druggable' targets.

Although a low signal-to-noise ratio is, as discussed above, likely to be the main reason for the absence of detectable treatment induced transcriptome changes, the apparent discrepancy between up- and downstream effects could be also be partially explained by the fact that our a priori assumption of the primary mechanism of action of PPAR agonists was incomplete. While it is apparent from many investigational studies with PPARγ agonists that changes in global gene expression in animal models or using human adipocyte cell-lines appear to occur [11;12], it is debatable whether these (small and often highly variable) changes play a major role in the primary and true mechanism(s) of action of these drugs *in vivo*. In fact, there is a good chance that many of the key 'downstream' effects of thiazolidinediones may actually be controlled by post-transcriptional, PPARγ-independent mechanisms, as has recently been shown for adiponectin [13;14]. Adiponectin is a cytokine produced and secreted by adipocytes and is regulated by thiazolidinediones. It is believed to be one of the key modulators of insulin sensitivity [15] by having an important insulin-independent effect on hepatic gluconeogenesis [16]. This means that, in theory, also other significant changes in 'downstream' parameters could be observed without changes in expression of the supposedly responsible PPARγ target genes.

Finally, our results appear to be corroborated by the findings from a recent study by Goldfine et al. [17]. The authors of this paper reported on the effects of 8-weeks rosiglitazone treatment on global gene expression profiles in adipose tissue and skeletal muscle of 6 drug-naive T2DM patients using a newly developed (more sensitive) array technology; Total Gene Expression Analysis (TOGA) [18]. Although this study used less rigorous statistical criteria for gene selection (concordant fold-change instead of p-values adjusted for multiple comparisons in our study) only two out of approximately 17,000 genes were found to be concordantly differently expressed after rosiglitazone treatment. The fact that independent authors using a more sensitive array method coupled with less stringent gene selection criteria only found two out of 17,000 transcripts to be modulated by thiazolidinedione treatment

strengthens our belief that many issues need to be resolved before gene expression profiles can be used as effective molecular biomarkers for the pharmacological effects of PPAR agonists *in vivo*.

As stated in the introduction, ideally, the combination and integration of all three established '-omics' subdisciplines as proposed in the 'systems biology' approach [19], should provide a more holistic view of the multiparametric response of a biological system to perturbation. Although the concept is appealing and has shown its successful application in *in vitro* cell-based and animal studies [20;21], with the currently available molecular profiling technologies and tested study designs, it appears to be a less fruitful approach for the assessment of global drug effects in small groups of human subjects. Hence, it appears that at least one of the pillars of systems biology, i.e. (cDNA microarray-based) transcriptomics, is currently unable to bear its share of weight in a clinical pharmacology setting.

Recently, and analogous to the above discussed issues regarding the PBLs transcriptome, it has become apparent that when studying heterogeneous tissue samples, cell-sorting is a necessary step for better and reproducible results in a microarray experiment [22]. It is expected that applying this step to human tissue samples will certainly improve the signal-to-noise ratio and will consequently deliver better results. In addition, new emerging technologies including laser capture micro-dissection, are currently being optimized, which will enable microarray researchers to study more pure target cell populations [23], or perform serial single cell collections directly from a research subject in a highly non-invasive manner [24]. Coupled with validated RNA amplification techniques [25-27], this may prove to be an adequate approach to resolve the issues related to tissue heterogeneity and relative invasiveness of tissue biopsies that clinical microarray scientists are currently facing. In addition, much effort is focused on improvements of the core microarray technology in terms of sensitivity and specificity of the arrays as well as the implementation of uniform standards, i.e. the minimal information about microarray experiments (MIAME), by a growing number of scientific journals as a requirement for publication [28;29]. In fact, these developments have already shown to allow better comparison of results between different laboratories, platforms and research groups [10;30-33]. Finally, the development of new bioinformatic protocols for the normalization of microarray data has recently gained substantial momentum, with new bioinformatic companies striving to develop better noise-reducing algorithms to improve the processing, analysis and interpretation of microarray generated data [34;35].

In conclusion, it appears that from the two '-omics' technologies evaluated in this thesis, ^{1}H NMR spectroscopy-based metabolomics of urine and plasma samples is currently the most promising '-omics' technology for the discovery of combinatorial biomarkers for PPAR-agonist action that may eventually (and obviously after independent validation) be applied to early clinical 'proof of concept' studies. Many of the spectral data derived from metabolomic analyses currently generate a large number of so-called 'black box' parameters, i.e. spectral peaks that can not be identified in public domain databases [36]. Nevertheless, it is expected that, analogous to ongoing developments in transcriptomics technology, when metabolomics and its accompanying bioinformatic tools mature, more and more data can be transformed into meaningful biological information. Adding this up to the easy and non-invasive sample collection, metabolomics will stand a very good chance of becoming one of the preferred methods for the identification of combinatorial biomarkers that can be successfully applied to future clinical 'proof of concept' studies.

However, until the '-omics' technologies and their accompanying bioinformatic protocols have evolved into more robust and clinically applicable tools, the measurement of 'traditional' biochemical plasma markers will continue to be the method of choice to evaluate which of the (PPAR agonist) drug-candidates in store will have the greatest chance of sweet success.

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