Human in vivo studies on the non-glycaemic effects of thiazolidinedione derivatives

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General introduction and outline of the thesis

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Background

This thesis describes the results of a number of clinical studies that explore the relationship between thiazolidinedione (TZD) treatment and fluid accumulation. Fluid accumulation includes conditions such as fluid retention, oedema formation and symptomatic heart failure, which, though being different clinical entities, are used interchangeably in the literature. While fluid accumulation has been recognized as one of the most important side effects of thiazolidinediones, the underlying mechanism of action remained unclear. In a series of experiments, we have tested various hypotheses.

All studies have been performed with *rosiglitazone* as a representative of the thiazolidinedione class. In this introduction, we will outline the scenery of diabetes mellitus and its treatment before the introduction of the thiazolidinediones. Then we will describe the clinical effects of thiazolidinediones and the techniques used in the studies. Finally, the outline of this thesis will be summarized.

Diabetes mellitus type 2 and insulin resistance

Diabetes is defined as a state of chronic hyperglycaemia and is classified into several subtypes. Type 1 diabetes is characterized by absolute insulin deficiency due to destruction of islet cells. Type 2 diabetes mellitus is defined as a combination of a decreased biological action of insulin (insulin resistance) and a defect in insulin production (insulin deficiency)[1]. Insulin is produced in the β-cells located in the islets of Langerhans in the pancreas and has its main influence on muscle, liver and fat tissue[2]. Insulin plays an important role in the energy balance of the body where it functions as anabolic hormone for carbohydrates, proteins and fats. Normally, insulin resistance is compensated by increased insulin production. If insulin production can no longer match the increased insulin need, (chronic) hyperglycaemia and thus diabetes will develop.

Diabetes type 2 is nowadays epidemic. In 2011 there were 801,000 people in the Netherlands diagnosed with diabetes mellitus of which around 90% had type 2 disease[3]. Worldwide, the number of people suffering from diabetes is estimated as close to 400 million[4]. Insulin resistance is not only associated with type 2 diabetes, but also with other metabolic disorders such as hypertension, dyslipidaemia and obesity, together clustered under the term insulin resistance syndrome or the metabolic syndrome[5].

Despite effective treatment for correction of acute hyperglycaemia, the morbidity and mortality burden of type 2 diabetes remains high especially due to chronic, cardiovascular complications[6]. Macrovascular complications, like myocardial and cerebral infarction, are caused by atherosclerosis which is accelerated by hyperglycaemia[7] and by other risk factors associated with the insulin resistance syndrome. The relative risk for myocardial infarction in patients with diabetes who are not intensively treated is between 2 and 4[8]. As a result of therapeutic improvements over the last two decades this relative risk declined, but is still 1.8[9].

The microvascular complications, retinopathy, nephropathy and neuropathy are strongly related to the duration and level of hyperglycaemia[10]. A subtype of peripheral neuropathy is diabetic autonomic neuropathy, in which the autonomic nerve fibers are damaged, and which can lead to dysfunction of several organ systems. The clinical manifestations are diverse, such as resting tachycardia, orthostatic hypotension, silent myocardial ischaemia, gastroparesis, and erectile dysfunction[11]. Especially cardiovascular autonomic neuropathy (CAN) has a serious impact on morbidity and mortality. Autonomic neuropathy is one of the most overlooked complications of diabetes[12].

Altogether, diabetes is associated with several, potentially serious complications, but the severity of the disease is often underestimated. The severity of the complications of diabetes mellitus are nicely illustrated in a phrase listed in the strategic planning report of the diabetes mellitus interagency coordinating committee: 'Diabetes kills with neither speed nor precision, but with stealth and the slow accumulation of insults. It can rob a person of the ability to see, feel, think, walk, and have sex'[13].

Treatment of type 2 diabetes

From the above, it will be clear that therapy in type 2 diabetes is aimed at prevention of the complications by (near) normalization of plasma glucose levels[14]. Lifestyle modification including weight reduction and increased physical exercise are first line interventions and the cornerstone of type 2 diabetes treatment[14]. This approach is effective on glucose lowering and moreover beneficial for the traditional cardiovascular risk factors, such as hypertension and dyslipidaemia and therefore reduce cardiovascular risk[15], however ineffective in the long term due to a high rate of weight regain[16]. For subjects with treatment failure on lifestyle

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modification, oral drug treatment is available from the 1950s. Sulphonylurea derivatives[17] were introduced first. These drugs stimulate pancreatic β-cell insulin secretion and are effective in lowering plasma glucose levels, however, it has not been proven that this class of drugs is effective in reducing cardiovascular disease[18]. A number of years after the sulphonylurea derivatives, the biguanides (with its main representative *metformin*) were introduced, which primarily lower hepatic glucose output[17] and additionally offer cardiovascular protection[19, 20]. Together with insulin treatment, these drug classes have dominated diabetes treatment in the pre-thiazolidinedione era. Although these drugs are effective in lowering glucose, it has also been found that type 2 diabetes is a progressive disease. Over time more and different compounds are needed to sustain good glycaemic control[21]. This phenomenon is explained by a progressive failure of the β-cells in the pancreas. Therefore, in the "pre-thiazolidinedione" era, there was a need to find better and more compounds to prevent chronic complications, β-cell failure and to meet tailor-made requirements. In addition, optimal therapy would not only lower blood glucose levels, but also decrease the cardiovascular risk.

Thiazolidinediones

Thiazolidinediones are synthetic ligands of the nuclear peroxisome proliferator-activated receptor-γ (PPAR-γ)[22]. After binding, PPAR-γ either activates or suppresses the expression of genes, including many genes involved in glucose and lipid metabolism[23]. The first thiazolidinedione that was clinically tested was *ciglitazone* (1982), but this compound was withdrawn from further development due to adverse effects on the liver[24]. *Troglitazone* reached the market in 1997 but was withdrawn in 2000 because of hepatotoxicity[25]. Finally, in 2000, *rosiglitazone* and *pioglitazone* were approved for the treatment of diabetes mellitus type 2. Thiazolidinediones lower plasma glucose[26, 27]. The many effects on gene expression in various target tissues was the reason for a scientific dispute on the key-mechanism of the insulin-sensitizing effects in humans[28]. The leading view is that hepatic and muscle sensitivity to insulin are increased. Interestingly, PPAR-γ is mainly expressed in adipocytes[22]. The accepted explanation for this apparent paradox is that thiazolidinediones stimulate free fatty acid storage in adipose tissue, leading to decreased substrate competition for glucose in the citric acid cycle of skeletal muscle. In addition, this will also reduce ectopic fat distribution in both liver and skeletal muscle[29].

As a consequence of the non-specific action on gene expression, thiazolidinediones have a broader therapeutic effect than improvement of glycaemic control only. Thiazolidinediones modulate the traditional cardiovascular risk factors, lipid profile[27, 30] and blood pressure[31, 32], but also have beneficial effects on inflammation[33] and on ischaemia-reperfusion injury[34].

Despite somewhat conflicting results and limited evidence, the general opinion at the start of this thesis project on thiazolidinediones was that this class was 'unremarkable in glucose lowering but potentially great in vascular prevention'. Notwithstanding this optimistic view, also adverse events were reported shortly after the introduction. Oedema formation[35] and especially chronic heart failure[36] were considered potentially serious side effects and viewed as the Achilles heel of this new class of drugs.

Thiazolidinediones and fluid accumulation

The incidence of oedema ranges from 3-5% during monotherapy with *rosiglitazone* or *pioglitazone*. In combination with other glucose lowering drugs the incidence is higher, for instance in combination with sulphonylureas 7-8% [36, 37] and with insulin 13-16%[38]. Apart from systemic oedema formation also cases with macula oedema[39] were described, but further discussion of this side effect is beyond the scope of this thesis.

The incidence of chronic heart failure is higher during thiazolidinedione treatment. The incidence in clinical trials during monotherapy is very low (<1%), but in combination with insulin up to 2-3%. Epidemiologic studies show a hazard ratio for developing heart failure of around 1.6 associated with the use of thiazolidinediones[36].

Potential mechanisms of fluid accumulation

At the start of this PhD fellowship several potential mechanisms for thiazolidinedione-related fluid accumulation had been postulated. In chapter 8, these hypothesized mechanisms are described in detail. The knowledge at the start of this project is briefly summarized below. First, thiazolidinedione-related reduction in cardiac function (cardiac hypothesis) was suggested, as this could explain both the development of oedema and of heart failure. However, no negative effects of *rosiglitazone* on cardiac systolic function were found[40]. Secondly, renal

sodium reabsorption (primary renal hypothesis) was proposed to be the initial event leading to oedema formation and symptomatic heart failure (Figure 1A). A shortcoming of this view is that it cannot explain the observed reduction in blood pressure during thiazolidinedione treatment. Finally, thiazolidinedione-induced arterial vasodilation (primary vascular hypothesis) with subsequent, secondary, renal sodium retention was proposed as initiating mechanism, explaining both blood pressure reduction and oedema formation (Figure 1B). Also other hypotheses were proposed, such as thiazolidinedione-increased sympathetic nervous system activity, or alterations in endothelial permeability[36].

At the start of this thesis there seemed to be scientific agreement that the vast majority of oedema cases was not caused by decreased cardiac function but that thiazolidinedione-induced fluid retention unmasked previously asymptomatic and unrecognized diastolic dysfunction. There was no scientific agreement on the cause of fluid accumulation but the leading views were primary renal sodium retention or arterial vasodilation with secondary renal sodium retention.

Figure 1A: Primary renal sodium retention raises plasma volume. In the local vascular bed this will elevate hydrostatic pressure with subsequent capillary leak and development of oedema. This rise in plasma volume will increase preload and increase blood pressure. The rise in preload can unmask asymptomatic heart failure.
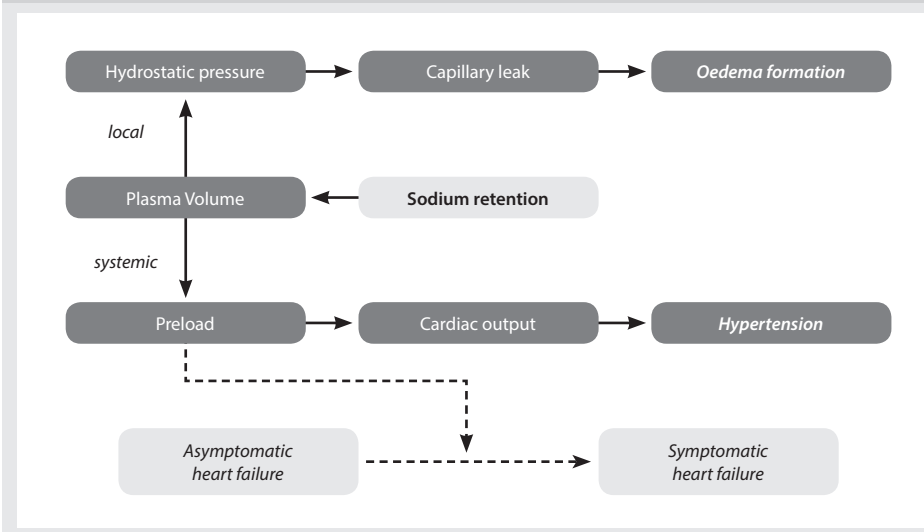
Figure 1B: Arterial vasodilation couples local increase in hydrostatic pressure and subsequent oedema development to systemic reduction in 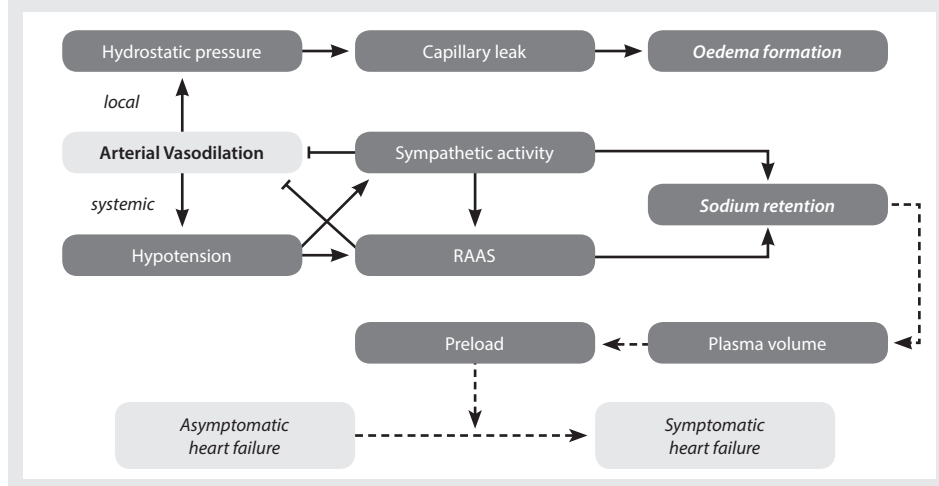blood pressure. As a consequence the Renin-Angiotensin-Aldosterone System (RAAS) will be activated and the sympathetic tone will be increased both inducing sodium retention. Sodium retention and subsequent rise in preload could again unmask asymptomatic heart failure.

Techniques to determine endovascular permeability and the size of body fluid compartments

Several studies in this thesis investigate the mechanism of *rosiglitazone*-induced fluid accumulation by measuring changes in plasma, extracellular and interstitial volumes and vascular permeability. The techniques used to measure these variables are briefly outlined below. Venous occlusion plethysmography is another important technique in several studies of this thesis, but we did not use it for measurements on vascular permeability or body fluid compartments. Therefore, we only briefly summarize some aspects in this part of the thesis. The keypoint of venous occlusion plethysmography is that the rate of volume increase of the arm during venous outflow impediment with unrestricted arterial inflow, is precisely measured with mercury-in-silastic strain gauges[41]. In this way, baseline forearm blood flow and the local vascular response to infusion of vasoactive drugs into the brachial artery **(chapter 2, 3, 6)** or the response to activation of the sympathetic nervous system **(chapter 6)** were measured.

Transcapillary escape rate of albumin (TERalb)[42]

The amount of albumin leaking out of the vascular system is a measure of capillary permeability. Assuming first order kinetics implies that, each hour the same percentage of total vascular albumin leaks out of the circulation. The technique involves intravenous injection of radioactively labeled human albumin, followed by drawing eight exactly timed blood samples from an arterial line over a one hour period. Arterial blood sampling prevents against sampling error by haemoconcentration due to venous occlusion[43]. After centrifugation, labeled albumin concentration is assessed by measuring radioactivity with a radioactivity counter. The measurements represent a declining exponential curve from which the fraction leaving the circulation per hour can be calculated. Plotting the extinction curve on a semi-logarithmic scale results in a straight line (Figure 2). The same procedure can be used to assess plasma volume. The theoretical peak concentration just after the injection is calculated from the extinction curve of the radioactivity in plasma. The injected amount of radioactivity divided by the calculated peak concentration results in the plasma volume.

Water displacement method for measurement of oedema in the foot[44]

Oedema usually occurs around the foot and ankles as a result of gravity and is generally limited to this region. Therefore, systemic measures of interstitial fluid

volume are not sensitive enough to detect mild depending oedema. Assuming that variation in the volume of the foot represents change in interstitial fluid, changes in measured foot volume are equal to the changes in interstitial fluid, ie oedema. The volume of parts of the body can be measured in a measuring cup using the water displacement method. However, due to the size of the foot, measuring cups will only provide a rough estimate of the foot volume as it needs to have a wide opening (Figure 3). In this thesis we have used a device that measures changes in water displacement indirectly but with a one milliliter accuracy. As shown in Figure 4, a Perspex water bath was placed on an electronic balance. To provide foot support, a stainless steel construction was suspended in the water bath. This construction rested fully on the floor on both sides of the balance. The water bath was filled with tap water until the balance indicated a weight of 15,000 grams. Immersion of the foot into the water resulted in water displacement and increase

Figure 2: On the left side of this Figure, the extinction curve for radioactivity is plotted on a semi-logarithmic scale. This results in a straight line due to the leakage of a constant percentage of albumin out of the circulation in each time interval, while the backflow from the interstitial space is not containing radioactivity during the first hour after injection. The right side of this panel represents the formula for calculation of TERalb. A(0) = concentration radioactive albumin at t=0 (just after injection). A(1hr) = concentration of radioactive albumin at 1 hour **(chapter 2, 3)**

radioactivity
Ln counts (cpm)
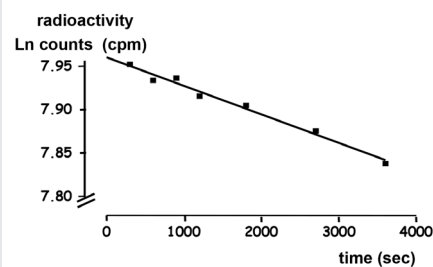
$$TERalb = \{ [A(0) - A(1hr)]/ A(0)\} \times 100\% \ (1)$$
$$TERalb = [A(0)/A(0) - A(1hr)/A(0)] \times 100\% \ (2)$$
$$TERalb = [1 - A(1hr)/A(0)] \times 100\% \ (3)$$
First order kinetic formula: $A(t) = A(0) \times e^{\beta x t}$ **(4)**
Logarithmic transformation: $Ln \ A(t) = Ln \ A(0) + \beta \times t$ **(5)**
β = slope **(6)**; see left panel
Rewrite formula **(4)**: $A(t) = A(0) \times e^{slope \times t}$ **(7)**
$A(1hr)/A(0) = e^{slope \times 3600}$ **(8)**
Then: $TERalb = [1- e^{3600 \times slope}] \times 100\%$ **(9)**

Figure 3: Two different methods to measure the volume of an object, for example two stones.
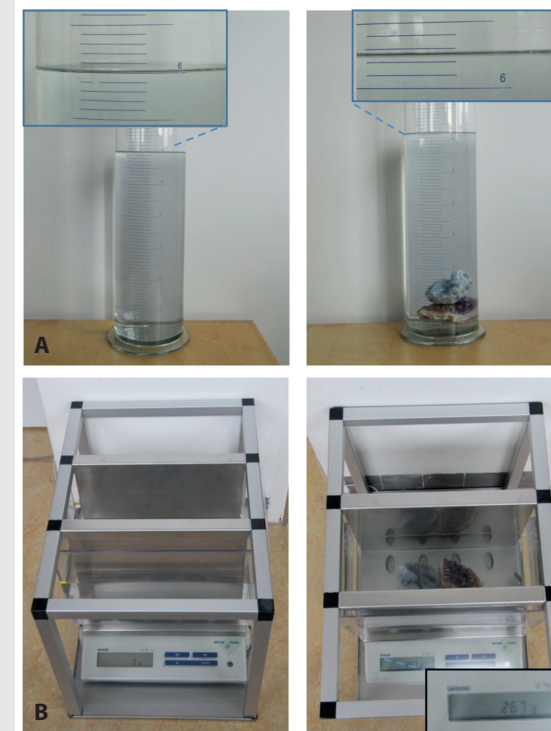
A) Traditional method to measure the volume of an object using a cylinder with water. This provides a rough estimate of volume by subtraction of the two measured volumes (250 to 270 ml).

B) Water displacement method. The amount of displaced water provides increased pressure on the electronic balance. The volume of the object is equal to the weight recorded on the balance divided by the relative weight of water. The volume of the stones is 267 ml.

in weight recorded on the balance (Archimedes principle). Foot volume is equal to the weight recorded on the balance divided by the relative weight of water (0.998 g/ml at 22 °C). To reduce immersion depth differences, the subject sat on a stable chair with an adjustable height. A standardized position was sought with the thigh in a horizontal position, with a 90° angle at the knee. The temperature of the water was always 22°C. A simple conversion of 1g to 1 ml was used, which gave systemic underestimation by only 0.2 %.

Measurment of body fluid compartments with bio-electrical impedance measurement[45]

Several methods exist to assess body fluid compartments. Most of these methods are based on dilution techniques and are invasive, time-consuming, expensive and require the use of radioactive substances. To overcome these problems, we used bio-electrical impedance with a soft tissue analyzer. Body compartments were measured indirectly with the use of specific formulas. Subjects rested supine for approximately 15 minutes to equalize fluid compartments. Four surface electrodes were applied (two to an arm and two to a leg). Bio-electrical impedance was measured by recording the voltage drop caused by body impedance modulus when applying an alternating and constant current. Phase sensitive sensors separate the components of the modulus into reactance and resistance. Extracellular water and total body water are then calculated from these values.

Figure 4: Measurement of foot volume
1) A: electronic balance; **B:** Perspex box filled with water; **C:** Foot support without either contact to Perspex box or electronics balance; **D:** Frame
2) Standard immersion of the foot
3) Accurate measurement of foot volume 1190g = 1190ml
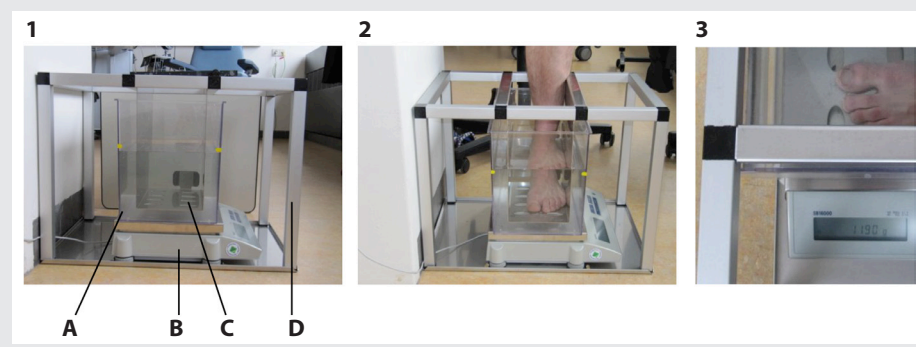
Outline of the thesis

In the present thesis we investigate the effects of thiazolidinediones beyond glycaemic control, especially the mechanism of action, risk factors and treatment of fluid retention and oedema formation. In addition, our investigations involve the influence of thiazolidinediones on autonomic neuropathy and ischaemia-reperfusion injury. We have performed four clinical trials with the thiazolidinedione *rosiglitazone*.

In this thesis, the primary hypothesis regarding the mechanism of thiazolidinedione-related fluid accumulation is arterial vasodilation, because it can explain the observed combination of blood pressure lowering and fluid retention associated with the use of thiazolidinediones (Figure 1B). Because thiazolidinedione treatment is associated with insulin sensitization[28], vasodilation during thiazolidinedione treatment may be either insulin-dependent or independent. In support of insulin-dependency is the notion that insulin itself has vasodilatory properties[46] and the observation that the oedema incidence is higher, when thiazolidinediones are combined with insulin[26]. We hypothesized that *rosiglitazone* amplifies insulin-dependent arterial vasodilation or insulin-dependent vascular permeability. This hypothesis was tested in a population characterized by insulin resistance and the results are described in **chapter 2**.

When thiazolidinediones indeed induce arterial vasodilation, this effect will normally be counterbalanced by activation of the autonomic nervous system. This would imply that the vascular effects of thiazolidinediones should be more pronounced in patients with autonomic neuropathy. In support of this assumption are the observations that the haemodynamic effects of insulin are exaggerated in subjects with autonomic neuropathy[47] and that insulin-induced oedema can be treated with *ephedrine*, an α-adrenergic agonist[48]. In **chapter 3**, we report the results of a study that tested this hypothesis in subjects with autonomic neuropathy. We assumed that these patients would be more prone to oedema formation when treated with *rosiglitazone* and insulin, due to an uncontrolled rise in capillary hydrostatic pressure followed by an exaggerated secondary renal fluid retention to restore systemic hypotension.

In the literature, evidence had emerged that primary renal sodium retention would be, at least in part, an important underlying pathophysiological mechanism of thiazolidinedione-related fluid accumulation. Several animal and in vitro experiments had found upregulation of the renal epithelial sodium transporter (ENaC) in thiazolidinedione-related fluid retention[49, 50] and found that *amiloride*, an inhibitor of ENaC, prevented oedema formation. Human in vivo evidence for such a mechanism, however, was lacking. An upregulation of ENaC by thiazolidinediones would indeed explain the clinical observation that thiazoldinedione-related oedema is resistant to therapy with loop diuretics[51]. In **chapter 4** we investigated whether *rosiglitazone* did upregulate ENaC and whether loop diuretic resistance developed in insulin-resistant subjects treated with *rosiglitazone*.

In clinical studies it has been widely suggested that thiazolidinedione-induced change in plasma volume is reflected by a change in haematocrit[26, 52]. Haematocrit is the ratio of erythrocyte volume and blood volume[53]. Haematocrit could be a marker for changes in plasma volume, but only if erythrocyte volume remains constant. Firm human in vivo data on the effects of thiazolidinediones on erythrocytes were lacking. Interestingly, an in vitro human cell study had shown suppression of erythroid-colony forming cells by thiazolidinediones[54]. In **chapter 5**, we explored whether changes in haematocrit were associated with changes in plasma volume during *rosiglitazone* treatment in a pooled data analysis of the studies described in **chapter 2 and 3**.

In our studies on thiazolidinedione-related fluid accumulation, we acquired extensive information regarding autonomic nervous system function as determined (non)invasively in patients with diabetes mellitus. In scientific literature cardiac autonomic neuropathy is an overlooked complication of diabetes, with a high impact on morbidity and mortality[11]. The diverse actions of the autonomic nervous system and the lack of a gold standard test panel may contribute to the paucity in data. So far, there is limited information available regarding the responsiveness of the sympathetic nervous system in patients with cardiovascular autonomic neuropathy in general and the effects of glucose lowering therapies on this response in particular. As we studied *rosiglitazone*-induced vascular leakage in the context of autonomic neuropathy **(chapter 3)**,

we were also able to assess the responsiveness of the sympathetic nervous system in patients with type 2 diabetes with and without autonomic neuropathy. We hypothesized that baseline sympathetic tone (α-adrenergic tone) and response to orthostatic stress would be decreased in patients with autonomic neuropathy. In addition, we were interested in the effects of *rosiglitazone*, as there is some evidence that PPAR-γ agonists like *rosiglitazone* may restore the autonomic imbalance in diabetes[55]. **Chapter 6** describes the results of these studies.

Several preclinical studies have shown beneficial effects of *rosiglitazone* on ischaemia-reperfusion injury[56,57], but thus far evidence from human in vivo studies was lacking. In **chapter 7** we tested whether *rosiglitazone* prevents against ischaemia-reperfusion injury in insulin-resistant subjects. In this study we applied annexin A5 scintigraphy[58] to visualize and quantify ischaemia-reperfusion injury in the skeletal muscle of the forearm of insulin-resistant subjects.

A summary of the results is provided in the first part of **chapter 8**. In the second part of this chapter the context and outcomes are put in perspective and used as a framework to construct an overall mechanistic pathway for thiazolidinedione-related fluid accumulation.

As noted, this thesis has applied *rosiglitazone*, to study the relationship between fluid accumulation and thiazolidinediones. There was no scientific guidance for choosing *rosiglitazone* over *pioglitazone*, and we believe the results described in this thesis are applicable to thiazolidinediones in general. Over the last decade, *rosiglitazone* has reached the market, created great expectations but finally has been withdrawn, at least in Europe, because of safety concerns. Although these issues were not the scope of this thesis, they are important in the context of our studies. In addition, *pioglitazone* is still widely used as is *rosiglitazone* in several parts of the world, and as such the relevance of the findings in this thesis has not changed. For this reason, we have included a paragraph in **chapter 8** in which we provide a summary of the dispute over rosiglitazone's cardiovascular unsafety. The last paragraph briefly illustrates the present position of thiazolidinediones and newer PPAR-γ agonists in view of the current drug treatment options for type 2 diabetes.

References

1. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 1997; 20:1183-1197.

2. Cahill GF, Jr. The Banting Memorial Lecture 1971. Physiology of insulin in man. *Diabetes* 1971; 20:785-799.

3. RIVM Volksgezondheid Toekomst Verkenning, Nationaal Kompas Volksgezondheid. In: http://www.nationaalkompas.nl; accessed 3rd of September 2013.

4. IDF Diabetes Atlas 5th edition 2012 update. In: www.idf.org/sites; accessed 3rd of September 2013.

5. Reaven GM. Banting lecture 1988. Role of insulin resistance in human disease. *Diabetes* 1988; 37:1595-1607.

6. Donahue RP, Orchard TJ. Diabetes mellitus and macrovascular complications. An epidemiological perspective. *Diabetes Care* 1992; 15:1141-1155.

7. Gerstein HC. 1997 Glucose: a continuous risk factor for cardiovascular disease. *Diabet Med* 1997; 14 Suppl 3:S25-31.

8. Kannel WB, McGee DL. 1979 Diabetes and cardiovascular disease. The Framingham study. *JAMA* 1979; 241:2035-2038.

9. Gregg EW, Li Y, Wang J, Burrows NR, Ali MK, Rolka D, Williams DE, Geiss L. Changes in diabetes-related complications in the United States, 1990-2010. *N Engl J Med* 2014; 370:1514-1523.

10. Ohkubo Y, Kishikawa H, Araki E, Miyata T, Isami S, Motoyoshi S, Kojima Y, Furuyoshi N, Shichiri M. Intensive insulin therapy prevents the progression of diabetic microvascular complications in Japanese patients with non-insulin-dependent diabetes mellitus: a randomized prospective 6-year study. *Diabetes Res Clin Pract* 1995; 28:103-117.

11. Vinik AI, Maser RE, Mitchell BD, Freeman R. Diabetic autonomic neuropathy. *Diabetes Care* 2003; 26:1553-1579.

12. Vinik AI, Ziegler D. Diabetic cardiovascular autonomic neuropathy. *Circulation* 2007; 115:387-397.

13. NIH Advances and emerging opportunities in diabetes research: a Strategic Planning report of the DMICC. In: http://www2.niddk.nih.gov/nr/rdonlyres/42b8d16a-eceb-4af4-b866-0eda67e5b7ba/0/dsp2011_11_DiabetesComplications_508.pdf; accessed 3rd of September 2013.

14. Blonde L. Current antihyperglycemic treatment guidelines and algorithms for patients with type 2 diabetes mellitus. *Am J Med* 2010; 123:S12-18.

15. Look ARG, Pi-Sunyer X, Blackburn G, Brancati FL, Bray GA, Bright R, Clark JM, Curtis JM, Espeland MA, Foreyt JP, Graves K, Haffner SM, Harrison B, Hill JO, Horton ES, Jakicic J, Jeffery RW, Johnson KC, Kahn S, Kelley DE, Kitabchi AE, Knowler WC, Lewis CE, Maschak-Carey BJ, Montgomery B, Nathan DM, Patricio J, Peters A, Redmon JB, Reeves RS, Ryan DH, Safford M, Van Dorsten B, Wadden TA, Wagenknecht L, Wesche-Thobaben J, Wing RR, Yanovski SZ. Reduction in weight and cardiovascular disease risk factors in individuals with type 2 diabetes: one-year results of the look AHEAD trial. *Diabetes Care* 2007; 30:1374-1383.

16. Nathan DM, Buse JB, Davidson MB, Ferrannini E, Holman RR, Sherwin R, Zinman B, American Diabetes A, European Association for Study of Diabtes. Medical management of hyperglycemia in type 2 diabetes: a consensus algorithm for the initiation and adjustment of therapy: a consensus statement of the American Diabetes Association and the European Association for the Study of Diabetes. *Diabetes Care* 2009; 32:193-203.

17. Kobayashi M. Effects of current therapeutic interventions on insulin resistance. *Diabetes Obes Metab* 1999; 1 Suppl 1:S32-40.

18. Intensive blood-glycaemic control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group. *Lancet* 1998; 352:837-853.

19. Effect of intensive blood-glycaemic control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). UK Prospective Diabetes Study (UKPDS) Group. *Lancet* 1998; 352:854-865.

20. Scarpello JH. Improving survival with metformin: the evidence base today. *Diabet Med* 2003; 29:6S36-43.

21. Turner RC, Cull CA, Frighi V, Holman RR. Glycemic control with diet, sulfonylurea, metformin, or insulin in patients with type 2 diabetes mellitus: progressive requirement for multiple therapies (UKPDS 49). UK Prospective Diabetes Study (UKPDS) Group. *JAMA* 1999; 281:2005-2012

22. Willson TM, Lambert MH, Kliewer SA. Peroxisome proliferator-activated receptor gamma and metabolic disease. *Annu Rev Biochem.* 2001; 70:341-367.

23. Chinetti G, Fruchart JC, Staels B. Peroxisome proliferator-activated receptors (PPARs): nuclear receptors at the crossroads between lipid metabolism and inflammation. *Inflamm Res* 2000; 49:497-505.

24. Day C. Thiazolidinediones: a new class of antidiabetic drugs. *Diabet Med* 1999; 16:179-192

25. Ikeda T. Drug-induced idiosyncratic hepatotoxicity: prevention strategy developed after the troglitazone case. *Drug Metab Pharmacokinet* 2011; 26:60-70.

26. Phillips LS, Grunberger G, Miller E, Patwardhan R, Rappaport EB, Salzman A, Rosiglitazone Clinical Trials Study Group. Once- and twice-daily dosing

with rosiglitazone improves glycemic control in patients with type 2 diabetes. *Diabetes Care* 2001; 24:308-315.

27. Aronoff S, Rosenblatt S, Braithwaite S, Egan JW, Mathisen AL, Schneider RL. Pioglitazone hydrochloride monotherapy improves glycemic control in the treatment of patients with type 2 diabetes: a 6-month randomized placebo-controlled dose-response study. The Pioglitazone 001 Study Group. *Diabetes Care* 2000; 23:1605-1611.

28. Yki-Jarvinen H. Thiazolidinediones. *N Engl J Med* 2004; 351:1106-1118.

29. Unger RH. Lipid overload and overflow: metabolic trauma and the metabolic syndrome. *Trends Endocrinol Metab* 2003; 14:398-403.

30. Nolan JJ, Jones NP, Patwardhan R, Deacon LF. Rosiglitazone taken once daily provides effective glycaemic control in patients with Type 2 diabetes mellitus. *Diabet Med* 2000; 17:287-294

31. Bakris GL, Dole JF, Porter LE, Huang C, Freed MI. Rosiglitazone improves blood pressure in patients with type 2 diabetes Mellitus. *Diabetes* 2000; 49:A96-A96.

32. Fullert S, Schneider F, Haak E, Rau H, Badenhoop K, Lubben G, Usadel KH, Konrad T. Effects of pioglitazone in non-diabetic patients with arterial hypertension: A double-blind, placebo-controlled study. *J Clin Endocr Metab* 2002; 87:5503-5506.

33. Cuzzocrea S, Mazzon E, Dugo L, Patel NSA, Serraino I, Di Paola R, Genovese T, Britti

D, De Maio M, Caputi AP, Thiemermann C. Reduction in the evolution of murine type II collagen-induced arthritis by treatment with rosiglitazone, a ligand of the peroxisome proliferator-activated receptor gamma. *Arthritis Rheum* 2003; 48:3544-3556

34. Wayman NS, Hattori Y, McDonald MC, Mota-Filipe H, Cuzzocrea S, Pisano B, Chatterjee PK, Thiemermann C. Ligands of the peroxisome proliferator-activated receptors (PPAR-gamma and PPAR-alpha) reduce myocardial infarct size. *FASEB J* 2002; 16:1027-1040

35. Diamant M, Heine RJ. Thiazolidinediones in type 2 diabetes mellitus: current clinical evidence. *Drugs* 2003; 63:1373-1405.

36. Nesto RW, Bell D, Bonow RO, Fonseca V, Grundy SM, Horton ES, Le Winter M, Porte D, Semenkovich CF, Smith S, Young LH, Kahn R. Thiazolidinedione use, fluid retention, and congestive heart failure: a consensus statement from the American Heart Association and American Diabetes Association. *Diabetes Care* 2004; 27:256-263.

37. Belcher G, Michel J. Tolerability profile of pioglitazone in combination with a sulphonylurea or metformin in controlled clinical trials. *Diabetes* 2001; 50:A416-A416.

38. Raskin P, Rendell M, Riddle MC, Dole JF, Freed MI, Rosenstock J, Rosiglitazone Clinical Trials Study Group. A randomized trial of rosiglitazone therapy in patients

with inadequately controlled insulin-treated type 2 diabetes. *Diabetes Care* 2001; 24:1226-1232.

39. Colucciello M. Vision loss due to macular oedema induced by rosiglitazone treatment of diabetes mellitus. *Arch Ophthalmol* 2005; 123:1273-1275.

40. Sutton MS, Rendell M, Dandona P, Dole JF, Murphy K, Patwardhan R, Patel J, Freed M. A comparison of the effects of rosiglitazone and glyburide on cardiovascular function and glycemic control in patients with type 2 diabetes. *Diabetes Care* 2002; 25:2058-2064.

41. Wilkinson IB, Webb DJ. Venous occlusion plethysmography in cardiovascular research: methodology and clinical applications. *Br J Clin Pharmacol* 2001; 52:631-646

42. Parving HH, Gyntelberg F. Albumin transcapillary escape rate and plasma volume during long-term beta-adrenergic blockade in essential hypertension. *Scand J Clin Lab Invest* 1973; 32:105-110.

43. Vervoort G, Lutterman JA, Smits P, Berden JH, Wetzels JF. Transcapillary escape rate of albumin is increased and related to haemodynamic changes in normo-albuminuric type 1 diabetic patients. *J Hypertens* 1999; 17:1911-1916.

44. van Hamersvelt HW, Kloke HJ, de Jong DJ, Koene RA, Huysmans FT. Oedema formation with the vasodilators nifedipine and diazoxide: direct local effect or sodium retention? *J Hypertens* 1996; 14:1041-1045.

45. Lukaski HC. Methods for the assessment of human body composition: traditional and new. *Am J Clin Nutr* 1987; 46:537-556.

46. Scherrer U, Randin D, Vollenweider P, Vollenweider L, Nicod P. Nitric oxide release accounts for insulin's vascular effects in humans. *J Clin Invest* 1994; 94:2511-2515.

47. Page MM, Watkins PJ. Provocation of Postural Hypotension by Insulin in Diabetic Autonomic Neuropathy. *Diabetes* 1976; 25:90-95.

48. Hopkins DFC, Cotton SJ, Williams G. Effective Treatment of Insulin-Induced Oedema Using Ephedrine. *Diabetes Care* 1993; 16:1026-1028.

49. Guan YF, Hao CM, Cha DR, Rao R, Lu WD, Kohan DE, Magnuson MA, Redha R, Zhang YH, Breyer MD. Thiazolidinediones expand body fluid volume through PPAR gamma stimulation of ENaC-mediated renal salt absorption. *Nat Med* 2005; 11:861-866

50. Zhang H, Zhang AH, Kohan DE, Nelson RD, Gonzalez FJ, Yang TX. Collecting duct-specific deletion of peroxisome proliferator-activated receptor gamma blocks thiazolidinedione-induced fluid retention. *Proc Natl Acad Sci USA* 2005; 102:9406-9411.

51. Niemeyer NV, Janney LM. Thiazolidinedione-induced oedema. *Pharmacotherapy* 2002; 22:924-929

52. Karalliedde J, Buckingham R, Starkie M, Lorand D, Stewart M, Viberti G.

Effect of various diuretic treatments on rosiglitazone-induced fluid retention. *J Am Soc Nephrol* 2006; 17:3482-3490

53. Gregersen MI, Rawson RA. Blood Volume. *Physiol Rev* 1959; 39:307-342.

54. Nagasawa E, Abe Y, Nishimura J, Yanase T, Nawata H, Muta K. Pivotal role of peroxisome proliferator-activated receptor gamma (PPAR gamma) in regulation of erythroid progenitor cell proliferation and differentiation. *Exp Hematol* 2005; 33:857-864.

55. Petrofsky J, Lee S, Cuneo-Libarona M, Apodaca P. The effect of rosiglitazone on orthostatic tolerance during heat exposure in individuals with type II diabetes. *Diabetes technol Ther* 2007; 9:377-386.

56. Wayman NS, Hattori Y, Mcdonald MC, Mota-Filipe H, Cuzzocrea S, Pisano B, Chatterjee PK, Thiemermann C. Ligands of the peroxisome proliferator-activated receptors (PPAR-gamma and PPAR-alpha) reduce myocardial infarct size. *FASEB J* 2002; 16:1027-1040

57. Khandoudi N, Delerive P, Berrebi-Bertrand I, Buckingham RE, Staels B, Bril A. Rosiglitazone, a peroxisome proliferator-activated receptor-gamma, inhibits the Jun NH2-terminal kinase/activating protein 1 pathway and protects the heart from ischaemie/reperfusion injury. *Diabetes* 2002; 51:1507-1514.

58. Rongen GA, Oyen WJG, Ramakers BP, Riksen NP, Boerman OC, Steinmetz N, Smits P. Annexin A5 scintigraphy of forearm as a novel in vivo model of skeletal muscle preconditioning in humans. *Circulation* 2005; 111:173-178.

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Fluid retention and vascular effects of
rosiglitazone in obese, insulin-resistant,

non-diabetic subjects

Abstract

Objective: The use of thiazolidinedione derivatives is associated with fluid retention, especially when combined with *insulin*. Since thiazolidinediones improve the metabolic effect of insulin, they may also reverse the blunted vascular response to insulin. We hypothesize that improvement of the action of insulin on vascular tone or permeability is the key mechanism of thiazolidinedione-related fluid retention.

Research design and methods: In a randomized, double-blind, placebo-controlled, crossover study in 18 obese, nondiabetic subjects with features of the metabolic syndrome, we investigated the effects of a 12-week treatment with 4 mg *rosiglitazone* twice a day on glucose disposal, haemodynamics (including forearm vasoconstrictor response to nitric oxide (NO) synthase inhibition by N-monomethyl-L-arginine-acetate, L-NMMA), vascular permeability (transcapillary escape rate of albumin) and plasma volume during a hyperinsulinaemia euglycaemic clamp (120 minutes, 120 mU/m^2/min).

Results: As expected, *rosiglitazone* increased glucose infusion rate during clamping. However, neither vascular permeability nor forearm blood flow response to hyperinsulinaemia or L-NMMA were affected by *rosiglitazone*. Compared with placebo, *rosiglitazone* decreased diastolic blood pressure by 5 mmHg (95% CI: 2.35, 6.87, $P = 0.0005$) and increased plasma volume by 255 ml/1.73m^2 (95% CI: 80, 430, $P = 0.007$). Interestingly, the positive effect of *rosiglitazone* on glucose disposal correlated with change in foot volume ($R^2 = 0.53$, $P = 0.001$).

Conclusions: *Rosiglitazone* improved insulin sensitivity but had no effect on NO-dependent vasodilation in the forearm or vascular permeability in obese, insulin-resistant, nondiabetic subjects. As such, thiazolidinedione-related fluid retention was not caused by improvement of the vascular actions of insulin. Nonetheless, *rosiglitazone*-induced improvement in insulin sensitivity appears to be correlated to oedema formation.

Introduction

Thiazolidinedione derivatives improve insulin sensitivity and hence are valuable in the treatment of type 2 diabetes[1]. Important adverse effects are fluid retention and peripheral oedema formation. The precise mechanism(s) of these adverse effects are unclear and probably are multifactorial[2]. Although multiple factors are involved, the existence of an initial trigger or main mechanism could be of clinical importance. In theory, the initial trigger of fluid retention may originate either from kidney, heart or peripheral circulation. As thiazolidinedione treatment is associated with a reduction in blood pressure[3-6], a primary renal mechanism seems unlikely. A primary cardiac origin also seems improbable, since long-term studies with *rosiglitazone* have not revealed any negative effect on myocardial structure or function[7].

The combination of blood pressure reduction, fluid retention and oedema formation is compatible with changes in the peripheral circulation resulting in capillary leakage. This may be induced by certain actions of thiazolidinediones, such as improved insulin-mediated vasodilation, direct vasoactive effects[8], or increased endothelial permeability[9]. Interestingly, the incidence of oedema increases substantially when *rosiglitazone*[10] or *pioglitazone*[11] are used in combination with *insulin*. A number of findings suggest that the tendency of fluid retention is coupled to the effect of thiazolidinediones on the metabolic actions of insulin. For example, both glycaemic efficacy and oedema formation are dose-dependent features of thiazolidinedione therapy[10]. Furthermore, peroxisome proliferator-activated receptor-γ (PPAR-γ agonists) with more potent glucose-lowering effects seem to be associated with a higher incidence of oedema formation[12]. Besides a metabolic effect, insulin has also important vascular properties on several sites of the vascular tree. For instance, insulin increases vascular permeability[13] and induces vasodilation in resistance arteries[14], venules[15], and precapillary arteriolae[16], thereby inducing capillary recruitment[17] and resulting in a decrease in capillary resistance. Acute hyperinsulinaemia has been reported to increase the transcapillary escape rate of albumin[13], consistent with a direct effect of insulin on arteries and capillaries promoting vascular leakage and therefore oedema formation. If thiazolidinediones augment both the metabolic and vascular effects of insulin, the effect on glycaemic control and fluid retention would indeed be coupled.

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In the present study, we investigated whether *rosiglitazone* treatment, besides improving the metabolic action of insulin, can also reverse the blunted vasodilator response to insulin[18] and/or change vascular permeability in insulin-resistant subjects. To avoid confounding by improved glycaemic control, we studied nondiabetic subjects with characteristics of the metabolic syndrome.

Research design and method

The study population consisted of 18 healthy, obese volunteers (BMI between 27 and 36 kg/m^2, age: 30-65 years) with either two or more features of the metabolic syndrome as defined by the National Cholesterol Education Program[19] or one of these features in combination with a first-degree relative having type 2 diabetes. Subjects were not eligible for inclusion if they had fasting plasma glucose > 7.0 mmol/L, glycosylated haemoglobin (HbA$_{1c}$) >6.5%, if they used non-steroidal anti-inflammatory drugs, fibrates, anticoagulants, antihypertensives, any investigational drug or a PPAR-γ agonist, or if they had just started lipid-lowering therapy. Additional exclusion criteria were: blood pressure exceeding 160/100 mmHg, unstable or severe angina or congestive heart failure, presence of clinically significant hepatic or renal disease or anaemia, pregnancy, lactation, lack of appropriate contraception for women of child-bearing potential, and alcohol or drug abuse. Study participants were selected by advertisement, received a payment and gave written informed consent. This study was approved by the hospital ethics committee and was performed according to good clinical practice guidelines.

Within six weeks after screening, participants were randomly assigned to either *rosiglitazone* (4 mg twice daily), or placebo for 12 weeks in a double-blind, crossover design. The primary end points of the study were measured at the end of each 12-week treatment period, and we considered this long enough to avoid a carryover effect. Therefore, we decided to not include an extra washout period between both treatment periods. At week 2 and 6 of each treatment period, adverse events and pill compliance were recorded. Physical examination was performed, foot volume was measured, and safety chemical, hematological, and glycaemic profiles were determined. At the end of each 12-week treatment period

the haemodynamic and metabolic effects of insulin were quantified during a hyperinsulinaemia euglycaemic clamp procedure. During this test, vascular permeability was assessed by measurement of the transcapillary escape rate of labeled albumin (TERalb). Two weeks after the final treatment period there was a follow-up visit. Participants were strictly advised to maintain their diet and not to change lifestyle.

Protocol experimental day

After an overnight fast of at least 10 hours the subject entered a quiet temperature-controlled room (23-24 ºC) at 8:00 A.M. A 20-gauge catheter (Angiocath, Becton Dickinson, Sandy, UT) was inserted into the left brachial artery under local anaesthesia (0.3-0.4 ml lidocaine HCl; 20 mg/ml), connected via an arterial pressure monitoring line to a Hewlett Packard 78353B Monitor and kept patent with saline and heparin (0.9% NaCl and 2units/ml heparin; NaCl, 3ml/h). This catheter was used for both intra-arterial drug infusion (automatic syringe infusion pump, type STC-521, Terumo, Tokyo, Japan) and for blood sampling. One venous catheter (Venflon, 20 G, 32 mm) was inserted antegrade into a deep arm vein for the infusion of *insulin* and glucose.

After a 30-minute equilibration period, the intra-arterial pressure wave signal was recorded for 5 minutes to calculate cardiac output and systemic vascular resistance using "model flow analysis"[20]. Subsequently, forearm blood flow (FBF)[21,22] was measured simultaneously in the experimental and control arm using mercury-in-Silastic strain-gauge venous occlusion plethysmography. The FBF of the contralateral arm was used as a time-control value to observe systemic effects. After these baseline measurements, the hyperinsulinaemia- euglycaemic clamp[23,24] was started. *Insulin* (Actrapid, Novo-Nordisk, Denmark) was infused intravenously at a dose of 720 pmol/m^2/min (120 mU/m^2/min). *Insulin* (50 units/ml) was diluted in 47.5 ml of 0.9% NaCl with the addition of 2 ml of the subject's blood to a concentration of 1unit/ml. Euglycaemia was maintained at 5.0 mmol/L by a variable infusion of 20% glucose solution, adjusted at 5-min intervals according to arterial glucose measurements. Glucose infusion rate (GIR) was defined as the GIR during the last 30 min of the clamp expressed in micromoles per kilogram per minute[25]. Potassium chloride (1 mmol/ml) was infused to prevent hypokalaemia.

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Throughout the clamp procedure, FBF measurements were performed, intra-arterial pulse wave was recorded and ^{125}I-albumin was injected for calculation of TERalb and plasma volume. Moreover, blood samples for insulin measurement were drawn. After two hours of hyperinsulinaemia-euglycaemic clamping, the specific nitric oxide (NO)-synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) was infused into the brachial artery at a rate of 0.4 mg/min/dL and the subsequent vasoconstrictor response was measured. L-NMMA (100 mg, Clinalpha, Läufelfingen, Switzerland) solution was freshly made with 25 ml 0.9% NaCl immediately before use. After the experiment was finished, glucose infusion was continued and the participants were served a carbohydrate-rich meal in order to avoid hypoglycaemic events after the test.

TERalb

At 60 minutes, an additional venous needle (BD Valu-set, 0.6 x 20 mm) was inserted and 2-4 μCi ^{125}I-albumin (Shering Nederland BV, Weesp, the Netherlands) was given as an intravenous bolus injection. During the next 60 minutes, seven plasma samples were collected from the arterial line for radioactivity measurements. Plasma volume and TERalb were calculated using the following formulas[26,27]:

Plasma volume (PV) (milliliters)/ 1.73 m^2 = [counts per minute injected / counts per minute t=0/milliliters] / surface (square meters)/ 1.73 m^2

TERalb = fraction of the intravascular mass of albumin leaving the vascular system per hour.

TERalb = [1- e$^{3600 \times slope}$] x 100% (%/h).

Analytical methods

Arterial plasma glucose was measured in duplicate with the glucose oxidation method (Beckman Glucose Analyzer 2, Beckman Instruments Inc, Fullerton, CA). Atrial natriuretic peptide (ANP) concentrations were analyzed by radioimmunoassay after cartridge extraction. Insulin levels were measured using the Perkin-Elmer AutoDELFIA Insulin kit with an automatic immunoassay system. C-peptide was analyzed with C-peptide double-antibody (^{125}I) radioimmunoassay kit.

Control visits

During all control visits (0, 2, 6, 14 and 18 weeks), blood pressure and heart rate were assessed after the subject had been sitting quietly for at least 5 minutes. Blood pressure was measured by auscultation method with the nondominant arm supported at heart level. Moreover, foot volume was assessed using the water displacement method which measures volume displacement in an indirect way with an electronic balance (coefficient of variation is 0.30%)[28]. The balance recorded the force necessary for a standardized immersion of the foot, which depends solely on the volume of the foot (Archimedes principle). The mean temperature of the water was 22.9 ºC and did not differ >1 ºC between visits of one subject.

Statistical analysis

The study was powered (90%) to detect a 50% increase in percentage change in FBF between the treatment groups with 16 evaluable subjects. All significance tests and confidence intervals (CI) were two-sided and the overall type I error was 5%. Descriptive statistics of population characteristics are presented as means ± SD. The comparison between *rosiglitazone* and placebo was conducted within each subject. The response was measured at the end of each treatment period, assuming any carryover from the first treatment period should be washed out. All data were analyzed using analysis of variance (ANOVA), with adjustment for period if applicable. We used paired Student's t-test or Wilcoxon rank test, if appropriate, and ANOVA repeated measures for sequential data to derive P-values. Treatment effects are presented as means ± SD or, for relative changes, as mean percentage change derived from the geometric mean with CIs. Correlations were calculated using Pearson's or Spearman's correlation tests if appropriate. All statistical analyses were performed using the SPSS personal computer software package.

Results

Included subjects represented an overweight (98±12 kg; BMI 32±3 kg/m^2), middle-aged (46±9 years) population of 11 men and 7 women. Obvious features of the metabolic syndrome present in our population were increased waist circumference (109±7 cm), diastolic blood pressure (DBP) (93±5 mmHg) and plasma triglyceride levels (1.9 ± 0.9 mmol/l). Other characteristics were systolic

blood pressure (SBP) (134 ± 10 mmHg), plasma total cholesterol (5.7 ± 1.0 mmol/l), plasma HDL levels (1.2 ± 0.3 mmol/l), plasma fasting plasma glucose (5.5 ± 0.4 mmol/l), and HbA_{1c} (5.50 ± 0.33%). Ten subjects were randomized to receive placebo first, and the remaining 8 subjects received *rosiglitazone* first. All subjects completed both treatment regimens. Drug compliance, measured by tablet counting, was excellent. Subjects reported only mild side effects, equally distributed between both treatments. One subject developed moderate oedema during *rosiglitazone* treatment.

Effect of *rosiglitazone* on insulin's metabolic actions
During *rosiglitazone*, the fasting values of plasma glucose (0.28 mmol/l [95% CI 0.05-0.50] *P* = 0.02), insulin and C-peptide concentrations (14 pmol/l [2-26] *P* < 0.05 and 0.13 nmol/l [0.01-0.25] *P* < 0.05, respectively) were significantly decreased as compared with placebo. During the final 30 minutes of the clamp procedure, blood glucose values were equal during *rosiglitazone* and placebo treatment (4.96 ± 0.12 and 4.96 ± 0.15 mmol/l, respectively) and stable (coefficients of variation; 4.36 ± 2.08 and 4.15 ± 1.96 %, respectively). Also, steady-state plasma insulin concentrations were similar (1,664 ± 533 pmol/l versus 1,795 ± 688 pmol/l *P* = 0.29). Insulin sensitivity, measured by GIR, significantly improved during *rosiglitazone* (39.6±9.2 μmol/kg/min) treatment compared to placebo (33.7 ± 11.7 μmol/kg/min) resulting in a period-adjusted treatment effect of 5.26 μmol/kg/min (95% CI 1.68-8.83, *P* = 0.007).

Effect of *rosiglitazone* on insulin's vascular actions
Hyperinsulinaemia (≈1,700 pmol/L) did not change FBF during either treatment, consistent with persistent vascular insulin resistance (treatment effect for *rosiglitazone*; −8.2% [95% CI-27.2 to 8.0], *P* = 0.318) (Figure 1A).

During L-NMMA infusion, blood flow decreased, but the reductions were similar during *rosiglitazone* and placebo treatment (−22.9% [-13.5 to -31.3] vs. −25.7% [-18.8 to -31.8], NS) (Figure 1A). *Rosiglitazone* had no effect on vascular permeability measured with TERalb (+ 0.27 %/hr [-1.21 to 1.75] *P* = 0.71) (Figure 1B)

Insulin infusion reduced systemic vascular resistance during placebo treatment (-6.2% [95% CI -9.1 to -3.2], *P* < 0.001) and not during *rosiglitazone* treatment (-4.5% [-10.2 to 1.6], *P* = 0.14), but these changes did not differ significantly between treatments (0.4% [-5.5 to 6.7], *P* = 0.68). Similarly, *insulin* increased cardiac output, but again these changes were not different between both treatments.

Effect of rosiglitazone on blood pressure
DBP was reduced during *rosiglitazone* treatment whether measured via ausculatory or intra-arterial methods (ausculatory -5 mmHg [95% CI -6.87 to -2.35], *P* = 0.0005; intra-arterially – 2 mmHg [-3.6 to -1.6], *P* = 0.03) (Figure 1C). *Rosiglitazone* seemed to reduce the calculated systemic vascular resistance, but the difference in this measure failed to reach statistical significance (-3.2% [-9.6 to 3.7], *P* = 0.28).

Effect of *rosiglitazone* on fluid compartments
During *rosiglitazone*, plasma volume increased by 255 ml/1.73m^2 (95% CI 80-430) (*P* = 0.007) compared with placebo (Figure 1D). Haematocrit decreased accordingly (−0.019 l/l [-0.03 to -0.01], *P* = 0.002). We observed an increase in plasma ANP with *rosiglitazone*, (12.1 pg/mL [0.7-23.4] *P* = 0.039; *rosiglitazone* vs. placebo). *Rosiglitazone* did not induce an increase in foot volume over placebo (0.37% [-0.80 to 1.50], NS). However, a period effect was detected, with greater relative differences from baseline during the second period, probably related to a seasonal increase in outside temperature throughout the study. Post hoc analyses revealed a significant correlation between changes in foot volume and GIR (Figure 2) (R^2 = 0.53, *P* = 0.001) and trends between changes in GIR and TERalb and between changes in GIR and DBP (R^2 = 0.23, *P* = 0.07 and R^2 = 0.15, *P* = 0.11, respective).

Characterization of subject with thiazolidinedione-induced oedema
One subject developed moderate oedema and showed an increase in body weight of 3.7 kg, in plasma volume of 544 ml/1.73 m^2, and in of foot volume 4.6% during *rosiglitazone* treatment. Compared to the whole study population this subject had an equivalent treatment response with regard to insulin-mediated vasodilation (-9% vs. −7.6% [95% CI -21.4 to+8.7], but a more pronounced response in insulin sensitivity (15.8% vs. 5.3 [1.7-8.8].

Figure 1:

A) Mean percentage change in FBF (experimental arm [mean and CI] during hyperinsulinaemia clamp and during subsequent infusion of L-NMMA into the brachial artery. There was no difference in response between placebo and *rosiglitazone*.

B) Intra-subject changes in transcapillary escape rate of albumin (means ± SE). There was no difference in vascular permeability between the two treatments (9.14 ± 0.52 vs. 9.41 ± 0.51%).

C) Absolute change in diastolic blood pressure (means ± SE) from the start of each treatment period. *Rosiglitazone* clearly reduced diastolic blood pressure.

D) Intrasubject changes in plasma volume adjusted for body surface (means ± SE). During *rosiglitazone* treatment the mean increase was 255 ml/1.73m^2 compared to placebo.

[n=14: Four subjects were excluded for this analysis. In one patient, no ^{125}I-albumin was available; in two cases the correlation between (ln)plasma radioactivity and time did not exceed 0.85; and in one case, we derived a nonphysiologic high plasma volume.]
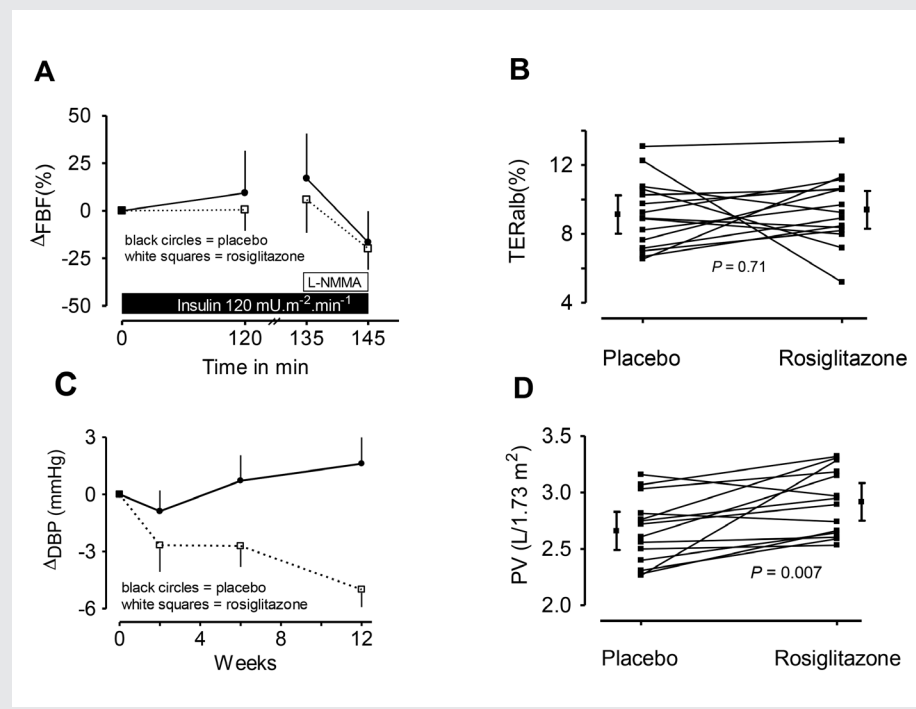
Figure 2: Plot of correlation between differences in foot volume and glucose infusion rate between *rosiglitazone* and placebo treatment. This correlation is not driven only by the subject with oedema (□), n = 18.
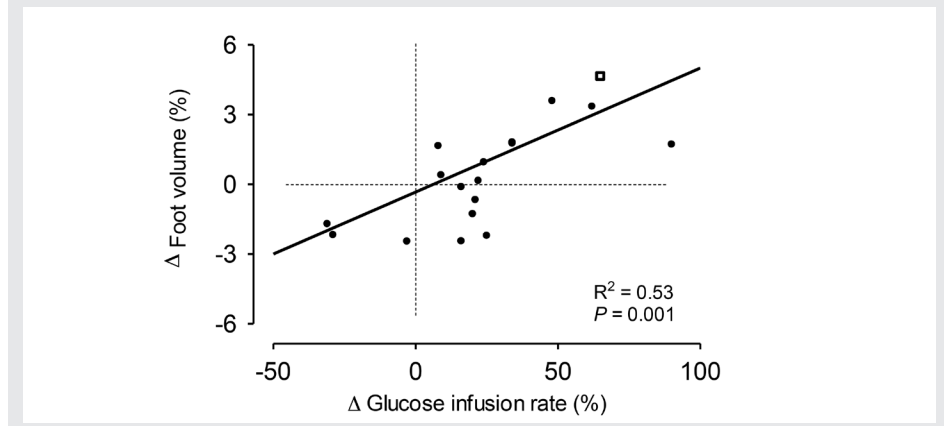
Conclusions

The first principal observation of the present study is that *rosiglitazone*, while improving the metabolic action of insulin, did neither affect vascular permeability nor the NO-dependent vascular responses to insulin. The second is that *rosiglitazone* significantly increased plasma volume and lowered diastolic blood pressure. Taken together, these findings do not support the hypothesis that potentiation of the vascular effects of insulin, being either vasodilation or increased vascular permeability, are the specific mechanism of thiazolidinedione-induced fluid retention. Nevertheless, since the change in insulin-induced glucose uptake appeared to be related to the change in foot volume, our study does support some relationship between the effects of *rosiglitazone* on glucose uptake and interstitial fluid content.

In this study, *rosiglitazone* did not affect the vascular actions of insulin. In contrast, Paradisi et al. found that *troglitazone* was able to reverse the blunted insulin-mediated vasodilation in subjects with polycystic ovary syndrome[29]. There are two important differences between Paradisi's study and ours, 1) the investigated population and 2) measurement of leg blood flow, while we measured

FBF. Since previous studies have shown that the vasodilator response to acute hyperinsulinaemia did not differ between the leg and the forearm vascular bed, our data may be extrapolated to the leg[30]. Someone might still argue that *rosiglitazone* could exert a different effect on the response to insulin in forearm versus leg. However, in agreement with our forearm observations, we did not find any treatment effect of *rosiglitazone* on calculated total peripheral vascular resistance during hyperinsulinaemia.

Two other studies are in complete agreement with our present findings. In a previous study, we did not find an effect of *troglitazone* on insulin-induced changes in FBF in obese subjects[23], neither did Natali et al. in patients with type 2 diabetes[31]. In both studies a lower *insulin* dose (60 and 40 mU/m²/min) was used. As such, the results of the present study confirm previous reports in obese or diabetic subjects using forearm measurements and extend it to high insulin infusion rates. Since our data are in contrast with observations in the polycystic ovary syndrome, the vascular mechanism of action of *rosiglitazone* may be different in this particular form of insulin resistance.

Our observation that *rosiglitazone* did not reverse insulin-mediated vasodilation seems to conflict with published reports showing a beneficial effect of *rosiglitazone* on NO-dependent vasodilation (and hence on endothelial function) measured with acetylcholine infusion. For example, Pistrosch et al. reported an increased vasodilator response to either acetylcholine alone or acetylcholine combined with locally infused insulin in *rosiglitazone*-treated patients compared to *nateglinide*-treated patients[32]. Likewise, Natali et al., found that *rosiglitazone* improved the vasodilator responses to acetylcholine but not to insulin in patients with type 2 diabetes[31]. Of note, Natali did not find any effect of *rosiglitazone* on the response to L-NMMA infusion, which is perfectly in line with our observations. It appears that insulin's activation of the NO-pathway is not strong enough to disclose *rosiglitazone's* favourable effects on endothelium and on insulin-mediated vasodilation and that either a large improvement of insulin sensitivity (Pistrosch et al. 85%) or additional infusion of acetylcholine is needed.

This was the first human *in vivo* study investigating the influence of *rosiglitazone*-treatment on TERalb. The finding that *rosiglitazone* did not change TERalb seems in contrast with an *in vitro* study with human pulmonary artery endothelial cells[9], but the discrepancy can be explained by clear differences in design and methodology. The absolute rate of TERalb in our population appeared to be rather high[33], although Pedrinelli et al. reported a similar rate (9.6%) in subjects with essential hypertension[34], and Hilsted et al. found TERalb rate of 9.9% in normal individuals during an hyperinsulinaemia-euglycaemic clamp[13,34]. Therefore, the observed high TERalb could either be the resultant of features of the metabolic syndrome such as hypertension or be due to the hyperinsulinaemia state. Please note that TERalb is a measure of total body protein permeability. As such, we cannot exclude from these data that *rosiglitazone* affects total body fluid filtration.

In the present study, *rosiglitazone* resulted in a decrease in DBP (but not SBP) when measured intra-arterially or ausculatory, which is in agreement with another study[31]. As DBP is primarily determined by peripheral resistance, the reduction in blood pressure during *rosiglitazone* treatment could be caused by systemic vasodilation. In support of this notion is our finding that the systemic vascular resistance was lower during *rosiglitazone* treatment before the start of the clamp. Interestingly, Shargorodsky et al. did report that *rosiglitazone* lowers systemic vascular resistance[35]. Apart from a potentiation of insulin effect, *rosiglitazone* may induce vasodilation by inhibition of calcium currents[36,37], reduction of endothelin-1 secretion[38], or down regulation of the sympathetic nervous system[39].

Several studies have reported a decrease in haematocrit in response to thiazolidinedione treatment, which has been interpreted as the result of an increase in plasma volume[40], but so far only one other study combined haematocrit with directly derived plasma volume measurements[41]. Indeed haematocrit decreased and plasma volume increased in our study, but, interestingly, we did not find a correlation between changes in haematocrit and changes in plasma volume. Also the observed elevation of plasma ANP levels during *rosiglitazone* treatment is consistent with plasma volume expansion. In healthy subjects, *rosiglitazone* increased plasma volume by only 1.8 ml/kg after 8 weeks treatment[42]. Apparently, the fluid-retaining effect of *rosiglitazone* is more pronounced in insulin-resistant subjects.

As there was no association between changes in metabolic and vascular actions of insulin, our results do not support the view that insulin-induced glucose disposal is the consequence of enhanced total muscle blood flow[18]. However, it should be acknowledged that opposing views exist in the literature, whether or not the vasodilator effects of (physiological levels of) insulin contribute to the effect of insulin on tissue glucose uptake[17]. The emerging view is that insulin may increase capillary recruitment and increase tissue perfusion, without necessarily increasing total blood flow[43]. This view could be the explanation for the correlation between change in foot volume and the metabolic but not vascular action of insulin, as found by post hoc analysis. Capillary recruitment will reduce systemic vascular resistance, and increase glucose transport and fluid filtration. Therefore, capillary recruitment couples oedema formation, reduced blood pressure and insulin sensitization. In line with this reasoning, Bakris et al. reported a correlation between the reduction of diastolic blood pressure and the improvement in insulin sensitivity during *rosiglitazone* treatment[44].

Altogether, our findings do not support the hypothesis that changes in the vascular effect of insulin, being either vasodilation measured in the forearm or increased vascular permeability, are *the* specific mechanism of thiazolidinedione-induced fluid retention. Although this conclusion is valid at the level of the whole study population, it also appears to be true for the single case with oedema.

This study included an insulin-resistant nondiabetic population, which enabled us to investigate whether *rosiglitazone* can reverse the blunted vascular response of insulin, without any interference from changes in glycaemic control. For example hyperglycaemia in itself could additionally impair endothelial function[45]. The main outcome of the present study, being no correlation between fluid retention (plasma volume) and changes in the vascular action of insulin, probably holds true for a diabetic population as well. The incidence of oedema may be expected to be higher in a diabetic population, for example because of autonomic neuropathy (sympathetic nervous system dysfunction) or because of heart failure. As such, in a diabetic population the correlation between improved insulin sensitivity and oedema formation could be less strong due to potential confounders.

The hypothetical framework of the present study lends heavily on capillary recruitment being the primary cause of oedema formation, but the pathogenesis of fluid retention is probably multifactorial[2]. At the moment, there are controversial reports about the potential of PPAR-γ agonists to stimulate the epithelial sodium channel (ENaC), which could play an important role in thiazolidinedione-related fluid retention[46-48].

In summary, this study provides no support for the view that thiazolidinediones increase transcapillary leakage of fluid as a result of either the augmentation of the NO-mediated vasodilator response to insulin or an increase of capillary permeability. The correlation between metabolic insulin sensitivity and oedema formation may point to an alternative mechanism of thiazolidinedione-related oedema formation, possibly increased capillary recruitment.

Acknowledgments

References

1. Yki-Jarvinen. H Thiazolidinediones. *N Engl J Med* 2004; 351:1106-1118.

2. Nesto RW, Bell D, Bonow RO, Fonseca V, Grundy SM, Horton ES, Le Winter M, Porte D, Semenkovich CF, Smith S, Young LH, Kahn R. Thiazolidinedione use, fluid retention, and congestive heart failure: a consensus statement from the American Heart Association and American Diabetes Association. October 7, 2003. *Circulation* 2003; 108:2941-2948.

3. Wang TD, Chen WJ, Lin JW, Chen MF, Lee YT. Effects of rosiglitazone on endothelial function, C-reactive protein, and components of the metabolic syndrome in nondiabetic patients with the metabolic syndrome. *Am J Cardiol* 2004 ; 93:362-365.

4. Raskin P, Rendell M, Riddle MC, Dole JF, Freed MI, Rosenstock J. A randomized trial of rosiglitazone therapy in patients with inadequately controlled insulin-treated type 2 diabetes. *Diabetes Care* 2001; 24:1226-1232.

5. Honisett SY, Stojanovska L, Sudhir K, Kingwell BA, Dawood T, Komesaroff PA. Rosiglitazone lowers blood pressure and increases arterial compliance in postmenopausal women with type 2 diabetes. *Diabetes Care* 2003; 26:3194-3195.

6. Fullert S, Schneider F, Haak E, Rau H, Badenhoop K, Lubben G, Usadel KH, Konrad T. Effects of pioglitazone in nondiabetic patients with arterial hypertension: a double-blind, placebo-controlled study. *J Clin Endocrinol Metab* 2002; 87:5503-5506.

7. St John SM, Rendell M, Dandona P, Dole JF, Murphy K, Patwardhan R, Patel J, Freed M. A comparison of the effects of rosiglitazone and glyburide on cardiovascular function and glycemic control in patients with type 2 diabetes. *Diabetes Care* 2002; 25:2058-2064.

8. Walker AB, Naderali EK, Chattington PD, Buckingham RE, Williams G. Differential vasoactive effects of the insulin sensitizers rosiglitazone (BRL 49653) and troglitazone on human small arteries in vitro. *Diabetes* 1998; 47:810-814.

9. Idris I, Gray S, Donnelly R. Rosiglitazone and pulmonary oedema: an acute dose-dependent effect on human endothelial cell permeability. *Diabetologia* 2003; 46:288-290.

10. Phillips LS, Grunberger G, Miller E, Patwardhan R, Rappaport EB, Salzman. A Once- and twice-daily dosing with rosiglitazone improves glycemic control in patients with type 2 diabetes. *Diabetes Care* 2001; 24:308-315.

11. Rubin C, Egan J, Schneider R. Combination therapy with pioglitazone and insulin in patients with type 2 diabetes. *Diabetes* 1999; 48:A110.

12. Saad MF, Greco S, Osei K, Lewin AJ, Edwards C, Nunez M, Reinhardt RR. Ragaglitazar improves glycemic control and lipid profile in type 2 diabetic subjects: a 12-week, double-blind, placebo-controlled dose-ranging study with an open pioglitazone arm. *Diabetes Care* 2004; 27:1324-1329.

13. Hilsted J, Christensen NJ, Larsen S. Effect of catecholamines and insulin on plasma volume and intravascular mass of albumin in man. *Clin Sci (Lond)* 1989; 77:149-155.

14. Scherrer U, Randin D, Vollenweider P, Vollenweider L, Nicod P Nitric oxide release accounts for insulin's vascular effects in humans. *J Clin Invest* 1994; 94:2511-2515.

15. Bouskela E, Cyrino FZ, Wiernsperger N. Effects of insulin and the combination of insulin plus metformin (glucophage) on microvascular reactivity in control and diabetic hamsters. *Angiology* 1997; 48:503-514.

16. Zhang L, Vincent MA, Richards SM, Clerk LH, Rattigan S, Clark MG, Barrett EJ. Insulin sensitivity of muscle capillary recruitment in vivo. *Diabetes* 2004; 53:447-453.

17. Coggins M, Lindner J, Rattigan S, Jahn L, Fasy E, Kaul S, Barrett E. Physiologic hyperinsulinemia enhances human skeletal muscle perfusion by capillary recruitment. *Diabetes* 2001; 50:2682-2690.

18. Laakso M, Edelman SV, Brechtel G, Baron AD Impaired insulin-mediated skeletal muscle blood flow in patients with NIDDM. *Diabetes* 1992; 41:1076-1083.

19. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *JAMA* 2001; 285:2486-2497.

20. Timmers HJ, Wieling W, Soetekouw PM, Bleijenberg G, Van Der Meer JW, Lenders JW. Haemodynamic and neurohumoral responses to head-up tilt in patients with chronic fatigue syndrome. *Clin Auton Res* 2002; 12:273-280.

21. Tack CJ, Smits P, Willemsen JJ, Lenders JW, Thien T, Lutterman JA Effects of insulin on vascular tone and sympathetic nervous system in NIDDM. *Diabetes* 1996; 45:15-22.

22. Whitney RJ. The measurement of volume changes in human limbs. *J Physiol* 1953;121:1-27.

23. Tack CJ, Ong MK, Lutterman JA, Smits P. Insulin-induced vasodilatation and endothelial function in obesity/insulin resistance. Effects of troglitazone. *Diabetologia* 1998; 41:569-576.

24. Defronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol* 1979; 237:E214-E223.

25. Bergman RN, Finegood DT, Ader M. Assessment of insulin sensitivity in vivo. *Endocr Rev* 1985; 6:45-86.

26. Parving HH, Gyntelberg F. Albumin transcapillary escape rate and plasma volume during long-term beta-adrenergic blockade in essential hypertension. *Scand J Clin Lab Invest* 1973; 32:105-110.

27. Vervoort G, Lutterman JA, Smits P, Berden JH, Wetzels JF. Transcapillary escape rate of albumin is increased and related to haemodynamic changes in normo-albuminuric type 1 diabetic patients. J *Hypertens* 1999; 17:1911-1916.

28. Van Hamersvelt HW, Kloke HJ, de Jong DJ, Koene RA, Huysmans FT. Oedema formation with the vasodilators nifedipine and diazoxide: direct local effect or sodium retention? *J Hypertens* 1996; 14:1041-1045.

29. Paradisi G, Steinberg HO, Shepard MK, Hook G, Baron AD. Troglitazone therapy improves endothelial function to near normal levels in women with polycystic ovary syndrome. *J Clin Endocrinol Metab* 2003; 88:576-580.

30. Yki-Jarvinen H, Utriainen T. Insulin-induced vasodilatation: physiology or pharmacology? *Diabetologia* 1998; 41:369-379.

31. Natali A, Baldeweg S, Toschi E, Capaldo B, Barbaro D, Gastaldelli A, Yudkin JS, Ferrannini E. Vascular effects of improving metabolic control with metformin or rosiglitazone in type 2 diabetes. *Diabetes Care* 2004; 27:1349-1357.

32. Pistrosch F, Passauer J, Fischer S, Fuecker K, Hanefeld M, Gross P. In type 2 diabetes, rosiglitazone therapy for insulin resistance ameliorates endothelial dysfunction independent of glycaemic control . *Diabetes Care* 2004; 27:484-490.

33. Nielsen S, Hermansen K, Rasmussen OW, Thomsen C, Mogensen CE. Urinary albumin excretion rate and 24-h ambulatory blood pressure in NIDDM with microalbuminuria: effects of a monounsaturated-enriched diet. *Diabetologia* 1995; 38:1069-1075.

34. Pedrinelli R, Penno G, Dell'Omo G, Bandinelli S, Giorgi D, Di B, V, Navalesi R, Mariani M. Microalbuminuria and transcapillary albumin leakage in essential hypertension. *Hypertension* 1999; 34:491-495.

35. Shargorodsky M, Wainstein J, Gavish D, Leibovitz E, Matas Z, Zimlichman R. Treatment with rosiglitazone reduces hyperinsulinemia and improves arterial elasticity in patients with type 2 diabetes mellitus. *Am J Hypertens* 2003; 16:617-622.

36. Song J, Walsh MF, Igwe R, Ram JL, Barazi M, Dominguez LJ, Sowers JR. Troglitazone reduces contraction by inhibition of vascular smooth muscle cell Ca2+ currents and not endothelial nitric oxide production. *Diabetes* 1997; 46:659-664.

37. Buchanan TA, Meehan WP, Jeng YY, Yang D, Chan TM, Nadler JL, Scott S, Rude RK, Hsueh WA. Blood pressure lowering by pioglitazone. Evidence for a direct vascular effect. *J Clin Invest* 1995; 96:354-360.

38. Martin-Nizard F, Furman C, Delerive P, Kandoussi A, Fruchart JC, Staels B, Duriez P. Peroxisome proliferator-activated receptor activators inhibit oxidized low-density lipoprotein-induced endothelin-1 secretion in endothelial cells. *J Cardiovasc Pharmacol* 2002; 40:822-831.

39. Yosefy C, Magen E, Kiselevich A, Priluk R, London D, Volchek L, Viskoper RJ, Jr. Rosiglitazone improves, while Glibenclamide worsens blood pressure control in treated hypertensive diabetic and dyslipidemic subjects via modulation of insulin resistance and sympathetic activity. *J Cardiovasc Pharmacol* 2004; 44:215-222.

40. Lebovitz HE. Differentiating members of the thiazolidinedione class: a focus on safety. *Diabetes Metab Res Rev 18 Suppl* 2002; 2:S23-S29.

41. Young MM, Squassante L, Wemer J, van Marle SP, Dogterom P, Jonkman JH. Troglitazone has no effect on red cell mass or other erythropoietic parameters. *Eur J Clin Pharmacol* 1999; 55:101-104.

42. Avandia (rosiglitazone maleate) [package insert]. 2000. Philidelphia, Pa: GlaxoSmithkline Pharmaceuticals.

43. Clerk LH, Vincent MA, Lindner JR, Clark MG, Rattigan S, Barrett EJ. The vasodilatory actions of insulin on resistance and terminal arterioles and their impact on muscle glucose uptake. *Diabetes Metab Res Rev* 2004; 20:3-12.

44. Bakris G, Viberti G, Weston WM, Heise M, Porter LE, Freed MI. Rosiglitazone reduces urinary albumin excretion in type II diabetes. *J Hum Hypertens* 2003;17:7-12.

45. Makimattila S, Virkamaki A, Groop PH, Cockcroft J, Utriainen T, Fagerudd J, Yki-Jarvinen H. Chronic hyperglycemia impairs endothelial function and insulin sensitivity via different mechanisms in insulin-dependent diabetes mellitus. *Circulation* 1996; 94:1276-1282.

46. Hong G, Lockhart A, Davis B, Rahmoune H, Baker S, Ye L, Thompson P, Shou Y, O'Shaughnessy K, Ronco P, Brown J . PPARgamma activation enhances cell surface ENaCalpha via up-regulation of SGK1 in human collecting duct cells. *FASEB J* 2003; 17:1966-1968.

47. Guan Y, Hao C, Cha DR, Rao R, Lu W, Kohan DE, Magnuson MA, Redha R, Zhang Y, Breyer MD. Thiazolidinediones expand body fluid volume through PPARgamma stimulation of ENaC-mediated renal salt absorption. *Nat Med* 2005; 11:861-866.

48. Nofziger C, Chen L, Shane MA, Smith CD, Brown KK, Blazer-Yost BL. PPARgamma agonists do not directly enhance basal or insulin-stimulated Na(+) transport via the epithelial Na(+) channel. *Pflugers Arch* 2005

3

Autonomic neuropathy predisposes to
rosiglitazone-induced vascular leakage in
insulin-treated patients with type 2 diabetes:
a randomized-controlled trial on
thiazolidinedione-induced vascular leakage

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Abstract

Aims/hypothesis: The mechanism of fluid-related complications caused by thiazolidinedione-derivatives is unclear. One potential mechanism is thiazolidinedione-induced arterial vasodilation, which results in vascular leakage and a fall in blood pressure, normally counterbalanced by sympathetic activation and subsequent renal fluid retention. We hypothesized that thiazolidinedione-induced vascular leakage will be particularly prominent in patients with autonomic neuropathy.

Methods: We conducted a randomized, double-blind, placebo-controlled, parallel study in 40 patients with type 2 diabetes on insulin-treatment recruited from a university medical centre. The randomization was performed by a central office using a randomization schedule. Both treatment groups, placebo (n = 21) and *rosiglitazone* (n = 19), were stratified for sex and level of autonomic neuropathy as assessed by Ewing score (<2.5 or 2.5). We investigated the effects of 16-week treatment with *rosiglitazone* 4 mg twice daily on vascular leakage (transcapillary escape rate of albumin, TERalb), body weight, extracellular volume and plasma volume.

Results: Thirty-nine patients were included in the analysis. In the patients with high Ewing scores (n = 16), *rosiglitazone* increased TERalb significantly (ΔTERalb: *rosiglitazone* +2.43±0.45%/hr vs. placebo -0.11±0.15%/hr, *P = 0.002*), while *rosiglitazone* had no effect in the patients with low Ewing scores (n = 23). *Rosiglitazone*-induced increase in TERalb and Ewing score at baseline were correlated (r = 0.65, *P* = 0.02). There was no correlation between Ewing score and *rosiglitazone*-induced changes in fluid variables. One subject was withdrawn from the study because of atrial fibrillation.

Conclusions/interpretation: *Rosiglitazone* may increase vascular leakage in insulin-treated patients with type 2 diabetes with autonomic neuropathy. Autonomic neuropathy did not exaggerate *rosiglitazone*-induced fluid retention. Therefore, autonomic neuropathy should be considered as a risk factor for thiazolidinedione-induced oedema, not for thiazolidinedione-induced fluid retention.

Introduction

Thiazolidinedione-derivatives are used in the treatment of type 2 diabetes as they improve insulin sensitivity and reduce blood glucose concentration[1,2]. Besides their effect on glycaemia, thiazolidinediones appear to have favourable effects on plasma lipids, blood pressure, fibrinolysis and inflammation, which might offer additional beneficial effects beyond glucose lowering with respect to the prevention of cardiovascular disease[3]. There is intense scientific dispute about whether thiazolidinediones, particularly *rosiglitazone* protect against cardiovascular disease or may even increase the risk of ischaemic cardiovascular events[4-7]. Part of the beneficial effects of thiazolidinediones may be outbalanced by side effects, especially fluid retention. For example, the PROspective pioglitAzone Clinical Trial In macroVascular Events (PROActive) study showed a trend towards a reduced risk of cardiovascular events with *pioglitazone* compared to placebo in subjects with type 2 diabetes, but this benefit was largely offset by fluid-related adverse events, oedema formation and heart failure[8]. The mechanism of fluid retention is unclear[9], but unraveling the mechanism would identify risk factors for the use of thiazolidinediones and enable prescribing thiazolidinedione therapy only to patients with a favourable benefit/risk ratio.

Based on the demonstration in preclinical studies that thiazolidinediones stimulate epithelial sodium channels in the renal collecting duct[10,11], it has been suggested that thiazolidinedione-related fluid retention in humans is caused by primary renal mechanisms, although a recent preclinical study showed opposing results[12] and human experimental data are lacking. Primary renal sodium retention would also increase blood pressure which is at odds with the observed blood pressure lowering effect of thiazolidinediones[13,14]. As the initiating key mechanism, thiazolidinediones-induced arterial vasodilation[9] explains both blood pressure reduction and fluid retention. The *local* microvascular consequence of arterial vasodilation is increased hydrostatic capillary pressure with more vascular leakage and formation of interstitial fluid. The *systemic* consequence is reduction in peripheral vascular resistance[15] and blood pressure, which is the driving force for secondary renal sodium retention. Meanwhile, the sympathetic nervous system counteracts the vasodilator effect[16], reducing the increment in vascular leakage and preventing an exaggerated fall in blood pressure and

consecutive increment in sodium retention. Sympathetic activation also directly stimulates rennin production which induces sodium retention. Therefore, the net effect of the increased sympathetic tone on renal sodium retention is unclear (Figure 1).

Patients with autonomic failure are not able to counterbalance haemodynamic changes effectively, which will result in an unopposed change in capillary hydrostatic force. Indeed, some case reports suggest that the haemodynamic effects of insulin are exaggerated in subjects with autonomic neuropathy[17,18], while blockade of the autonomic nervous system may increase insulin-induced vascular leakage[19].

Epidemiological data support this mechanistic line of reasoning. It has been reported that the incidence of oedema is higher when thiazolidinediones are combined with *insulin*[9]. This may be due to a combined effect of thiazolidinediones and *insulin* or to complications accompanying longstanding diabetes[9]. In a previous study in insulin-resistant people without diabetes, we found no evidence for adverse vascular effects of the combined use of *rosiglitazone* and *insulin*[20], suggesting that it is not *insulin* itself, but probably complications associated with longstanding diabetes that render patients prone to oedema formation. Autonomic neuropathy is a typical complication of longstanding diabetes[21,22].

In the present study in *insulin*-treated patients with type 2 diabetes, we investigated whether thiazolidinedione-induced microvascular leakage is more pronounced in patients with autonomic neuropathy. We also investigated whether autonomic neuropathy affects thiazolidinedione-induced fluid retention.

Methods

The study population consisted of 40 participants with type 2 diabetes, who had received *insulin* treatment for at least 6 months. Further inclusion criteria were age between 30 and 75 years, body mass index below 40 kg/m², and fasting plasma glucose (FPG) between 7.0 and 15 mmol/l. Participants were not eligible for inclusion if HbA$_{1c}$ was higher than 12%, if they used over 200 units *insulin*

Figure 1: Schematic representation of hypothesis
The local consequence of *rosiglitazone*-induced vasodilation will be increased hydrostatic pressure leading to an elevation in capillary filtration (vascular leakage), which predisposes to oedema formation (top). An intact sympathetic nervous system counteracts the vasodilator effect which will prevent increased vascular leakage.
The systemic consequence of vasodilation is reduction of blood pressure (bottom), leading to renin production and sodium retention. An intact sympathetic nervous system on the one hand prevents reduction in blood pressure immediately, on the other sympathetic activation directly stimulates renin production, the result is diminished and elevated sodium retention respectively.
RAAS: Renin-Angiotensin-Aldosterone-System
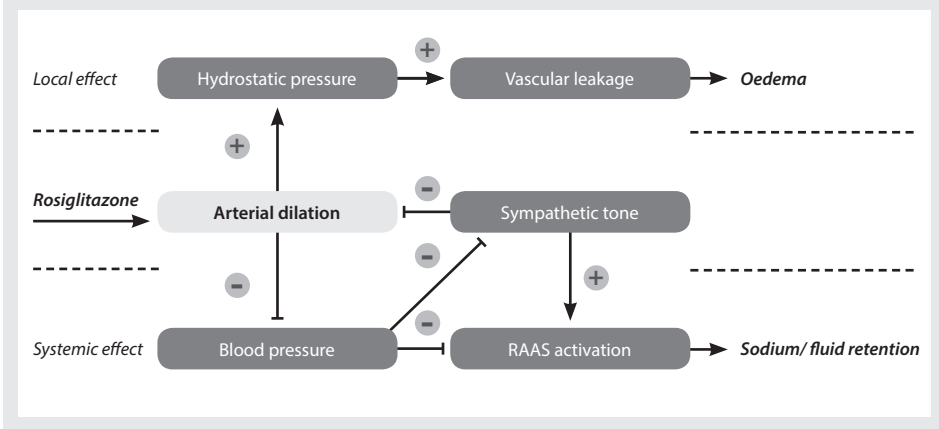
a day, if they used oral hypoglycaemic drugs other than *metformin*, if they used any investigational drug or had used a peroxisome proliferator activated-receptor gamma (PPAR-γ) agonist within 4 months before the start of the study, or had a significant history of hypersensitivity to a PPAR-γ agonist. Additional exclusion criteria were blood pressure exceeding 160/90 mmHg, symptomatic postural hypotension, diuretic therapy for oedema, unstable or severe angina or congestive heart failure, any cardiovascular event in the last six months before entry to the study, presence of clinically significant hepatic disease or anaemia, calculated creatinine clearance below 40 ml/min, pregnancy, lactation, women of childbearing potential without appropriate contraception, and alcohol or drug abuse. To overcome confounding in the interpretation of the Ewing score, all participants using alpha or beta-blockers were excluded. Study participants were either selected by advertisement or invited by their own physician at the outpatient clinics of the Radboud University Nijmegen Medical Centre, Rijnstate

or Catharina Hospital. The participants received a payment and gave written informed consent. The study was approved by the hospital ethics committee, registered at clinical trials.gov (NCT00422955) and performed according to Good Clinical Practice guidelines.

Procedure

This was a randomized, placebo-controlled, double-blind, single-centre, parallel study with 4 weeks of single-blind run-in. At screening, the Ewing score was determined to quantify autonomic neuropathy[22]. In short, continuous finger arterial pressure and cardiac cycle duration (R-R interval) were recorded on a PC-based data-acquisition system during 5 standardized tests. Heart rate responses to the Valsalva maneuver (longest R-R interval after the manoeuvre divided by the shortest R-R interval during the maneuver; normal values are >1.21), to deep breathing (maximum-minimum heart rate during a breathing cycle; normal ≥15 bpm) and to standing up (ratio: longest R-R interval (±30th beat) divided by the shortest R-R interval (±15th beat); normal values are ≥1.04) were measured to determine parasympathetic function, and blood pressure response to standing up (fall in systolic blood pressure (SBP): normal values ≤10mmHg) and to sustained hand grip (increase in diastolic blood pressure (DBP): normal values ≥16mmHg) were determined as a measure of sympathetic function. Each result was compared with the normal response and scored as normal, borderline or abnormal which were allocated 0, ½ or 1 point respectively. The total score ranges from 0 to 5. The participants were divided into two groups, with either Ewing scores ≥2.5 or <2.5. Throughout this article the groups are referred high versus low Ewing scores or established autonomic neuropathy versus mild or no autonomic neuropathy. Four weeks after screening (week –4), eligible participants were randomized to either *rosiglitazone* 4mg twice daily or placebo for 16 weeks in a 1:1 ratio (week 0) and balanced for the two Ewing score groups within 40-60% boundaries. The participants were assigned to study treatment in accordance with the randomisation schedule via the automatic GlaxoSmithKline Registration and Medication Ordering System, which could be reached by phone. At week 0 and week 16, primary endpoint experiments were performed. Vascular leakage was assessed as the transcapillary escape rate of albumin (TERalb). During all visits, including the control visits in week 4, 8 and 12, adverse events and pill compliance were recorded and blood glucose lowering pharmacotherapy

was adjusted. If a participant repeatedly had measurements below 4 mmol/L, the *metformin* dose was decreased, as it was our intention to keep the *insulin* dose as constant as possible during the study. In addition, we performed a physical examination, measured body composition using bio-impedance and foot volume, and chemical, haematological, and glycaemic safety profiles were determined. There was a final follow-up visit in week 18. Participants were strictly advised to maintain their diet and not to change lifestyle throughout the study.

Experimental day

Each participant attended the hospital after an overnight fast without taking *insulin* or oral blood glucose lowering pharmacotherapy in the morning. The procedure started at 8.00a.m. in a quiet temperature-controlled room (23-24ºC) with the participant in supine position. A venous catheter (Venflon, 20G, 32mm, Becton Dickinson, Sandy, Utah, USA) was inserted for the infusion of either *insulin* or glucose to keep the glucose level between 5 and 12 mmol/l. During the experiment, plasma glucose was measured every twenty minutes (Glucocard memory 2, Menarini, Florence). The participant was asked to inject his or her normal morning *insulin* dose. Then, a 20-gauge catheter (Angiocath, Becton Dickinson) was inserted into the left brachial artery under local anaesthesia (0.3-0.4 ml lidocaine HCl 20mg/ml, Braun AG, Melsungen, Germany), connected to an arterial pressure monitoring line and kept patent with *heparin* in saline 0.9% (2 U/ml; 3 ml/h) (NaCl 0.9%, Baxter, Utrecht; heparin, Leo Pharma, Ballerup, Denmark). This catheter was used for blood sampling and blood pressure measurement. After 30 minutes of supine rest, blood was drawn for hormone analysis (atrial natriuretic peptide (ANP), aldosterone and renin) and for baseline TERalb measurement. An additional venous needle (BD Valu-set, 0.6 x 20 mm, Becton Dickinson) was inserted and 7.4×10^4-14.8×10^4 Bq ^{125}I-albumin (Shering Nederland BV, Weesp, the Netherlands) was given as an i.v. bolus injection at 0 min. Over the next 60 min, seven plasma samples were collected from the arterial line for radioactivity measurements.

Bioimpedance

During all visits, total body water (TBW) and extracellular volume (ECV) were assessed using an Akern 2000 bioelectrical impedance analyser[23] (Akern, Florence, Italy).

Foot volume

Foot volume was assessed by the water displacement method using an electronic balance (coefficient of variation: 0.30%)[24]. The balance recorded the force necessary for a standardized immersion of the foot, which depends solely on the volume of the foot (Archimedes' principle).

Analytical methods

Plasma ANP was analyzed by radioimmunoassay after cartridge extraction (Phoenix Pharmaceuticals, Inc., Burlingame, California, USA). Plasma renin was determined with a two-site immunochemiluminometric assay (Diagnostic System Laboratories (DSL), Webster, Texas, USA). Plasma aldosterone was analyzed using antibody-coated tubes and competing radiolabeled aldosterone (Diagnostic Products Corp., Los Angeles, California, USA).

Calculations

For each TERalb test the measured radioactivity was plotted over time. An extinction curve was drawn assuming first-order kinetics. Slope and the extrapolated peak plasma concentration at t=0 were calculated using Microsoft Excel. Plasma volume and TERalb were calculated using the following formulas[20,25]:

Plasma volume (ml)/1.73m^2 = [cpm injected/cpm t=0/ml]/surface(m^2)/1.73m^2

TERalb = [1- e $^{3600 \times slope}$]x100(%/h).

To ensure reliable results, calculated plasma volume and TERalb were excluded from primary endpoint analysis when the correlation coefficient between the extinction curve and the actual measured time points was below 80%. Creatinine clearance was calculated with the Cockcroft formula[26].

Statistical analysis

The groups were balanced for sex and Ewing score. For variables measured at baseline and in week 16, only participants with paired observations were included. For variables with additional assessments, intention-to-treat populations both with and without last observation carried forward were analyzed. The difference between the two treatment groups in either the total population or in one of the Ewing score subgroups was estimated by analysis of covariance with terms for treatment, sex, baseline measurement, and, if applicable, Ewing score. Similarly, the difference in treatment effect between participants from different Ewing score subgroups was estimated with an analysis of covariance with terms for sex, baseline, and Ewing score. For the assessment of various relationships, the partial correlation coefficient was estimated adjusted for sex. All significance tests were two-sided and the overall type I error was 5%. Descriptive statistics are presented as mean and standard deviation or as percentage. Treatment effects are presented as mean with standard error. All statistical analyses were performed using the SAS software package (SAS Institute, Cary, NC, USA).

Results

A total of 40 subjects were included in the study; 21 participants received placebo and 19 *rosiglitazone* (Figure 2). One participant in the *rosiglitazone* group was withdrawn after 8 weeks because of atrial fibrillation and heart failure. Thirteen participants in the *rosiglitazone*-treated group and seventeen participants in the placebo-treated group met the reliability criteria for TERalb-measurements and were included in the analysis of the primary endpoint.

There were no clinically significant differences in baseline characteristics between the treatment groups in the total population, nor were there differences between the randomised subgroups, except for Ewing score (Table 1). Sixteen participants were classified with Ewing scores ≥2.5.

Drug compliance was excellent. The 39 participants who finished the study reported only mild side effects. Oedema was more prevalent in the *rosiglitazone* group compared to placebo (63% vs. 24%, *P*<0.05), but always mild, and not different between participants with high or low Ewing scores.

Figure 2: Enrolment of study participants and distribution among subgroups and flow of participants through the study. [a]To assure reliable results, calculated transcapillary escape rate of albumin (TERalb) with correlation coefficients below 80% between the extinction curve and the actual measured time points were excluded from this analysis. FPG: fasting plasma glucose. BP: blood pressure.
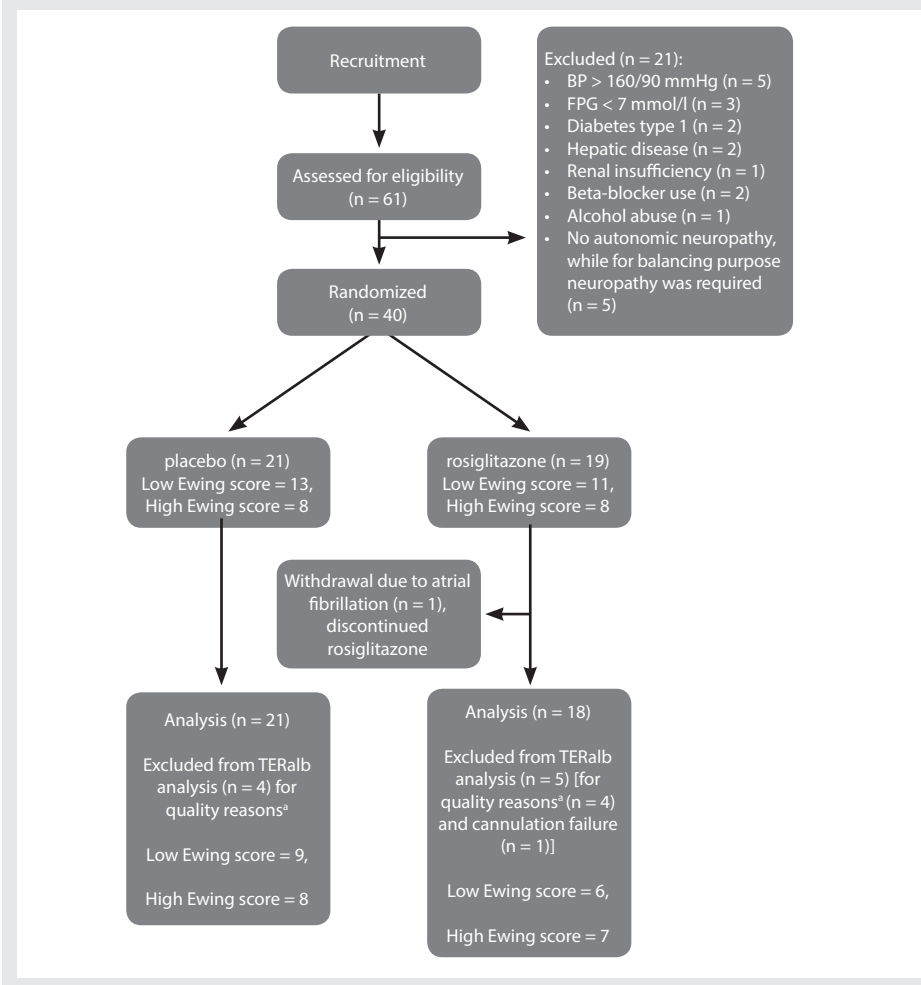
Table 1: Baseline characteristics.

	Total Population				Ewing score < 2.5		Ewing score ≥ 2.5	
	Rosiglita-zone	Placebo	Ewing score < 2.5	Ewing score ≥ 2.5	Rosiglita-zone	Placebo	Rosiglita-zone	Placebo
	(n=19)	(n=21)	(n=24)	(n=16)	(n=11)	(n=13)	(n=8)	(n=8)
Age (years)	58 ± 8	59 ± 10	59 ± 8	58 ± 10	58 ± 9	60 ± 8	59 ± 6	57 ± 13
Male (%)	58	57	63	50	64	62	50	50
BMI (kg/m²)	29 ± 4	29 ± 6	28 ± 4	31 ± 6[d]	28 ± 4	27 ± 5	30 ± 3	32 ± 8
Waist (cm)	101 ± 10	101 ± 17	97 ± 13	105 ± 14	98 ± 10	97 ± 15	104 ± 9	107 ± 19
SBP (mmHg)	132 ± 15	132 ± 15	132 ± 12	132 ± 18	131 ± 13	133 ± 13	133 ± 19	131 ± 19
DBP (mmHg)	80 ± 6	80 ± 7	80 ± 6	80 ± 7	80 ± 4	80 ± 8	79 ± 8	80 ± 6
Heart rate (1/min)	73 ± 10	75 ± 11	73 ± 12	76 ± 7	70 ± 10	75 ± 13	77 ± 7	75 ± 8
Calculated creatinine clearance (ml/min)	89 ± 16	85 ± 30	86 ± 19	88 ± 32	92 ± 15	81 ± 21	84 ± 17	92 ± 42
Urinary albumin/ creatinine ratio (mg/mmol creat)	0.9 ± 1.4	3.9 ± 13.3	0.9 ± 1.3	5.1 ± 15.5	1.0 ± 1.9	0.8 ± 0.8	0.9 ± 0.5	10.7 ± 23.5
Duration of diabetes (years)	12.8 ± 7.8	14.1 ± 5.7	13.1 ± 6.9	14.0 ± 6.5	11.2 ± 8.0	14.6 ± 5.8	14.9 ± 7.4	13.1 ± 5.9
HbA1c (%)	7.7 ± 1.4	8.1 ± 1.1	7.7 ± 1.2	8.3 ± 1.3	7.5 ± 1.3	7.8 ± 1.0	8.0 ± 1.4	8.6 ± 1.1
Insulin dose (U/day)	60 ± 40	64 ± 33	58 ± 28	69 ± 45	57 ± 24	58 ± 32	64 ± 56	74 ± 34
Metformin (%)	32	43	29	50	18	38	50	50
Ewing score	2.1 ± 1.3	1.7 ± 0.9	1.2 ± 0.6	3.0 ± 0.8[c]	1.2 ± 0.6	1.1 ± 0.6	3.3 ± 0.9 [a]	2.7 ± 0.5[b]

Data are mean ± SD
[a] $P < 0.05$ vs. *rosiglitazone*, Ewing score < 2.5
[b] $P < 0.05$ vs. placebo, Ewing score < 2.5
[c] $P < 0.001$ vs. Ewing score < 2.5
[d] $P < 0.05$ vs. Ewing score < 2.5

Total population

In this section the model-adjusted treatment effects of *rosiglitazone* and placebo are given. A summary of the raw endpoint data at baseline and after treatment for the total population is provided in Table 2.

3

Table 2: Endpoint variables at baseline and after treatment in the total population (mean±SE).

	Rosiglitazone				Placebo				Rosiglitazone vs placebo
	n	Before	After	Adjusted effect	n	Before	After	Adjusted effect	*P*-value
TERalb (%/hr)	13	6.75 ± 0.51	8.36 ± 0.48	1.38 ± 0.46	17	7.38 ± 0.38	7.46 ± 0.40	0.29 ± 0.40	0.09
Plasma volume (ml)	13	2663 ± 108	2912 ± 109	220 ± 71	16	2746 ± 84	2706 ± 83	-21 ± 64	<0.05
Body weight (kg)	18	85.5 ± 1.9	87.8 ± 2.0	2.4 ± 0.5	21	88.5 ± 5.1	88.8 ± 5.0	0.5 ± 0.4	<0.01
TBW (l)	18	43.7 ± 1.4	44.8 ± 1.5	1.0 ± 0.3	21	45.1 ± 2.1	45.4 ± 2.0	0.4 ± 0.3	0.12
ECV (l)	18	19.3 ± 0.6	20.1 ± 0.6	0.8 ± 0.2	21	19.7 ± 0.9	20.0 ± 0.8	0.4 ± 0.2	0.18
Haemato-crit	17	0.39 ± 0.01	0.36 ± 0.01	-0.02 ± 0.00	20	0.39 ± 0.01	0.38 ±0.01	-0.00 ± 0.00	<0.01
ANP (ng/l)	17	121 ± 14	180 ± 27	64 ± 20	21	108 ± 13	108 ±13	-1 ± 18	<0.05
DBP intra-arterial (mmHg)	17	74.3 ± 2.5	72.1 ±2.0	-2.3 ± 1.1	17	73.2 ±1.8	74.9 ±1.9	1.4 ± 1.1	<0.05
SBP intra-arterial (mmHg)	17	149.5 ± 5.1	150.8 ± 4.5	1.0 ± 2.3	17	147.4 ±3.9	152.1 ±3.8	4.1 ± 2.3	0.34
Insulin dose (units/day)	18	61 ± 10	50 ± 8	-11 ± 2	21	64 ± 7	66 ± 7	2 ± 2	<0.001
HbA1c (%)	17	7.79 ± 0.34	7.22 ± 0.22	-0.67 ± 0.15	21	8.12 ± 0.24	7.83 ± 0.20	-0.24 ± 0.14	<0.05

Data are mean ±SE

Effect of *rosiglitazone* on glycaemic control

Rosiglitazone improved glycaemic control (HbA$_{1c}$; *rosiglitazone*: -0.67±0.15% vs. placebo: -0.24 ±0.14%, *P*<0.05) despite a significant reduction in the daily *insulin* dose (*rosiglitazone*: -11±2 U/day vs. placebo: +2±2 U/day, *P*<0.0001) and a slight decrease of background *metformin* treatment.

Effect of *rosiglitazone* on vascular leakage and diastolic blood pressure

There was a trend for *rosiglitazone* to increase vascular leakage (TERalb; *rosiglitazone*: +1.38±0.46%/hr vs. placebo: +0.29±0.40%/hr, *P*=0.09). *Rosiglitazone* decreased the intra-arterially measured diastolic blood pressure (*rosiglitazone*: -2.3 ± 1.1mmHg vs. placebo: +1.4±1.1mmHg, *P* = 0.02)

Effect of *rosiglitazone* on fluid parameters and vascular hormones

During *rosiglitazone* treatment, plasma volume (*rosiglitazone*: 220±71 ml/1.73m^2 vs. placebo: -21 ± 64 ml/1.73m^2, *P* = 0.02) and body weight (*rosiglitazone*: +2.4 kg ± 0.5 vs. placebo: +0.5 ± 0.4 kg, *P* = 0.004) increased, while haematocrit decreased (*rosiglitazone*: -0.024 ± 0.005 vs. placebo: -0.005 ± 0.005, *P* = 0.007). *Rosiglitazone* did not increase ECV (*rosiglitazone*: +0.8 ± 0.2 l vs. placebo: +0.4±0.2 l, *P* = 0.18) and TBW (*rosiglitazone*: +1.0 ± 0.3 l vs. placebo: +0.4±0.3 l, *P* = 0.12), and had no effect on foot volume. In addition, *rosiglitazone* increased plasma ANP (*rosiglitazone*: +64 ± 20 ng/l vs. placebo: -1 ± 18 ng/l, *P* = 0.02) but did not influence plasma renin and aldosterone levels.

Low and High Ewing score subgroups

A summary of raw endpoint data and model-adjusted treatment effects of *rosiglitazone* and placebo within these Ewing score subgroups is provided in Table 3.

Effect of *rosiglitazone* on glycaemic control

The changes in glycaemic control and *insulin* requirements did not differ between the two Ewing score subgroups.

3

Table 3A: Endpoint variables at baseline and after treatment in the subgroups with either high or low Ewing score.

Ewing Score < 2.5

Variable	Rosiglitazone				Placebo				Rosiglitazone vs placebo
	n	Before	After	[a]Adjusted effect	n	Before	After	[a]Adjusted effect	*P*-value
TERalb (%/hr)	6	6.41 ± 0.48	7.15 ± 0.66	-0.44 ± 0.72	9	7.94 ±0.42	8.11 ± 0.49	1.04 ± 0.56	0.16
Plasma volume (ml)	6	2681 ± 210	2989 ± 201	287 ± 82	9	2716 ± 131	2617 ±61	-98 ± 66	<0.01
Body weight (kg)	10	84.8 ± 2.9	87.1 ± 3.1	2.5 ± 0.6	13	82.0 ± 4.7	82.0 ± 4.6	0.0 ± 0.5	<0.01
TBW (l)	10	44.9 ± 1.8	46.2 ± 2.0	1.3 ± 0.4	13	43.6 ± 2.2	43.8 ± 2.2	0.3 ± 0.3	0.04
ECV (l)	10	19.5 ± 0.7	20.3 ± 0.7	0.9 ± 0.3	13	19.0 ± 0.9	19.1 ± 0.8	0.2 ± 0.3	0.12
Haematocrit	10	0.39 ± 0.01	0.37 ± 0.02	-0.02 ± 0.01	13	0.39 ± 0.01	0.39 ± 0.01	0.00 ± 0.00	<0.01
DBP intra-arterial (mmHg)	10	73.5 ± 2.5	72.2 ± 1.8	-2.2 ± 1.7	11	73.5 ±2.2	75.2 ± 2.7	1.6 ± 1.5	0.11
SBP intra-arterial (mmHg)	10	147.7 ± 4.6	150.2 ± 4.5	1.4 ± 3.3	11	149.9 ±3.8	155.0 ± 3.6	5.2 ± 3.0	0.39
ANP (ng/l)	10	109 ± 16	152 ± 24	37 ± 22	13	117 ± 17	125 ± 17	9 ± 19	0.35
Aldosterone [b] (nmol/l)	5	0.161 ± 0.022	0.144 ± 0.028	-0.014 ± 0.016	4	0.125 ± 0.018	0.083 ± 0.011	-0.058 ± 0.021	0.18
Renin[b] (mU/l)	10	103 ± 64	76 ± 46	-15 ± 37	9	27 ± 12	38 ± 14	-14 ± 37	0.99
Insulin dose (units/day)	10	58 ± 8	45 ± 8	-13 ± 3	13	58 ± 9	60 ± 9	2 ± 3	<0.01
HbA1c(%)	10	7.59 ± 0.43	7.17 ± 0.35	-0.45 ± 0.17	13	7.83 ± 0.28	7.68 ± 0.22	-0.11 ± 0.15	0.15

Data are mean±SE
[a]Adjusted effect is raw value after treatment minus raw value before treatment using model-adjustments (see Methods). The data in this Table are model-adjusted for the comparison between *rosiglitazone* and placebo within the same Ewing score subgroup. Therefore, we cannot derive the model-adjusted difference in TERalb change (+1.96%/hr) due to treatment with *rosiglitazone* between the participants with high (2.54±0.49%/hr) and low Ewing score (0.58±0.53%/hr) from this Table.
[b]The number of valid observations is low because many participants had aldosterone and renin levels below the detection level.

Table 3B: Endpoint variables at baseline and after treatment in the subgroups with either high or low Ewing score.

Ewing Score ≥ 2.5

Rosiglitazone				Placebo				Rosiglitazone vs placebo
n	Before	After	[a]Adjusted effect	n	Before	After	[a]Adjusted effect	*P*-value
7	7.04 ± 0.88	9.41 ± 0.40	2.43 ± 0.45	8	6.75 ± 0.61	6.73 ± 0.56	-0.11 ± 0.42	<0.01
7	2648 ± 108	2845 ± 116	171 ± 110	7	2786 ± 103	2821 ± 172	132 ± 117	0.82
8	86.3 ± 2.5	88.6 ± 2.6	2.1 ± 0.7	8	99.0 ± 10.4	99.8 ± 10.1	1.0 ± 0.7	0.33
8	42.2 ± 2.3	43.1 ± 2.2	0.7 ± 0.6	8	47.5 ± 4.3	48.0 ± 4.0	0.8 ± 0.6	0.92
8	19.2 ± 1.1	19.9 ± 0.9	0.7 ± 0.3	8	20.8 ± 1.8	21.4 ± 1.7	0.7 ± 0.3	1.00
7	0.38 ± 0.01	0.35 ± 0.01	-0.02 ±0.01	7	0.38 ±0.02	0.37 ± 0.01	-0.01 ± 0.01	0.40
7	75.3 ± 5.1	71.9 ± 4.2	-3.1 ± 1.2	6	72.6 ± 3.4	74.1 ± 2.7	1.2 ± 1.3	0.03
7	152.2 ± 10.9	151.7 ± 9.2	0.7 ± 3.7	6	142.9 ± 9.0	146.9 ± 8.7	3.0 ± 4.0	0.68
7	138 ± 26	220 ± 55	81 ± 45	8	95 ± 18	79 ± 17	-20 ± 29	<0.05
3	0.296 ± 0.098	0.139 ± 0.028	-0.059 ± 0.039	6	0.203 ± 0.083	0.129 ± 0.028	-0.085 ± 0.024	0.57
5	44 ± 20	27 ± 8	-10 ± 3	6	27 ± 11	20 ± 7	-12 ± 2	0.56
8	64 ± 20	55 ± 15	-10 ± 2	8	74 ± 12	75 ± 11	2 ± 2	<0.01
7	8.09 ± 0.59	7.29 ± 0.23	-0.97 ± 0.31	8	8.60 ± 0.40	8.09 ± 0.40	-0.37 ± 0.28	0.19

Effect of *rosiglitazone* on vascular leakage and blood pressure

In the subgroup of patients with high Ewing scores, *rosiglitazone* significantly increased TERalb (*rosiglitazone*: 2.43 ± 0.45%/hr vs. placebo: -0.11 ± 0.42%/hr, $P = 0.002$), while *rosiglitazone* had no effect in the subgroup of patients with low Ewing scores (*rosiglitazone*: -0.44 ± 0.72%/hr vs. placebo: 1.04 ± 0.56%/hr, P = not significant, Figure 3A). As a result, *rosiglitazone* significantly increased TERalb in patients with high Ewing scores compared to those with low Ewing scores (high Ewing score: 2.54 ± 0.49%/hr vs. Low Ewing score: 0.58±0.53%/hr $P = 0.03$).

Figure 3: *Rosiglitazone* induced vascular leakage in the autonomic neuropathy subgroups.

A) (top): Mean model-adjusted change in transcapillary escape rate of albumin (TERalb)(SE) during treatment with either *rosiglitazone* or placebo within the subgroup of participants with a high Ewing score (left) and with a low Ewing score (right) (dichotomous). Values are means with SE. The data in this Figure are model-adjusted for the comparison between *rosiglitazone* and placebo within a Ewing score subgroup. Therefore, we cannot derive the exact model-adjusted difference in TERalb change (+1.96%/hr) due to the treatment with *rosiglitazone* between the participants with high and low Ewing score from this Figure (see Methods). Black bars: *rosiglitazone*; white bar: placebo. [a]P=0.002;[b]P=0.03; [c] not significant

B) (bottom): Correlation (r =0.65, P=0.02) between baseline Ewing score (continuous) and change in TERalb (individual raw data, therefore not model-adjusted) during treatment with *rosiglitazone*.

In the *rosiglitazone*-treated patients the reduction in intra-arterial diastolic blood pressure tended to be more pronounced in participants with high Ewing scores (*rosiglitazone*: -3.1 ± 1.2 mmHg vs. placebo: +1.2 ± 1.3 mmHg, $P = 0.03$) than with low Ewing scores (*rosiglitazone*: -2.2 ± 1.7 mmHg vs. placebo: +1.6 ± 1.5 mmHg, $P = 0.11$).

Effect of *rosiglitazone* on fluid parameters and vascular hormones

In the subgroup of patients with a high Ewing score, *rosiglitazone* did not increase fluid parameters and decrease haematocrit significantly, while in the patients with low Ewing scores *rosiglitazone* seemed to increase plasma volume, TBW, body weight, and did decrease haematocrit (Table 3). The difference in effect of *rosiglitazone* over placebo in both subgroups was partly driven by the different response to placebo in these subgroups.
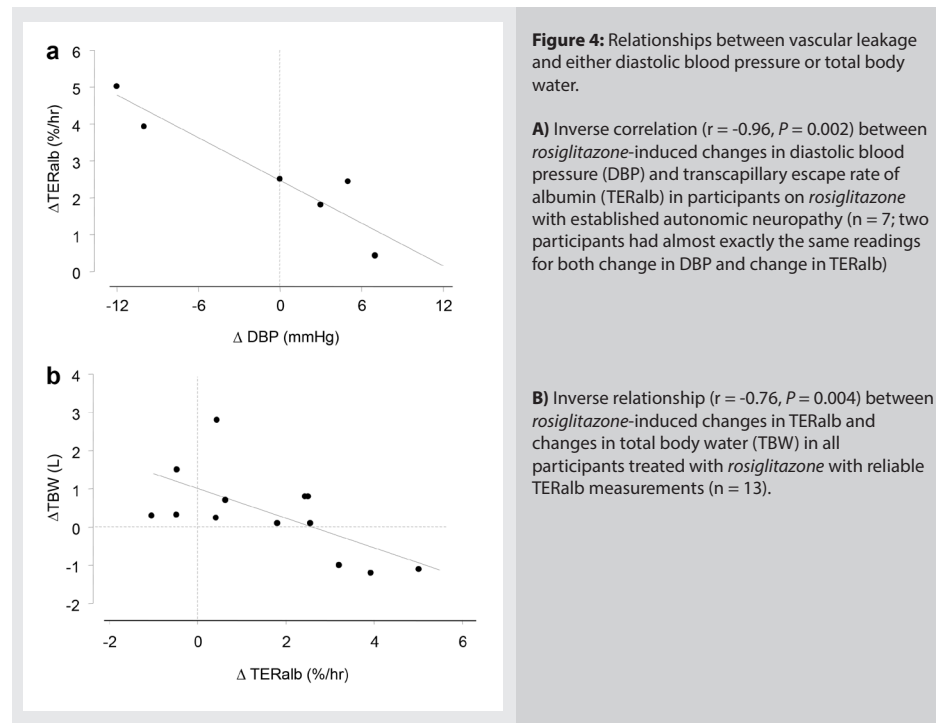
Correlations

Baseline Ewing score and change in vascular leakage or fluid parameters

In patients randomized to *rosiglitazone*, the increase in TERalb was highly correlated with the Ewing score at baseline (r = 0.65, $P = 0.02$, Figure 3B), while the change in TERalb during placebo treatment was not (r = 0.12, $P = 0.66$). The correlation between the increase in TERalb during *rosiglitazone* treatment and baseline Ewing score was even more robust after inclusion of all the TERalb results (r = 0.72, $P = 0.002$), showing that the exclusion of qualitative weak TERalb measurements neither biased nor caused the relationship. As participants with a high Ewing score had a significantly higher BMI we also evaluated the correlation between the change in TERalb during *rosiglitazone* treatment and baseline BMI (r = 0.11) but this correlation was not significant. Furthermore, the changes in intra-arterial DBP while taking *rosiglitazone* were not significantly correlated with baseline Ewing score (r = -0.28). In addition, also the changes in body weight, haematocrit, plasma volume, TBW and ECV during *rosiglitazone* treatment were not correlated with the Ewing score at baseline.

Correlation between change in vascular leakage, and change in diastolic blood pressure or fluid parameters

As expected from the vascular hypothesis, changes in diastolic blood pressure in patients receiving *rosiglitazone* were strongly inversely correlated to changes in vascular leakage in participants with high Ewing scores (r = -0.96, $P = 0.002$, Figure 4A), indicating that participants with a large blood pressure drop had a large increase in vascular leakage. This correlation was not significant in participants with low Ewing scores.

In patients taking *rosiglitazone*, the changes in vascular leakage were inversely correlated to changes in TBW (r = -0.76, $P = 0.004$, Figure 4B) and ECV (r = -0.65, $P = 0.02$).

Figure 4: Relationships between vascular leakage and either diastolic blood pressure or total body water.

A) Inverse correlation (r = -0.96, *P* = 0.002) between *rosiglitazone*-induced changes in diastolic blood pressure (DBP) and transcapillary escape rate of albumin (TERalb) in participants on *rosiglitazone* with established autonomic neuropathy (n = 7; two participants had almost exactly the same readings for both change in DBP and change in TERalb)

B) Inverse relationship (r = -0.76, *P* = 0.004) between *rosiglitazone*-induced changes in TERalb and changes in total body water (TBW) in all participants treated with *rosiglitazone* with reliable TERalb measurements (n = 13).

Discussion

There are two main clinically relevant findings in the present study. First, in the presence of autonomic neuropathy, *rosiglitazone* induces vascular leakage in *insulin*-treated patients with type 2 diabetes. Second, neither autonomic neuropathy nor the increase in vascular leakage in itself led to increased fluid retention. Together these findings suggest that, in established autonomic neuropathy, thiazolidinediones will lead to exaggerated vascular leakage, but not necessarily to more pronounced fluid retention. Nevertheless, an increased vascular leak will render these patients more susceptible to oedema formation.

We postulate that thiazolidinediones have a vasodilator action, which subsequently promotes vascular leakage into interstitial tissues. In the present study, vascular leakage indeed tended to increase during treatment with *rosiglitazone*, although not statistically significant. Predefined subgroup analyses, however, showed a clear increase in vascular leak following treatment with *rosiglitazone* in participants with established autonomic neuropathy but not in participants with absent or mild autonomic neuropathy. This was confirmed by the positive correlation between Ewing score and change in TERalb. The findings suggest that autonomic nerve damage in diabetic participants prevents sympathetic nerve stimulation from counteracting the vasodilator effects of *rosiglitazone*. This concept (Figure 1) is supported by the strong inverse correlation between changes in diastolic blood pressure and TERalb in participants with established autonomic neuropathy (Figure 4A). All in all, the findings fit with the concept that defective counter regulation of haemodynamic changes caused by autonomic neuropathy exaggerates vascular leakage induced by thiazolidinediones. In persons with none or mild autonomic neuropathy, *rosiglitazone* did not increase vascular leakage, which is in accordance with previous human[20] and with preclinical[27] findings.

The notion that autonomic neuropathy results in a defective counterbalance towards *rosiglitazone*-induced vasodilation and subsequent vascular leakage, has two consequences. First, people prone to vascular leak should be protected against excessive plasma volume expansion, because excess fluid would leak out of the plasma compartment. Indeed, we did not observe either a disproportional increase in plasma volume or decrease in haematocrit during *rosiglitazone* treatment in participants with established autonomic neuropathy. Second, the excessive leakage should result in an increase in TBW and ECV. This, however, was not observed in the present study: if anything changes in TBW an ECV were lower in the established autonomic neuropathy group. This apparent discrepancy can be explained by the complicated relation between sympathetic counter regulation and renal sodium retention. The sympathetic nervous system responds to *systemic* hypoperfusion, both by direct renal sodium retention and by activation of the Renin-Angiotensin-Aldosterone-System[28]. In fact, after drug-induced vasodilation an intact sympathetic nervous system protects the body against *local* vascular leak by reflex vasoconstriction and against *systemic* hypoperfusion by sodium retention. Consequently, a defective sympathetic nervous system will lead to vascular leak without much sodium retention (Figure 1). In both situations there should be an inverse correlation between vascular leak and ECV and TBW, and this indeed is in complete agreement with our findings (Figure 4B). The clinical implication of our observations is that *insulin*-treated

patients with diabetic autonomic neuropathy may be partly protected from fluid overload by sodium retention induced by thiazolidnediones, but on the other hand will be more prone to vascular oedema, as a consequence of microvascular imbalance.

The prevalence of oedema was much higher in our study than in another study in *insulin*-treated patients performed by Raskin et al.[13], both during placebo treatment (present study: 24%, Raskin et al.: 4%) and during *rosiglitazone* treatment (present study: 64%, Raskin et al.: 17%). A potential explanation for this difference is that we were more focused on the development of oedema because oedema was a main outcome in our study. Another explanation could be the high prevalence of autonomic neuropathy in our population, although there was no correlation between Ewing score and the clinical finding of oedema. The reason we found differences in vascular leakage but not in oedema formation between the autonomic neuropathy subgroups during *rosiglitazone* treatment seems to be the absence of a reliable clinical test to quantitatively assess changes in total body interstitial fluid (oedema) in a chronic setting. Vascular leakage was measured as mean of total body capillary leakage while oedema was measured only locally in the foot. For instance, changes in visceral vascular leakage will not influence foot volume.

In the present study, the glycaemic effect of rosiglitazone was moderate (a decrease in HbA_{1c} of 0.42%) and seemingly less than expected. For example, Raskin et al. reported a treatment effect of -1.3%[13]. This difference may be explained by the shorter treatment period, the lower baseline HbA_{1c}, the reduction in *insulin* dose, the slight decrease of background *metformin* use during *rosiglitazone*, and the marked glycaemic improvements in the placebo group.

The participant who was withdrawn from this study with atrial fibrillation had used beta blocker therapy for subjective palpitations in the past. She discontinued this therapy one month before entry into the study. Atrial fibrillation and oedema resolved quickly after re-institution of beta blocker therapy and discontinuation of *rosiglitazone*. Despite correction of the rhythm, the participant was withdrawn as beta blocker therapy would have been a confounder in the final analyses.

To assess the degree of autonomic neuropathy, different methods each

with important limitations, are being used. We have used the Ewing score, as it is well validated, widely accepted, non-invasive, and suitable for screening. A disadvantage of the Ewing score is that there is no international consensus how to adjust the tests for aging. We have our own age-adjusted reference tables but these have not been published. Therefore, we used in this study the internationally accepted Ewing tests without age adjustment. We did perform a post-hoc analysis after adjusting Ewing score for age, but this did not result in any change in study outcome (data not shown).

We have also measured baseline arterial plasma noradrenaline, adrenaline and calculated the noradrenaline appearance rate (data not shown), which tended to be lower in the group with established autonomic neuropathy, suggesting that the higher Ewing score did indeed reflect autonomic neuropathy.

While sympathetic dysfunction is most relevant for our hypothesis, the Ewing score contains parasympathetic as well as sympathetic tests[22]. In most patients the initial manifestation of autonomic disease is an abnormal response to the parasympathetic tests, followed by abnormal sympathetic tests in more severe autonomic neuropathy[21]. The increased frequency of abnormalities found with the parasympathetic tests may reflect both an earlier involvement of parasympathetic damage and a better sensitivity of the parasympathetic tests. To maximize the separation of participants with and without sympathetic neuropathy, we divided our population on the basis of the Ewing score in two categories with a cut off value of 2.5, in slight deviation with the original four categories described by Ewing (normal, early, definite and severe). Indeed, more participants in the ≥2.5 group had abnormalities in the sympathetic nerve tests than in the <2.5 group (data not shown). In line with the concept of sympathetic counterbalance, *rosiglitazone* increased vascular leakage in participants with the combination of a high Ewing score *and* sympathetic disturbances in a post hoc analysis. On the contrary, *rosiglitazone* did not influence vascular leakage in participants with the combination of a low Ewing score and no sympathetic disturbances.

In conclusion, in *insulin*-treated type 2 diabetes patients who have autonomic neuropathy, thiazolidinediones increase vascular leakage and render the patient susceptible to development of oedema. Autonomic neuropathy in itself does not exaggerate thiazolidinedione-induced fluid retention. Therefore, autonomic

neuropathy should be considered as a risk factor for thiazolidinedione-induced oedema, not for fluid retention.

Acknowledgments

References

1. Yki-Jarvinen H. Thiazolidinediones. *N.Engl.J.Med* 2004; 351: 1106-1118.

2. Kahn SE, Haffner SM, Heise MA, et al. Glycemic durability of rosiglitazone, metformin, or glyburide monotherapy. *N.Engl.J.Med* 2006; 355: 2427-2443.

3. Parulkar AA, Pendergrass ML, Granda-Ayala R, Lee TR, Fonseca VA. Nonhypoglycemic effects of thiazolidinediones. *Ann.Intern.Med* 2001; 134: 61-71.

4. Nissen SE, Wolski K. Effect of rosiglitazone on the risk of myocardial infarction and death from cardiovascular causes. *N.Engl.J.Med* 2007; 356: 2457-2471.

5. Home PD, Pocock SJ, Beck-Nielsen H, et al. Rosiglitazone evaluated for cardiovascular outcomes--an interim analysis. *N.Engl.J.Med* 2007; 357: 28-38.

6. Gerstein HC, Miller ME, Byington RP, et al. Effects of intensive glucose lowering in type 2 diabetes. *N.Engl.J.Med* 2008; 358: 2545-2559.

7. Home PD, Pocock SJ, Beck-Nielsen H, et al. Rosiglitazone evaluated for cardiovascular outcomes in oral agent combination therapy for type 2 diabetes (RECORD): a multicentre, randomised, open-label trial. *Lancet* 2009; 373: 2125-2135.

8. Dormandy JA, Charbonnel B, Eckland DJ, et al. Secondary prevention of macrovascular events in patients with type 2 diabetes in the PROactive Study (PROspective pioglitAzone Clinical Trial In macroVascular Events): a randomised controlled trial. *Lancet* 2005; 366: 1279-1289.

9. Nesto RW, Bell D, Bonow RO, et al . Thiazolidinedione use, fluid retention, and congestive heart failure: a consensus statement from the American Heart Association and American Diabetes Association. October 7, 2003. *Circulation* 2003; 108: 2941-2948.

10. Guan Y, Hao C, Cha DR, et al. Thiazolidinediones expand body fluid volume through PPARgamma stimulation of ENaC-mediated renal salt absorption. *Nat.Med* 2005; 11: 861-866.

11. Hong G, Lockhart A, Davis B, et al. PPARgamma activation enhances cell surface ENaCalpha via up-regulation of SGK1 in human collecting duct cells. *FASEB J* 2003; 17: 1966-1968.

12. Vallon V, Hummler E, Rieg T, et al. Thiazolidinedione-Induced Fluid Retention Is Independent of Collecting Duct {alpha}ENaC Activity. *J.Am.Soc.Nephrol* 2009.

13. Raskin P, Rendell M, Riddle MC, Dole JF, Freed MI, Rosenstock J. A randomized trial of rosiglitazone therapy in patients with inadequately controlled insulin-treated type 2 diabetes. *Diabetes Care* 2001 ; 24: 1226-1232.

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14. Shimkets RA, Warnock DG, Bositis CM, et al. Liddle's syndrome: heritable human hypertension caused by mutations in the beta subunit of the epithelial sodium channel. *Cell* 1994; 79: 407-414.

15. Shargorodsky M, Wainstein J, Gavish D, Leibovitz E, Matas Z, Zimlichman R. Treatment with rosiglitazone reduces hyperinsulinemia and improves arterial elasticity in patients with type 2 diabetes mellitus. *Am.J.Hypertens* 2003; 16: 617-622.

16. Lindqvist M, Kahan T, Melcher A, Hjemdahl P. Acute and chronic calcium antagonist treatment elevates sympathetic activity in primary hypertension. *Hypertension* 1994; 24: 287-296.

17. Page MM, Watkins PJ. Provocation of postural hypotension by insulin in diabetic autonomic neuropathy. *Diabetes* 1976; 25: 90-95.

18. Seyer-Hansen K. Postprandial hypotension. *Br.Med.J* 1977; 2: 1262.

19. Hilsted J, Frandsen H, Christensen NJ, Nielsen SL. Plasma volume changes during hypoglycaemia: the effect of autonomic blockade. *Eur.J.Clin.Invest* 1991; 21: 22-26.

20. Rennings AJ, Smits P, Stewart MW, Tack CJ. Fluid retention and vascular effects of rosiglitazone in obese, insulin-resistant, nondiabetic subjects. *Diabetes Care* 2006; 29: 581-587.

21. Vinik AI, Maser RE, Mitchell BD, Freeman R. Diabetic autonomic neuropathy. *Diabetes Care* 2003; 26: 1553-1579.

22. Ewing DJ, Martyn CN, Young RJ, Clarke BF. The value of cardiovascular autonomic function tests: 10 years experience in diabetes. *Diabetes Care.* 1985; 8: 491-498.

23. Lukaski HC. Methods for the assessment of human body composition: traditional and new. *Am.J.Clin.Nutr* 1987; 46: 537-556.

24. van Hamersvelt HW, Kloke HJ, de Jong DJ, Koene RA, Huysmans FT. Oedema formation with the vasodilators nifedipine and diazoxide: direct local effect or sodium retention? *J.Hypertens* 1996; 14: 1041-1045.

25. Parving HH, Gyntelberg F. Albumin transcapillary escape rate and plasma volume during long-term beta-adrenergic blockade in essential hypertension. *Scand.J.Clin.Lab Invest* 1973; 32: 105-110.

26. Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. *Nephron* 1976; 16: 31-41.

27. Cosson E, Cohen-Boulakia F, Tarhzaoui K, et al. Capillary endothelial but not lymphatic function is restored under rosiglitazone in Zucker diabetic fatty rats. *Microvasc.Res* 2009; 77: 220-225.

28. Osborn JL, Holdaas H, Thames MD, DiBona GF. Renal adrenoceptor mediation of antinatriuretic and renin secretion responses to low frequency renal nerve stimulation in the dog. *Circ.Res* 1983; 53: 298-305.

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Preserved response to diuretics in *rosiglitazone*-treated subjects with insulin resistance: a randomized double-blind placebo-controlled crossover study

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Abstract

Thiazolidinediones are associated with fluid retention, that has been suggested to be resistant to treatment with loop diuretics. This resistance is thought to be caused by upregulation of renal epithelial sodium channels (ENaCs). In this study, we tested whether these mechanisms are of clinical significance.

We conducted a well-controlled study in 12 insulin-resistant nondiabetic participants, who received treatment for 9 weeks with either *rosiglitazone* at a dosage of 4 mg b.i.d. or placebo The aim of the study was to investigate whether upregulation of ENaCs by *rosiglitazone* reduces *furosemide's* natriuretic response and enhances the response to the ENaC inhibitor *amiloride*. The natriuretic response to *furosemide* and *amiloride,* and the amount of α-ENaC in urinary exosomes were quantified. *Rosiglitazone* neither reduced *furosemide*-induced natriuresis nor changed *furosemide's* concentration-effect curve. Furthermore, *rosiglitazone* did not change either *amiloride*-induced natriuresis nor the amount of urinary α-ENaC.

This study challenges previous findings regarding thiazolidinedione-related ENaC upregulation and suggests that thiazolidinedione-induced fluid retention should respond normally to loop diuretics.

Introduction

Thiazolidinedione derivatives (TZDs) are used in the treatment of type 2 diabetes because they improve insulin sensitivity and reduce plasma glucose levels[1,2]. Besides their effect on glycaemia, thiazolidinediones appear to have favourable effects on plasma lipids, blood pressure, fibrinolysis, and inflammation that are thought to offer additional cardiovascular benefit[3,4]. However, there is controversy about the use of thiazolidinediones, particularly *rosiglitazone*, as possibly being associated with higher risks of ischaemic cardiovascular events[5-8]. Such concerns recently resulted in the suspension of marketing authorization for *rosiglitazone* in Europe. The well-documented side effect of fluid retention associated with the use of thiazolidinediones, which doubles the incidence of heart failure, significantly contributes to the vigorous discussion about the benefit-to-risk ratio of this class of drugs. The incidence of oedema formation during *rosiglitazone* treatment ranges from -4.8% when given as monotherapy to almost 15% during concomitant use of *insulin*[9]. For example, the PROActive (Prospective Pioglitazone Clinical Trial in Macrovascular Events) study showed a trend towards a reduced risk of cardiovascular events with the use of *pioglitazone* as compared with placebo in subjects with type 2 diabetes, but this benefit was largely offset by fluid retention-related adverse events, namely, oedema formation and heart failure[10].

The findings from experiments in animals and *in vitro* cell experiments suggest that thiazolidinediones upregulate epithelial sodium channels (ENaCs) in the renal collecting duct[11,12]. Consequently, thiazolidinedione-related fluid retention in humans may be caused by primary renal mechanisms. ENaC is a membrane-bound channel that specializes in the reabsorption of sodium, regulated by aldosterone and insulin[13] and pharmacologically inhibited by the diuretic drug *amiloride*[14]. The concept that thiazolidinediones upregulate ENaC is appealing and explains the following two observations.

First, despite *furosemide's* higher natriuretic potential, *spironolactone*, an aldosterone receptor antagonist and indirect inhibitor of ENaC, was superior in the treatment of *rosiglitazone*-induced fluid retention[15]. This is similar to observations in cirrhotic patients with ascites, in whom hyperaldosteronism[16] and subsequent ENaC upregulation results in increased distal sodium reabsorption.

Using this line of reasoning, the direct ENaC-inhibitor *amiloride* might be the drug of choice to treat thiazolidinedione-induced fluid overload[17]. In healthy individuals, *amiloride* is not a potent natriuretic drug, but upregulation of ENaCs may lead to an increased natriuretic potential similar to what has been observed in patients with a gain-of-function mutation of ENaC, the so called Liddle's syndrome[18].

Second, thiazolidinedione-induced oedema is reported to be resistant to loop diuretic therapy. Several case studies on thiazolidinedione-related pulmonary oedema observed resistance to loop diuretics[19,20]. Niemeyer et al. presented a clinical cohort study showing that up to 30% of thiazolidinedione-induced oedema was refractory to *furosemide* treatment[21]. Resistance to loop diuretics has been studied extensively in the past, but not specifically with thiazolidinediones. Loop diuretics specifically inhibit the apical Na-K-2Cl-cotransporter in the ascending limb of the loop of Henle[22]. Extensive distal renal sodium reabsorption, for example as a result of ENaC upregulation, is one of the potential pharmacodynamic causes of resistance to loop diuretics[23].

This concept is of great interest and needs clinical proof. Therefore, we addressed the hypothesis that treatment of insulin-resistant subjects with rosiglitazone upregulates ENaCs in the renal collecting duct, thereby reducing the diuretic response to *furosemide* while stimulating the diuretic response to *amiloride*. To this end, we measured the effect of *rosiglitazone* on *furosemide*- and *amiloride*-induced natriuresis and the amount of ENaC in urinary exosomes in humans *in vivo* in a double-blind, placebo-controlled, randomized clinical trial.

Results

Thirteen participants with insulin resustance were enrolled in the study (Figure 1). One participant decided to withdraw two weeks after randomization because of personal reasons. Therefore, the final statistical analysis was performed on end-point data for 12 participants (6 participants in each treatment sequence) (Table 1).

Adherence to the study drug (as measured by tablet counting) was excellent during both treatment regimens (*rosiglitazone*: 96%, range 80-102%; placebo:

97%, range 92-101%). The participants reported slightly more adverse events during *rosiglitazone* treatment (total 32 events, 92% of subjects) as compared with placebo (total: 25 events, 100% of subjects). All events were mild including oedema (ankle oedema that did not interference with daily activities) (*rosiglitazone*: 42%; placebo: 25%, $P = 0.32$) and anaemia (defined as haemoglobin levels of < 8.1 mmol/L for men and < 7.3 mmol/L for women) (*rosiglitazone*: haemoglobin: 8.2 ± 0.2 mmol/L; placebo: haemoglobin 8.5 ± 0.2 mmol/L, $P = 0.08$). There was no difference in natriuretic responses between period 1 and 2 either in the total population or in placebo treatment. Therefore, it seems unlikely that either a carryover or a period effect confounded the primary outcome data.

Figure 1: Enrolment of study participants
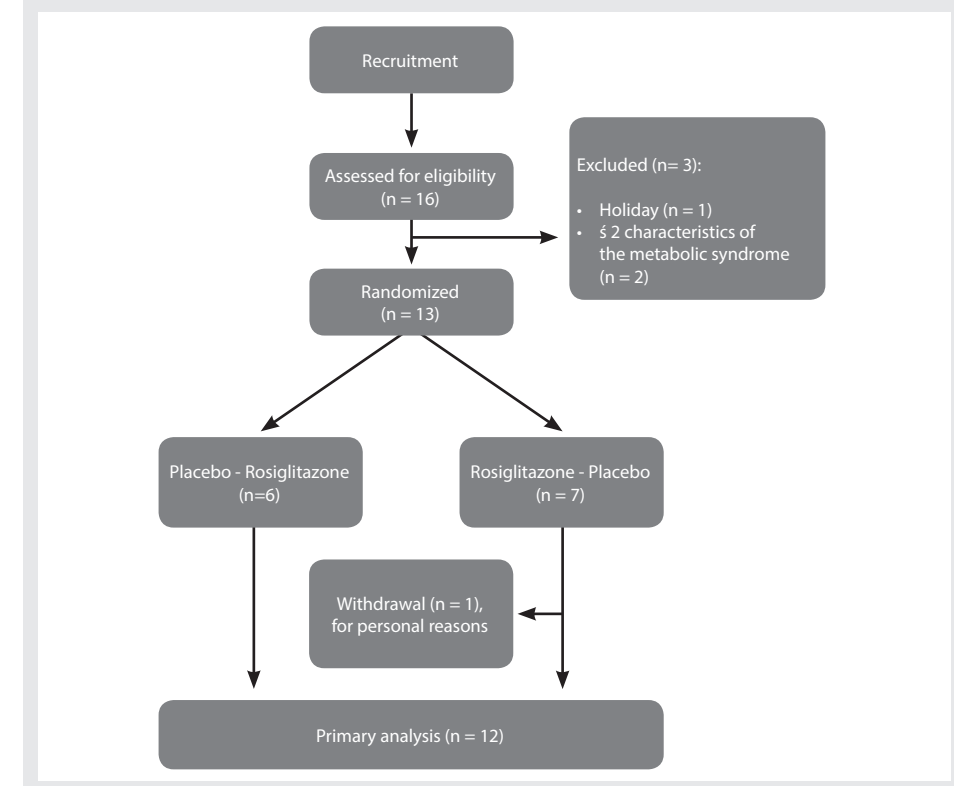
The effect of *rosiglitazone* on metabolic, haemodynamic and fluid variables

Relative to placebo *rosiglitazone* reduced insulin levels (*rosiglitazone*: 8.0 ± 1.0 mU/l *versus* placebo: 11.3 ± 1.5 mU/l, $P < 0.05$) and insulin resistance (Homeostasis model assessment index (HOMA): *rosiglitazone*: 1.67 ± 0.22 *versus* placebo: 2.41 ± 0.34, $P < 0.05$). As expected, given that the participants were not diabetic, *rosiglitazone* did not lower fasting plasma glucose (*rosiglitazone*: 4.6 ± 0.1 mmol/l *versus* placebo: 4.7 ± 0.1 mmol/l, P=NS) or glycosylated haemoglobin (HbA$_{1c}$) (*rosiglitazone*: 5.5 ± 0.1 % *versus* placebo: 5.4 ± 0.1 %, P = NS). In addition, *rosiglitazone* tended to lower alanine transaminase levels (*rosiglitazone*: 26 ± 34 U/l *versus* placebo: 32 ± 4 U/l, P = 0.07).

As compared with placebo, *rosiglitazone* tended to decrease systolic blood pressure (*rosiglitazone*: 121 ±2 mmHg *versus* placebo: 126 ± 3 mmHg, P = 0.09) as well as diastolic blood pressure (*rosiglitazone*: 82 ± 2 mmHg *versus* placebo: 85 ± 2 mmHg, P = 0.04) after 8 weeks of treatment.

As compared with placebo, *rosiglitazone* treatment did not result in an increase in body weight, foot volume, extracellular water concentration or total body water concentration or a decrease in haematocrit level. After 8 weeks of *rosiglitazone* treatment, we did not find differences in plasma concentrations of atrial natriuretic peptide, brain natriuretic peptide, vascular endothelial growth factor, aldosterone, or renin in plasma samples.

Table 1: Baseline characteristics of the 12 evaluable subjects

Variable	Mean±SD
Age (year)	51 ± 8
Male (%)	58
Body Mass Index (kg/m^2)	32.6 ± 3.5
Systolic blood pressure (mmHg)	143±8
Diastolic blood pressure (mmHg)	92 ± 7
Waist circumference (cm)	109 ± 9
Plasma Triglycerides (mmol/L)	1.91 ± 1.24
HDL-cholesterol (mmol/L)	1.20 ± 0.31
Fasting Plasma Glucose (mmol/L)	5.0 ± 0.4
HOMA-index (mU.mmol/L^2)	3.2 ± 1.7
Cockcroft (ml/min)	108 ± 26
Criteria metabolic syndrome	
2 criteria (n)	5
3 criteria (n)	4
4 criteria (n)	3
Medication	
Statin (%)	17
Antihypertensive medication (%)	17

HDL, High Density Lipoprotein
HOMA, Homeostasis model assessment index
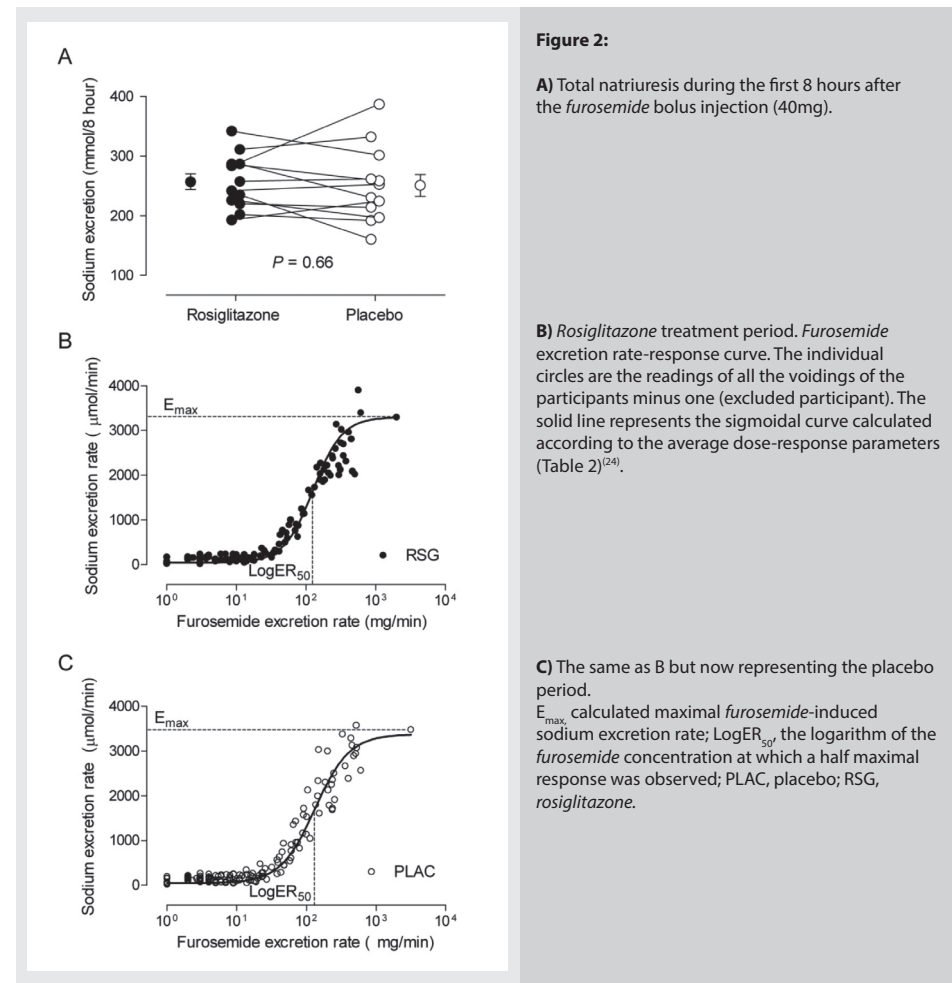
Figure 2:

A) Total natriuresis during the first 8 hours after the *furosemide* bolus injection (40mg).

B) *Rosiglitazone* treatment period. *Furosemide* excretion rate-response curve. The individual circles are the readings of all the voidings of the participants minus one (excluded participant). The solid line represents the sigmoidal curve calculated according to the average dose-response parameters (Table 2)[24].

C) The same as B but now representing the placebo period.
E$_{max}$, calculated maximal *furosemide*-induced sodium excretion rate; LogER$_{50}$, the logarithm of the *furosemide* concentration at which a half maximal response was observed; PLAC, placebo; RSG, *rosiglitazone*.

Natriuretic effects of *furosemide*

Furosemide induced a clear natriuretic response, but sodium excretion over 8 hours was not different between *rosiglitazone* and placebo (257 ± 13 mmol and 251 ± 18 mmol respectively, P = 0.66; Figure 2A) nor were there differences in the natriuretic response as assessed by comparing the *furosemide* excretion rate-response curves (Table 2; Figure 2B,C). The analyses of these *furosemide* excretion rate-response curves were performed after exclusion of one participant, for whom nonlinear regression analysis resulted in an unrealistically maximum

sodium excretion rate. Post-hoc, we reanalyzed the data without excluding this particiapant's data, but this did not change the conclusion that there was no difference in natriuretic effect of *furosemide*.

Furosemide concentrations, in both plasma and urine, were similar for both treatment periods (Table 3). The peak value obtained for *furosemide* concentration was consistent with previous studies by our group[24]. In addition, the value for area under the curve was in line with observations of Beermann et al.[25]. Except for blood pressure, which was slightly lower during *rosiglitazone*, the baseline values before the *furosemide* experiments were similar during both treatment periods (Table 4).

Table 2: Indexes of the natriuretic and diuretic response to *furosemide*

Parameter	Rosiglitazone	Placebo	P-value
Urine 8 hours sodium output (mmol)	257 ± 13	251 ± 18	NS
Urine 24 hours sodium output (mmol)	297 ± 12	296 ± 26	NS
Baseline sodium excretion rate (µmol/min)	46.9 ± 8	44.6 ± 12	NS
Sodium E_{max} (µmol/min)	3299 ± 490	3480 ± 300	NS
Furosemide ER_{50} (µg/min)	127 ± 5	133 ± 4	NS
Hill slope	1.98 ± 0.2	1.71 ± 0.2	NS

Data are mean ± SE; n = 12
E_{max}: Calculated maximal *furosemide*-induced sodium excretion rate. ER_{50}: calculated from the analysis of the *furosemide* excretion rate-response curves. The ER_{50} is the *furosemide* excretion rate at which a half-maximal response was observed. NS, nonsignificant

Natriuretic effects of *amiloride*

Amiloride induced a clear natriuretic response, but sodium excretion over 24 hours was not different between *rosiglitazone* and placebo (313 ± 21 mmol and 315 ± 20 mmol, respectively; $P = 0.90$, Figure 3A).

Amiloride concentrations in both plasma and the urine were similar for *rosiglitazone* and placebo treatment periods (Table 3). The steady-state concentration of *amiloride* (42 ng/ml) was within the chosen range. In addition, the baseline parameter valueprior to commencing *amiloride* experiments were

similar for the *rosiglitazone* and placebo treatment periods, except for the blood pressure readings, which were slightly lower during *rosiglitazone* treatment (Table 4).

Table 3: Exposition to *furosemide* and *amiloride* during the diuretic experiments.

Parameter	Amiloride		Furosemide	
	rosiglitazone	placebo	rosiglitazone	placebo
Peak plasma level (ng/ml; µg/ml)	96 ± 7	103 ± 8	2.9 ± 0.3	3.2 ± 0.2
Plasma steady state (ng/ml)	42 ± 2	43 ± 2	NA	NA
AUC plasma concentration (µg.ml^{-1}.min^1)	22.5 ± 1.1	22.5 ± 1.3	166 ± 13	166 ± 13
Peak urinary concentration (µg/ml)	18.4 ± 2.9	18.5 ± 2.7	453 ± 52	482 ± 59
Mean urinary concentration (µg/ml)^a	7.8 ± 1.6	6.8 ± 0.6	NA	NA

Data are mean ± SE; n = 12; No significant differences observed
^{a}Total amount of *amiloride* divided by total volume of urine during the first 8 hours.
AUC, Area under curve; NA, not applicable

Table 4: Baseline conditions before the diuretic experiments.

Parameter	Amiloride		Furosemide	
	rosiglitazone	placebo	rosiglitazone	placebo
Baseline 24-hours sodium output (mmol)	204±40	184±29	151±12	139±19
Plasma creatinin (µmol/L)	73±3	73±3	73±4	71±3
Systolic blood pressure (mmHg)	123±3^a	129±2	121±2	126±3
Diastolic blood pressure (mmHg)	80±2^a	85±2	82±2^a	85±2
Aldosterone (nmol/L)	0.11±0.02	0.13±0.03	0.14±0.03	0.22±0.08
Renin (mE/L)	10±2	9±2	10±2	12±2
Atrial natriuretic peptide (pmol/L)	16±2	16±2	15±2	12±1

Data are mean ± SE; n = 12;
$^a P < 0.05$

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Figure 3:

A) Total natriuresis during 24 hours after the start of the *amiloride* infusion in the individual participant during both periods.

B) An equal amount of urinary exosome proteins from the individual participants (depicted by treatment number), was blotted for α-ENaC both during *rosiglitazone* (black bars) and placebo treatment (broken line). Signals were quantified by densitometry and individually normalized for placebo-treated α-ENaC levels (100%). ENaC: epithelial sodium channel.

α-ENaC abundance in exosomes

When normalized for the total amount of urinary exosome protein and for the individual placebo-treated α-ENaC exosome abundance, *rosiglitazone* did not increase α-ENaC exosome abundance (*rosiglitazone*: 83 ± 12%; placebo: 100%; $P = 0.15$) (see Figure 3B for the individual data). Also, there was no influence of *rosiglitazone* on α-ENaC abundance normalized for the individual urinary creatinine concentration (*rosiglitazone*: 282±123%; placebo: 100%; $P = 0.40$). We were able to measure α-ENaC abundance in 7 out of the 12 participants. In two participants the urine was too dilute to isolate enough protein for immunoblotting, and in three others we were unable to detect α-ENaC even though sufficient protein was avaiable.

Discussion

The most important observation in this study was the lack of any alteration in the diuretic response to *furosemide* and *amiloride* in insulin-resistant subjects treated with *rosiglitazone*. This observation is robust because the natriuretic responses to *furosemide* and *amiloride* were quantified and analyzed with high accuracy in this trial. Moreover, the findings are relevant, because the outcome parameters (natriuresis and diuresis) are of obvious clinical significance. Altogether, the results convincingly show that treatment with *rosiglitazone* does not reduce the diuretic response to *furosemide* in subjects with insulin resistance.

Interestingly, the hypothesis of our study was also addressed by an elegant clinical study of Karalliedde et al.[15]. Their conclusion was that *spironolactone* was superior to *furosemide* in the treatment of *rosiglitazone*-induced oedema in diabetic patients. This suggests, at the minimum, that *furosemide* was less effective than expected. The authors viewed their finding as compatible with the upregulation of ENaCs in the renal collecting ducts. However, their study did not compare the diuretic responses during *rosiglitazone* and placebo; instead, it investigated the diuretic responses to *spironolactone*, *furosemide* and *hydrochlorothiazide* separately in an open-label parallel study. Therefore, differences in the dose of these three diuretic drugs chosen could have been responsible for the observed differences in outcome.

There are also a few case reports or case series suggestive for resistance to loop diuretics during thiazolidinedione treatment[19-21]. Obviously, these case reports were not placebo-controlled , and leave ample room for alternative explanations for the described resistance to loop diuretics. For instance, the subjects had established diabetes, with more insulin resistance and overt fluid retention in comparison with our study population. In order to support the clinical relevance of our data we performed a post hoc analysis whether oedema formation or degree of insulin resistance influences the natriuretic response to *furosemide* during *rosiglitazone* treatment. Within our population we could not detect such an influence. However, in actual practice, subjects may have additional problems (e.g., autonomic neuropathy) in addition to their original metabolic disease, which may influence the water and sodium homeostasis[26]. This makes us careful with the extrapolation of our results to the clinic.

In the present study, we thoroughly investigated the pharmacodynamic properties of *furosemide* during placebo and *rosiglitazone* phases under similar baseline conditions (Table 4). Therefore, pharmacodynamic mechanisms of thiazolidinedione-induced *furosemide* resistance, such as ENaC upregulation, increments in renin-angiotensin-aldosterone system (RAAS) activity or atrial natriuretic peptide secretion, including decreased tubular *furosemide* secretion (pharmacokinetic) are more or less excluded by our study. However, other differences in pharmacokinetics, such as changes in bioavailability of the orally administered drug, were not investigated and are beyond the scope of this study.

As mentioned, Karalliedde et al.[15] suggested superiority of *spironolactone* over *furosemide* in the treatment of thiazolidinedione-induced oedema. On the basis of the concept of ENaC upregulation we hypothesized that the optimal treatment for thiazolidinedione-induced oedema would be the ENaC-inhibitor *amiloride*[17]. It is important to stress that our study was not designed to address whether *amiloride* is superior to *furosemide* in the treatment of thiazolidinedione-induced oedema. The higher 24-hours natriuresis during *amiloride* is the result of the dosing schedules chosen. The clinical message that emerges from our study regarding diuretic therapy in thiazolidinedione-induced oedema is that we found no thiazolidinedione-induced *furosemide* resistance.

Apart from the lack of any effect of *rosiglitazone* on the response to *furosemide*, we also observed that the response was unaffected by the ENaC-inhibitor *amiloride*. This argues against a relevant upregulation of ENaCs in the renal collecting ducts in *rosiglitazone*-treated subjects. This clinical observation is further supported by direct measurements of ENaC expression in urinary exosomes.

To date, the relationship between *rosiglitazone* and ENaCs has been studied only in animals. Guan et al.[11] provided *in vivo* evidence for upregulation of ENaC in a mouse model with *pioglitazone*, and Hong et al.[12] had previously shown comparable results for both *rosiglitazone* and *pioglitazone*. Therefore, it is not likely that our negative findings are compound-related. This is in line with the joined consensus statement by the American Heart Association and the American Diabetes Association that considered fluid retention to be a class effect of thiazolidinediones rather than an effect of the individual compound[27]. Interspecies differences could be responsible for the discrepancy between the animal experiments and those of the present study.

Three elegant animal *in vivo* studies have thoroughly investigated whether inhibition of ENaC could reduce oedema formation. Guan et al.[11] provided evidence for the primary role of ENaC upregulation, by using *amiloride* in mice without peroxisome proliferator activated-receptor gamma (PPAR-γ) in their collecting duct cells. On the other hand, neither Chen et al.[28] using *amiloride* for direct ENaC inhibition, nor Vallon et al.[29], using a collecting-duct-specific gene inactivation of ENaCs, were able to reduce thiazolidinedione-induced oedema. Our clinical results related to the response to *amiloride* and *furosemide*, as well as our data on ENaC exosome abundance are in line with the latter two studies and a subsequent editorial[30] and challenge the concept of thiazolidinedione-induced ENaC upregulation with subsequent oedema formation. It could be argued that the findings in our mildly insulin-resistant non-diabetic population without fluid retention, are not representative of patients in actual practise. Although our *post hoc* analysis did not detect ane effect of insulin resistance and oedema formation on the response to *amiloride*, we admit that there are still some limitations to the extrapolation of our findings to daily practice.

Our data on α-ENaC exosome abundance must be interpreted with caution for a number of reasons. First, we were able to measure α-ENaC in only seven participants. Second, we assume that α-ENaC exosome abundance provides a measure of the ENaC expression in the kidney. Although data from studies in animals data showed a correlation between exosome abundance and kidney expression with respect to other transporters,[31] proof that such a correlation exists with respect to ENaCs is lacking. In addition, we did not measure independent confounders of this correlation, such as production and degradation rate of ENaCs[32]. In contrast, our observations on the diuretic response to *furosemide* and *amiloride* during *rosiglitazone* compared with placebo were very accurate and complete, and can be interpreted without any caution. Therefore, our complete data set argues against upregulation of renal ENaCs after *rosiglitazone* treatment in these mildly insulin-resistant nondiabetic subjects. An alternative hypothesis for thiazolidinedione-induced fluid retention is vasodilation[27], and this could unify our observations with the clinical observations on *furosemide*-resistant thiazolidinedione-induced oedema. Oedema related to microvascular imbalance will not resolve adequately by *furosemide* treatment even despite normal natriuresis could be obtained after a single dose. An illustrative example is oedema associated with *nifedipine* treatment, which is thought to be due to

microvascular imbalance. It develops despite the natriuretic characteristics of *nifedipine* and is resistant to additional diuretic treatment[33, 34].

The result of the tablet counts suggests that there was optimal compliance with *rosiglitazone* treatment. This is supported by the fact that *rosiglitazone* treatment ameliorated insulin sensitivity and lowered systolic and diastolic blood pressure, which is in line with our previous findings in such a population[35] and argues against primary renal fluid retention. As expected in this nondiabetic population, plasma glucose concentrations and HbA_{1c} levels did not change after *rosiglitazone* treatment. Consequently, our data on natriuresis have not been confounded by diuretic effects of changes in glucosuria as a result of the intervention with *rosiglitazone*. In theory, the slightly lower blood pressure during *rosiglitazone* treatment could have affected the natriuretic response to *furosemide* and *amiloride*. However, the lowering in blood pressure was mild, and well within the range of autoregulation; we therefore assume this did not influence our results. Finally, anaemia was more common in *rosiglitazone*-treated subjects, which may be compatible with the presence of some fluid retention in these individuals.

In conclusion, the results of this study in insulin-resistant nondiabetic subjects challenge the concept that, in humans, thiazolidinediones by themselves reduce the diuretic response to *furosemide*. The lack of any effect of *rosiglitazone* on the diuretic response to *amiloride* and on the ENaC abundance in urinary exosomes, argues against an effect of thiazolidinediones on ENaCs in the renal collecting duct.

Methods

Subjects

The study population consisted of 13 nondiabetic subjects and was characterized by two features of the metabolic syndrome (American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI) criteria)[36]. Their age ranged from 30 to 70 years. Diabetes was excluded because the expected reduction of glucosuria as a result of *rosiglitazone* would have seriously confounded our results on natriuresis and diuresis. Nonetheless, we tried to get as close as possible to the target population for thiazolidinedione treatment, and therefore we selected

insulin-resistant subjects who had not developed diabetes.

Subjects were not eligible for inclusion if fasting glucose was >7.0 mmol/L, they used hypoglycaemic agents, had a documented significant hypersensitivity to a peroxisome proliferator-activated receptor-γ agonist, or were simultaneously participating in another study. Additional exclusion criteria were clinically significant liver disease or anaemia, angina or heart failure (New York Heart Association classification I-IV); calculated creatinine clearance below 40 ml/min; abuse of alcohol, liquorice or drugs; and pregnancy or lactation. Study participants were selected by advertisement, and each gave written informed consent. Subjects who participated in this study received financial compensation. The study protocol was approved by the hospital ethics committee, was in accordance with the declaration of Helsinki, was registered at clinical trials.gov (NCT00285805) and performed according to Good Clinical Practice guidelines.

Study design

This was a randomized, placebo-controlled, double-blind, single-centre, crossover study comparing *rosiglitazone* 4 mg b.i.d. with placebo for 9-week treatment periods (washout period, 4 weeks). The medication used in the study was provided by GlaxoSmithKline (Brentford, United Kingdom). Randomization of the treatment sequence was computer-generated, with a sequentially driven allocation. Randomization and blinding were performed at the department of Clinical Pharmacy of Radboud University Nijmegen Medical Centre. In both treatment periods, the end-point experiments were performed with *furosemide* and *amiloride* at the end of weeks 8 and 9, respectively. During all the visits (week 0, 4, 8, 9) of each period, adverse events and compliance with treatment regimen (assessed by counting tablets) were recorded. In addition, physical examination, foot-volume and bioimpedance measurements were performed and safety-related biochemical and hematological profiles were determined. Only at start and at 8 weeks in each period, glucose, insulin and HbA_{1c} levels measured. All visits and interventions were performed at the Clinical Research Centre of the Radboud University Nijmegen Medical Centre.

Furosemide end-point experiment

Each participant visited the hospital at 8 a.m. after an overnight fast and having abstained from consumption of alcohol and caffeine for 20 hours, for 24-hour urine collection and the morning urine collection. On the three previous days each participant had to adhere to an individualized diet containing 150 mmol of sodium and 80 mmol of potassium prescribed by a dietician[24]. First, blood was collected to measure fasting glucose and insulin concentrations. Next, each subject was given an individualized breakfast along with one cup of water. Afterward, a brachial vein was cannulated and connected to a Braun infusion pump (B. Braun Medical, Sheffield, UK)(10 ml/hr NaCl 0.9%), and blood samples were drawn for safety and vascular hormone measurements (levels of aldosterone, atrial natriuretic peptide, brain natriuretic peptide, vascular endothelial growth factor and renin).

A bolus of 40 mg *furosemide* (Centrafarm, Etten-Leur, the Netherlands) was injected through a small cannule in a vein of the contra-lateral arm, immediately after emptying of the bladder. Venous blood samples were drawn at 0, 15, 30, 45, 60, 90,120, 150, 180, 240, 300, 360, 420 and 480 minutes after bolus injection to measure *furosemide* concentrations in plasma. The participants were asked to urinate regularly, at least once every hour. The exact time of voiding and the urine volume passed were recorded. After each voiding, the urine was separated into two samples. In one sample, sodium and creatinine concentrations were measured while the other sample was light-protected and immediately frozen for measurement of *furosemide* concentrations later on. To prevent dehydration each participant had to drink tap water equal in volume of diuresis in the previous hour[24]. During the test, the participant was in a sitting position on a bed. At noon, the participant was offered an individualized lunch. After 8 hours, each participant left the hospital with instructions regarding adherence to the prescribed diet but without restrictions on fluid intake. They were asked to collect their urine for up to 24 hours after the start of the experiment.

Amiloride end-point experiment

The initial setup for the *amiloride* experiment resembled the *furosemide* experiment. Until *amiloride* infusion, the procedures were similar. At time point 0, venous infusion of a loading dose of *amiloride* was started (150 μg/kg in 60 minutes) followed by maintenance infusion (0.20 μg/kg/min) for 4 hours.

Amiloride (Duchefa pharma BV, Haarlem, The Netherlands) was obtained as a sterile powder which was dissolved directly before use in NaCl 0.9% up to a concentration of 1 mg/ml. The solution was filtered through a 0.22 μm Millipore filter (Millipore, Billerica, MA). For measuring the concentration levels of *amiloride* in plasma, venous blood samples were drawn at 60, 180, 300 and 420 minutes. All the other procedures were similar to those in the *furosemide* experiment.

Pharmacokinetic considerations related to *amiloride* dosage

According to the literature, after the oral intake of 10 or 20 mg *amiloride,* peak concentration levels in plasma are reached after 3 to 4 hours and measure 20 μg/L[37] and 38-40 μg/L, respectively[38]. These concentrations are well below the half-maximal inhibitory concentration (IC_{50}) of *amiloride* for Na^+/H^+ and Na^+/Ca^{2+}-transporters and for the α_1-adrenergic receptor, but well above the IC_{50} for ENaC[39]. Using the pharmacokinetic characteristics of *amiloride*[37], we calculated the required dosing schedule of *amiloride* infusion required to reach a steady-state concentration between 30-45 μg/L.

Exosome extraction and quantification of α-ENaC

Urinary exosomes originate as the internal vesicles of multivesicular bodies and are delivered to the urine from all renal epithelial cell types. Exosomes contain a collection of cytosolic and apical plasma membrane proteins, including ENaCs from renal cortical collecting duct cells[32]. ENaC is a heteromultimeric protein containing three homologous subunits (α, β, and γ)[14]. Urinary exosomes were isolated by ultracentrifugation and α-ENaC abundance was measured by immunoblotting, as previously described[40, 41] and normalized to urine creatinine levels. Next, four μg of protein lysed in Laemmli buffer was loaded on 8% SDS-PAGE. Gel electrophoresis, blotting, and blocking of the polyvinylidene difluoride membranes were carried out as previously described[40]. The membrane was incubated with 1:4000-diluted affinity-purified rabbit α-ENaC antibody (kindly provided by BC Rossier, Lausanne, Switzerland), followed by 1:5,000-diluted goat anti-rabbit immunoglobulin G as secondary antibody coupled to horseradish peroxidase. Blotting signals were visualized using enhanced chemiluminescence (ECL, Pierce, Rockford, IL). The results were normalized for the expression level of α-ENaC during placebo treatment and calculated as percentages.

Analytical methods

The concentration levels of *furosemide* and *amiloride* concentration in plasma and urine were measured using high-performance liquid chromatography[42,43]. Plasma insulin was measured by a radioimmunoassay. HOMA was used to determine insulin resistance with the following formula: fasting plasma insulin (µU/ml) x fasting plasma glucose (mmol/l)/22.5[44].

Plasma atrial natriuretic peptide was analyzed by radioimmunoassay (Euro diagnostica BV, Arnhem, The Netherlands), brain natriuretic peptide using a fluorescence method with a Triage Meter Plus (Biosite, San Diego, CA), renin by means of an immunoradiometric assay (Cis Bio International, Gif-sur-Yvette, France), aldosterone by radioimmunoassay, and vascular endothelial growth factor by enzyme-linked immunosorbent assay.

Bioimpedance and foot volume

During each visit, total body water and extracellular volume were assessed in all the participants using the Akern 2000 bioelectrical impedance analyzer[45] (Akern, Florence, Italy). Foot volume was assessed using the water-displacement method[46].

Data analysis

The study was powered (80%) to detect a *rosiglitazone*-induced increase in natriuretic response of 40 mmol/24hours during *amiloride*, or a *rosiglitazone*-induced reduction of the half maximal response (ER_{50}) in the *furosemide* excretion rate of 20 µgram/min. All statistical tests and confidence intervals were two-sided and the overall type I error was 5%. Descriptive statistics of population characteristics are presented as mean with standard deviation. Treatment effects are presented as mean with standard error; these were statistically analyzed by the paired Student's t-test. The response was measured at the end of each treatment period, with the assumption that any carry-over effects from the previous treatment period would have been washed out. Responses to *furosemide* were evaluated by plotting the natriuretic response against the *furosemide* excretion rate. These *furosemide* excretion rate–response curves were analyzed according to a sigmoidal curve with variable slope by nonlinear regression using GraphPad Prism5.03 (GraphPad Software, San Diego, CA)[24]. All statistical analyses were performed using the SPSS personal computer software package16.0 (SPSS, Chicago, IL).

Acknowledgments

References

1. Yki-Jarvinen H. Thiazolidinediones. *N Engl J Med* 2004; 351(11):1106-18.

2. Kahn SE, Haffner SM, Heise MA, Herman WH, Holman RR, Jones NP et al. Glycemic durability of rosiglitazone, metformin, or glyburide monotherapy. *N Engl J Med* 2006; 355(23):2427-43.

3. Parulkar AA, Pendergrass ML, Granda-Ayala R, Lee TR, Fonseca VA. Nonhypoglycemic effects of thiazolidinediones. *Ann Intern Med* 2001; 134(1):61-71.

4. Rennings AJ, Meijer P, van Uden DJ, Tack CJ, Smits P, Boerman OC et al. Rosiglitazone reduces ischaemia-reperfusion injury in patients with the metabolic syndrome. *Eur Heart J* 2010; 31(8):983.

5. Nissen SE, Wolski K. Effect of rosiglitazone on the risk of myocardial infarction and death from cardiovascular causes. *N Engl J Med* 2007; 356(24):2457-71.

6. Home PD, Pocock SJ, Beck-Nielsen H, Curtis PS, Gomis R, Hanefeld M et al. Rosiglitazone evaluated for cardiovascular outcomes in oral agent combination therapy for type 2 diabetes (RECORD): a multicentre, randomised, open-label trial. *Lancet* 2009; 373(9681):2125-35.

7. Gerstein HC, Miller ME, Byington RP, Goff DC, Jr., Bigger JT, Buse JB et al. Effects of intensive glucose lowering in type 2 diabetes. *N Engl J Med* 2008; 358(24):2545-59.

8. Graham DJ, Ouellet-Hellstrom R, Macurdy TE, Ali F, Sholley C, Worrall C et al. Risk of Acute Myocardial Infarction, Stroke, Heart Failure, and Death in Elderly Medicare Patients Treated With Rosiglitazone or Pioglitazone. *JAMA* 2010.

9. Werner AL, Travaglini MT. A review of rosiglitazone in type 2 diabetes mellitus. *Pharmacotherapy* 2001; 21(9):1082-99.

10. Dormandy JA, Charbonnel B, Eckland DJ, Erdmann E, Massi-Benedetti M, Moules IK et al. Secondary prevention of macrovascular events in patients with type 2 diabetes in the PROactive Study (PROspective pioglitAzone Clinical Trial In macroVascular Events): a randomised controlled trial. *Lancet* 2005; 366(9493):1279-89.

11. Guan Y, Hao C, Cha DR, Rao R, Lu W, Kohan DE et al. Thiazolidinediones expand body fluid volume through PPARgamma stimulation of ENaC-mediated renal salt absorption. *Nat Med* 2005; 11(8):861-6.

12. Hong G, Lockhart A, Davis B, Rahmoune H, Baker S, Ye L et al. PPARgamma activation enhances cell surface ENaCalpha via up-regulation of SGK1 in human collecting duct cells. *FASEB J* 2003; 17(13):1966-8.

13. Loffing J, Korbmacher C. Regulated sodium transport in the renal connecting tubule (CNT) via the epithelial sodium channel (ENaC). *Pflugers Arch* 2009; 458(1):111-35.

14. Rossier BC, Canessa CM, Schild L, Horisberger JD. Epithelial sodium channels. *Curr Opin Nephrol Hypertens* 1994; 3(5):487-96.

15. Karalliedde J, Buckingham R, Starkie M, Lorand D, Stewart M, Viberti G. Effect of various diuretic treatments on rosiglitazone-induced fluid retention. *J Am Soc Nephrol* 2006; 17(12):3482-90.

16. Perez-Ayuso RM, Arroyo V, Planas R, Gaya J, Bory F, Rimola A et al. Randomized comparative study of efficacy of furosemide versus spironolactone in nonazotemic cirrhosis with ascites. Relationship between the diuretic response and the activity of the renin-aldosterone system. *Gastroenterology* 1983; 84(5 Pt 1):961-8.

17. Semenkovich CF. TZDs and diabetes: testing the waters. *Nat Med* 2005; 11(8):822-4.

18. Jeunemaitre X, Bassilana F, Persu A, Dumont C, Champigny G, Lazdunski M et al. Genotype-phenotype analysis of a newly discovered family with Liddle's syndrome. *J Hypertens* 1997; 15(10):1091-100.

19. Hirsch IB, Kelly J, Cooper S. Pulmonary oedema associated with troglitazone therapy. *Arch Intern Med* 1999; 159(15):1811.

20. Thomas ML, Lloyd SJ. Pulmonary oedema associated with rosiglitazone and troglitazone. *Ann Pharmacotheraphy* 2001; 35(1):123-4.

21. Niemeyer NV, Janney LM. Thiazolidinedione-induced oedema. *Pharmacotherapy* 2002; 22(7):924-9.

22. Odlind B, Beermann B. Renal tubular secretion and effects of furosemide. *Clin Pharmacol Ther* 1980; 27(6):784-90.

23. Brater DC. Diuretic therapy. *N Engl J Med* 1998; 339(6):387-95.

24. van Meyel JJ, Smits P, Russel FG, Gerlag PG, Tan Y, Gribnau FW. Diuretic efficiency of furosemide during continuous administration versus bolus injection in healthy volunteers. *Clin Pharmacol Ther* 1992; 51(4):440-4.

25. Beermann B. Kinetics and dynamics of furosemide and slow-acting furosemide. *Clin Pharmacol Ther* 1982; 32(5):584-91.

26. Rennings AJ, Smits P, Stewart MW, Tack CJ. Autonomic neuropathy predisposes to rosiglitazone-induced vascular leakage in insulin-treated patients with type 2 diabetes: a randomised, controlled trial on thiazolidinedione-induced vascular leakage. *Diabetologia* 2010; 53(9):1856-66.

27. Nesto RW, Bell D, Bonow RO, Fonseca V, Grundy SM, Horton ES et al. Thiazolidinedione use, fluid retention, and congestive heart failure: a consensus statement from the American Heart Association and American Diabetes Association. October 7, 2003. *Circulation*

2003; 108(23):2941-8.

28. Chen L, Yang B, McNulty JA, Clifton LG, Binz JG, Grimes AM et al. GI262570, a peroxisome proliferator-activated receptor {gamma} agonist, changes electrolytes and water reabsorption from the distal nephron in rats. *J Pharmacol Exp Ther* 2005; 312(2):718-25.

29. Vallon V, Hummler E, Rieg T, Pochynyuk O, Bugaj V, Schroth J et al. Thiazolidinedione-Induced Fluid Retention Is Independent of Collecting Duct {alpha}ENaC Activity. *J Am Soc Nephrol* 2009.

30. Nofziger C, Blazer-Yost BL. PPARgamma agonists, modulation of ion transporters, and fluid retention. *J Am Soc Nephrol* 2009; 20(12):2481-3.

31. Esteva-Font C, Wang X, Ars E, Guillen-Gomez E, Sans L, Gonzalez S, I et al. Are Sodium Transporters in Urinary Exosomes Reliable Markers of Tubular Sodium Reabsorption in Hypertensive Patients? *Nephron Physiol* 2010; 114(3):25-34.

32. Gonzales P, Pisitkun T, Knepper MA. Urinary exosomes: is there a future? *Nephrol Dial Transplant* 23 2008; (6):1799-801.

33. Wetzels JF, Wiltink PG, Hoitsma AJ, Huysmans FT, Koene RA. Diuretic and natriuretic effects of nifedipine in healthy persons. *Br J Clin Pharmacol* 1988; 25(5):547-53.

34. van der Heijden AG, Huysmans FT, van Hamersvelt HW. Foot volume increase on nifedipine is not prevented by pretreatment with diuretics. *J Hypertens* 2004; 22(2):425-30.

35. Rennings AJ, Smits P, Stewart MW, Tack CJ. Fluid retention and vascular effects of rosiglitazone in obese, insulin-resistant, nondiabetic subjects. *Diabetes Care* 2006; 29(3):581-7.

36. Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA et al. Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. *Circulation* 2005; 112(17):2735-52.

37. Jones KM, Liao E, Hohneker K, Turpin S, Henry MM, Selinger K et al. Pharmacokinetics of amiloride after inhalation and oral administration in adolescents and adults with cystic fibrosis. *Pharmacotherapy* 1997; 17(2):263-70.

38. Weiss P, Hersey RM, Dujovne CA, Bianchine JR. The meetabolism of amiloride hydroloride in man. *Clin Pharmacol Ther* 1969; 10(3):401-6.

39. Pickkers P, van BM, Hughes AD, Russel FG, Thien T, Smits P. Presence and mechanism of direct vascular effects of amiloride in humans. *J Cardiovasc Pharmacol* 1999; 34(3):388-93.

40. Kamsteeg EJ, Wormhoudt TA, Rijss JP, van Os CH, Deen PM. An impaired routing of wild-type aquaporin-2 after tetramerization with an aquaporin-2 mutant explains dominant nephrogenic diabetes insipidus. *EMBO J* 1999; 18(9):2394-400.

41. Pisitkun T, Shen RF, Knepper MA. Identification and proteomic profiling of exosomes in human urine. *Proc Natl Acad Sci U S A* 2004; 101(36): 13368-73.

42. Russel FG, Tan Y, Van Meijel JJ, Gribnau FW, Van Ginneken CA. Solid-phase extraction of furosemide from plasma and urine and subsequent analysis by high-performance liquid chromatography. *J Chromatogr* 1989; 496(1):234-41.

43. Forrest G, McInnes GT, Fairhead AP, Thompson GG, Brodie MJ. Simple high-performance liquid chromatographic method for the measurement of amiloride in body fluids. *J Chromatogr* 1988; 428(1):123-30.

44. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985; 28(7):412-9.

45. Lukaski HC. Methods for the assessment of human body composition: traditional and new. *Am J Clin Nutr* 1987; 46(4):537-56,.

46. van Hamersvelt HW, Kloke HJ, de Jong DJ, Koene RA, Huysmans FT. Oedema formation with the vasodilators nifedipine and diazoxide: direct local effect or sodium retention? *J Hypertens* 1996; 14(8):1041-5.

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Change in haematocrit does not reflect change in plasma volume in individuals on chronic treatment with thiazolidinediones

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Abstract

Thiazolidinediones (TZDs) expand plasma volume, which can cause oedema and heart failure. The current assumption is that the increase in plasma volume is reflected by a decrease in haematocrit. However, this is only valid when the total red blood cell volume is constant and thiazolidinediones can influence erythropoiesis. We used data from two double-blind placebo-controlled randomized trials with insulin-resistant subjects and diabetic subjects treated with *rosiglitazone* for 12-16 weeks to test this relationship. Plasma volume was measured directly by the dilution of ^{125}I-labelled human albumin. *Rosiglitazone* increased plasma volume by approximately 9% (ratio: 1.09 ± 0.02; $P < 0.001$), and decreased haematocrit by approximately 5% (ratio: 0.95 ± 0.01; $P < 0.001$), but there was no correlation at all between the change in plasma volume and the change in haematocrit (r = 0.009; $P = 0.96$). Thus, the observed change in haematocrit during treatment with *rosiglitazone* does not reflect changes in plasma volume and implicates that the reduction in haematocrit observed during thiazolidinedione treatment is, at least partly, explained by a decrease in red blood cell volume. It also shows that measurement of haematocrit cannot be used to monitor plasma volume expansion in individuals on thiazolidinedione treatment.

Introduction

Treatment with thiazolidinediones (TZDs) is associated with fluid retention and plasma volume expansion[1-3]. This important side effect can lead to oedema and heart failure and is one of the reasons for the limited risk benefit ratio for this class of drugs. The observed drop in haematocrit during thiazolidinedione treatment[4] is often attributed to plasma volume expansion and reported as a marker for fluid retention[5,6], but this has never undergone direct testing. Changes in haematocrit and plasma volume will only be correlated if total red blood cell volume remains constant[7]. This may not necessarily be the case, as an in-vitro human cell study has shown suppression of erythroid-colony forming cells by *troglitazone* and *pioglitazone*[8]. This notion is supported by a case report describing hypoerythropoetinemia due to *rosiglitazone*[9]. Also, it has recently become clear that thiazolidinediones induce osteopenia, which is partly explained by bone marrow fat hyperplasia[10,11]. So, the drop in haematocrit during thiazolidinedione treatment may be explained by a decreased red blood cell volume. This possibility is further supported by small drops in white blood cell counts observed during thiazolidinedione treatment[12] and by findings in older reports that gradual changes in plasma volume are not reflected at all by changes in haematocrit[7,13].

Plasma volume can be measured directly by dilutional methods, like measurement of the volume of distribution of radioactive iodinated albumin[2,3,14,15]. The advantage of this technique is that the measurements are not confounded by changes in red blood cell production and/or degradation.

Table 1: Baseline characteristics of evaluable subjects and patients (means±SD)

Characteristics	Study 1	Study 2	
	Rosiglitazone n = 15	Rosiglitazone n = 13	Placebo n = 16
Male (%)	60	54	50
Age (year)	46 ± 8	57 ± 8	59 ± 10
BMI (kg/m^2)	32 ± 3	29 ± 4	30 ± 7
Waist (cm)	109 ± 8	98 ± 10	103 ± 18
Systolic blood pressure (mmHg)	137 ± 10	129 ± 14	131 ± 16
Diastolic blood pressure (mmHg)	94 ± 4	79 ± 7	80 ± 8
Calculated creatinin clearance	96 ± 30	90 ± 16	85 ± 33
Duration of diabetes (years)		14.2 ± 7	14.2 ± 6
HbA1c (%)	5.5 ± 0.3	8.0 ± 1.3	8.2 ± 1.2
Insulin dose (U/day)		62 ± 46	72 ± 33

BMI: Body Mass Index
HDL: High Density Lipoprotein
HbA1c: Glycosylated haemoglobin

Table 2: Treatment effect on endpoints in ratio (mean±SE)

Endpoint	Study 1	Study 2		Pooled analysis
	Rosiglitazone	Rosiglitazone	Placebo	Rosiglitazone
Plasma volume	1.09±0.03*	1.10± 0.02**	0.99±0.03	1.09±0.02#
Haematocrit	0.96±0.01*	0.94±0.02**	0.99±0.01	0.95±0.01#
Plasma albumin	0.99±0.01	0.98± 0.02	0.99±0.01	0.98±0.01
TBW	1.00 ±0.01	1.02± 0.01	1.01±0.01	1.01±0.01

* $P < 0.05$; ** $P < 0.01$; # $P < 0.001$
MCV: Mean Corpuscular Volume
TBW: Total Body Water

Results

In this study we investigate whether changes in plasma haematocrit correlate to changes in directly measured plasma volume (^{125}I-labelled human albumin) in individuals who were treated with *rosiglitazone* for at least 3 months in the context of two previously reported trials. In the first study, overweight, insulin-

resistant subjects with obvious features of the metabolic syndrome were included. Fifteen subjects had paired plasma volume measurements. One subject in this study developed moderate oedema during *rosiglitazone* treatment[2].

The second study consisted of insulin-treated patients with type 2 diabetes who were additionally treated with *rosiglitazone* or placebo. In this study paired plasma volume measurements were obtained in thirteen patients in the *rosiglitazone*-treated group and sixteen patients in the placebo-treated group. Here, oedema was more prevalent in the *rosiglitazone* group compared to placebo (63% vs. 24%, $P < 0.05$), but always mild[3]. Baseline characteristics of the study populations are depicted in Table 1. Drug adherence, measured by tablet counting, was excellent in both studies and supported by expected effects of *rosiglitazone* on glycaemic control and blood pressure.

In both trials *rosiglitazone* increased plasma volume (Study 1: 223 ± 83 ml/1.73 m^2; Study 2: 248 ± 51 ml/1.73 m^2) and reduced haematocrit (Study 1: 0.019 ± 0.006; Study 2: 0.021 ± 0.006), while placebo had no effect (Table 2). *Rosiglitazone* had no effect on plasma albumin concentration, total body water (TBW) or mean corpuscular volume (MCV) of the red blood cells. There was no correlation between the

Figure 1: Plots of correlation between change in plasma volume and either haematocrit **(Panel A)** or plasma albumin **(Panel B)** during treatment with *rosiglitazone*. **Panel C:** correlation between plasma volume and haematocrit in patients using placebo only.
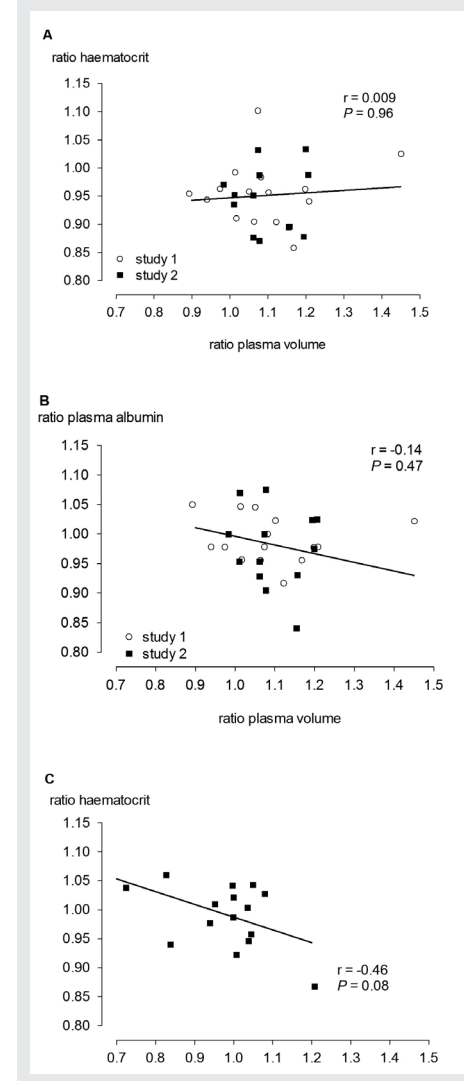
change in plasma volume and haematocrit, neither in the pooled population of participants treated with *rosiglitazone* (r = 0.009; *P* = 0.96; Figure 1, panel A), nor in the split populations (insulin-resistant subjects: r = 0.14; *P* =0.61; diabetes: r = –0.008; *P* = 0.98). Interestingly, in patients treated with placebo, changes in haematocrit and plasma volume did seem to correlate (r = –0.46; *P* = 0.08. Figure 1, panel C).

Discussion

The main finding of this pooled post-hoc analysis is that there is no correlation whatsoever between change in haematocrit and change in plasma volume during chronic treatment with *rosiglitazone* neither in subjects with insulin resistance nor in patients with diabetes. From a scientific point of view the uncoupling of haematocrit and plasma volume changes is important, as it suggests *rosiglitazone* has additional effects on top of plasma volume expansion, probably a reduction in total red blood cell volume. From a clinical point of view these findings suggest that changes in haematocrit, do not reflect fluid-related complications in an individual patient on chronic thiazolidinedione treatment.

The direct measurement of plasma volume by using ^{125}I-labelled human albumin in combination with measurements of haematocrit in subjects on chronic treatment with *rosiglitazone* are rather unique features of this study. Only one other study has measured plasma volume with a direct method during thiazolidinedione treatment in human subjects. Young et al. used ^{131}I-labelled human albumin to calculate plasma volume in 16 healthy subjects either treated with *troglitazone* 600 mg or placebo for 6 weeks in a parallel design[16]. *Troglitazone* non-significantly expanded the plasma volume by 8% and did not change haematocrit. The study has, however, a relatively short treatment interval and a small sample size. Two other human studies, both from Berria et al., investigated the correlation between changes in haematocrit and body fluid compartments. In the first study, 50 patients with diabetes type 2 were treated with either placebo or *pioglitazone* (45 mg a day) for 16 weeks[17]. Total body water and extracellular water but not plasma volume were measured using ^3H_2O dilution and bio-impedance. *Pioglitazone* induced a significant weight increase mainly driven by an increase in fat mass. These authors did not observe an

increase in TBW, decrease in haematocrit, nor a correlation (0.01) between both endpoints which is in agreement with our data. In the second study, subjects with the polycystic ovary syndrome were treated with *pioglitazone* which resulted in a fall in haematocrit without an increase in TBW, again suggesting that it is not haemodilution that drives the reduction in haematocrit[18]. In these studies plasma volume was not directly measured and there was no reduction in haematocrit in the diabetes study. Recently, Li et. al. observed that in patients with type 2 diabetes after interruption of *pioglitazone* treatment body weight decreased while haematocrit level did not change[19]. Thus, a number of earlier observations support our current findings.

Theoretically, at least on the short run, one would expect that an increase in plasma volume would result in a decrease in haematocrit. Interestingly, there was a strong indication for such a relationship in the placebo-treated group. Therefore our data suggest that it is *rosiglitazone* in itself that disrupts this relationship in the individual patient. A potential explanation is that *rosiglitazone* changes either the total number of red blood cells or the mean size of the individual erythrocyte. This last suggestion can be rejected as MCV did not change. As mentioned before, thiazolidinediones have been found to suppress erythroid-colony forming cells in human in-vitro cell experiments[8], but in short clinical trials they did not alter erythrocyte production nor destruction[16, 20]. To overcome potential confounding by influences on the red blood cell life cycle we analyzed in the present study whether changes in plasma albumin can be used as indirect marker for change in plasma volume. However, we did not find a correlation between these endpoints.

The present study has weaknesses. Because the plasma volume calculations assume first order kinetics for injected human albumin, we excluded measurements in which there was a correlation coefficient of less than 0.8 between extinction curve and time. However, also when all measurements were included, the results were unchanged. Second, this is a post-hoc analysis, although performed on predefined important endpoints. Finally, the present study only included *rosiglitazone*, but for a number of reasons, our results can be extrapolated to *pioglitazone*, as the effects on red blood cell production in in-vitro cell experiments have been described for *pioglitazone*[8]. Furthermore, a previous joined consensus statement considered fluid retention to be a class effect of thiazolidinediones[21].

In conclusion, we find no relationship between changes in haematocrit and changes in directly measured plasma volume in patients on thiazolidinedione treatment. These results implicate that haematocrit measurements are not useful for monitoring fluid-related complications during chronic thiazolidinedione treatment in an individual patient. They also suggest that thiazolidinediones have additional haematocrit lowering effects by interference with total red blood cell volume.

Concise methods

Study design and population

The current analysis merged data from two randomized placebo-controlled trials, investigating the mechanism and risk factors of fluid retention during treatment with *rosiglitazone*. The first trial was a crossover study in which 18 insulin-resistant subjects were treated with *rosiglitazone* 4 mg bd and placebo during 12 weeks[2] (No clinical trial registration as it was performed in 2003). The second, more recently published trial[3] (NCT00422955) had a parallel design and included 40 patients with diabetes type 2 treated with either *rosiglitazone* 4 mg bd or placebo for 16 weeks. As per inclusion, 16 subjects in this trial had autonomic neuropathy. Both study protocols were approved by the hospital ethics committee, in accordance with the declaration of Helsinki, and performed according to Good Clinical Practice.

Endpoint procedures

Plasma volume, haematocrit and Total Body Water (TBW) were measured in the morning, starting at 8.00 a.m. in a quiet temperature-controlled room (23-24ºC). TBW was assessed using the Akern 2000 bioelectrical impedance analyzer[22] (Akern, Florence, Italy) with the patient in supine position. A 20-gauge catheter (Angiocath, Becton Dickinson) was inserted into the left brachial artery under local anaesthesia (0.3-0.4 ml lidocaine HCl 20mg/ml, Braun AG, Melsungen, Germany), and connected with an arterial pressure monitoring line and kept patent with heparin in saline 0.9% (2 U/ml; 3 ml/h) (NaCl 0.9%, Baxter, Utrecht; heparin, Leo Pharma, Ballerup, Denmark). Blood was drawn for measurement of haematocrit, parameters of glycaemic control and safety parameters.

An additional venous needle (BD Valu-set, 0.6 x 20 mm, Becton Dickinson) was inserted and 2-4 μCi ^{125}I-albumin (Shering Nederland BV, Weesp, the Netherlands) was given as an intravenous bolus injection. Over the next 60 minutes, seven plasma samples were collected from the arterial line for radioactivity measurements.

Calculations

To assess plasma volume the measured radioactivity (counts per minute, cpm) was plotted over time. An extinction curve was drawn assuming first order kinetics. Slope and the extrapolated peak plasma concentration at t = 0 were calculated using Excel. Plasma volume was calculated using the following formula[2, 3, 23]:

Plasma volume (ml)/1.73m^2 = [cpm injected/cpm t=0/ml] /surface(m^2)/1.73m^2

To assure reliable results, calculated plasma volume was excluded from analysis when the correlation coefficient between the extinction curve and the actual measured time points was below 80%.

Statistical analysis

We used the paired student t-test or Wilcoxon rank test, if appropriate, to calculate statistical significances. Correlations were calculated using Pearson's or Spearman's correlation tests. Descriptive statistics of population characteristics are presented as mean with standard deviation. Treatment effects are presented as mean with standard error. All significance tests are two-sided with a Type I error of 5%. All statistical analyses were performed using the SPSS personal computer software package.

Acknowledgments

References

1. Yki-Jarvinen H. Thiazolidinediones. *N Engl J Med* 2004; 351(11): p. 1106-1118.

2. Rennings AJ, Smits P, Stewart MW, Tack CJ. Fluid retention and vascular effects of rosiglitazone in obese, insulin-resistant, nondiabetic subjects. *Diabetes Care* 2006; 29(3): p. 581-587.

3. Rennings AJ, Smits P, Stewart MW, Tack CJ. Autonomic neuropathy predisposes to rosiglitazone-induced vascular leakage in insulin-treated patients with type 2 diabetes: a randomised, controlled trial on thiazolidinedione-induced vascular leakage. *Diabetologia* 2010; 53(9): p. 1856-1866.

4. Diamant M, RJ Heine. Thiazolidinediones in type 2 diabetes mellitus: current clinical evidence. *Drugs* 2003; 63(13): p. 1373-1405.

5. Phillips LS, Grunberger G, Miller E, Patwardhan R, Rappaport EB, Salzman A. Once- and twice-daily dosing with rosiglitazone improves glycemic control in patients with type 2 diabetes. *Diabetes Care* 2001; 24(2): p. 308-315.

6. Karalliedde J, Buckingham R, Starkie M, Lorand D, Stewart M, Viberti G. Effect of various diuretic treatments on rosiglitazone-induced fluid retention. *J Am Soc Nephrol* 2006; 17(12): p. 3482-3490.

7. Gregersen MI, RA Rawson. Blood volume. *Physiol Rev* 1959; 39(2): p. 307-342.

8. Nagasawa E, Abe Y, Nishimura J, Yanase T, Nawata H, Muta K. Pivotal role of peroxisome proliferator-activated receptor gamma (PPARgamma) in regulation of erythroid progenitor cell proliferation and differentiation. *Exp Hematol* 2005; 33(8): p. 857-864.

9. Clevidence DE, MB Juckett, MJ Lucarelli. Marrow suppression with myelodysplastic features, hypoerythropoetinemia, and lipotrophic proptosis due to rosiglitazone. *WMJ* 2009; 108(9): p. 462-465.

10. Debiais F. Thiazolidinediones: antidiabetic agents with effects on bone. *Joint Bone Spine* 2009; 76(3): p. 221-223.

11. Grey A, Beckley V, Doyle A, Fenwick S, Horne A, Gamble G, Bolland M. Pioglitazone increases bone marrow fat in type 2 diabetes: results from a randomized controlled trial. *Eur J Endocrinol* 2012.

12. Buckingham RE. Thiazolidinediones: Pleiotropic drugs with potent anti-inflammatory properties for tissue protection. *Hepatol Res* 2005; 33(2): p. 167-170.

13. Painter EE, JH Holmes, MI Gregersen. Exchange and distribution of fluid in dehydration in the dog. *Am J Physiol* 1948; 152(1): p. 66-76.

14. Parving HP, F Gyntelberg. Transcapillary escape rate of albumin and plasma volume in essential hypertension. *Circ Res* 1973; 32(5): p. 643-651.

15. Gibson JG, A.M. Seligman. The distribution of red cells and plasma in large and minute vessels of the normal dog, determined by radioactive isotopes of iron and iodine. *J Clin Invest* 1946; 25(6): p. 848-857.

16. Young MM, Squassante L, Wemer J, van Marle SP, Dogterom P, Jonkman JH. Troglitazone has no effect on red cell mass or other erythropoietic parameters. *Eur J Clin Pharmacol* 1999; 55(2): p. 101-104.

17. Berria R, Glass L, Mahankali A, Miyazaki Y, Monroy A, De Filippis E, Cusi K, Cersosimo E, Defronzo RA, Gastaldelli A. Reduction in haematocrit and haemoglobin following pioglitazone treatment is not hemodilutional in Type II diabetes mellitus. *Clin Pharmacol Ther* 2007; 82(3): p. 275-281.

18. Berria R, Gastaldelli A, Lucidi S, Belfort R, De Filippis E, Easton C, Brytzki R, Cusi K, Jovanovic L, DeFronzo R. Reduction in haematocrit level after pioglitazone treatment is correlated with decreased plasma free testosterone level, not hemodilution, in women with polycystic ovary syndrome. *Clin Pharmacol Ther* 80 2006; (2): p. 105-114.

19. Lin KD, Lee MY, Feng CC, Chen BK, Yu ML, Shin SJ. Residual effect of reductions in red blood cell count and haematocrit and haemoglobin levels after 10-month withdrawal of pioglitazone in patients with Type 2 diabetes. *Diabet Med* 2014.

20. Dogterom P, J HG Jonkman, SE Vallance. Rosiglitazone: No effect on erythropoiesis or premature red cell destruction, in *Diabetes* 1999; p. A98 424.

21. Nesto RW, Bell D, Bonow RO, Fonseca V, Grundy SM, Horton ES, Le Winter M, Porte D, Semenkovich CF, Smith S, Young LH, Kahn R. Thiazolidinedione use, fluid retention, and congestive heart failure: a consensus statement from the American Heart Association and American Diabetes Association. October 7, 2003. *Circulation* 2003; 108(23): p. 2941-2948.

22. Lukaski HC. Methods for the assessment of human body composition: traditional and new. *Am J Clin Nutr* 1987; 46(4): p. 537-556.

23. Parving HH, F Gyntelberg. Albumin transcapillary escape rate and plasma volume during long-term beta-adrenergic blockade in essential hypertension. *Scand J Clin Lab Invest* 1973; 32(2): p. 105-110.

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Preserved α-adrenergic tone but blunted sympathetic response to orthostatic stress in diabetic neuropathy; effects of *rosiglitazone*

Abstract

Aim/background: Autonomic neuropathy is a common complication of longstanding diabetes and leads to clinical manifestations in a variety of organ systems. Cardiovascular autonomic neuropathy (CAN) is an important form of diabetic autonomic neuropathy in clinical practice. In this study we investigated whether the baseline sympathetic tone is changed in CAN and whether *rosiglitazone* can restore the expected blunted response to orthostatic stress.

Methods: We investigated the forearm vasodilator response to infusion of the α -adrenergic receptor antagonist *phentolamine* into the brachial artery (baseline α -adrenergic tone) and the forearm vasoconstrictor response to sympathetic stimulation (Lower Body Negative Pressure, LBNP) after either *rosiglitazone* 4 mg twice daily or placebo. Data were derived from a previously performed randomized, double-blind, placebo-controlled study in 40 patients with type 2 diabetes who were treated with insulin.

Results: In patients with CAN baseline *phentolamine*-induced flow, ie α-adrenergic tone, appeared to be intact (forearm blood flow in CAN: 6.7 ± 1.6 ml/dl/min; no CAN: 5.4 ± 0.7 ml/dl/min; $P = 0.46$), but the sympathetic responsiveness to LBNP was blunted (Change in diastolic blood pressure: no CAN: +2.1 ± 0.8 mmHg; CAN: -1.9 ± 1.8 mmHg, $P < 0.05$). *Rosiglitazone* did not affect baseline α-adrenergic tone, but improved the sympathetic response to LBNP (LBNP-induced change in forearm blood flow ($P = 0.02$): placebo week 0: -0.95 ± 0.25 ml/dl/min, week 16: -0.25 ± 0.14 ml/dl/min; *rosiglitazone* week 0:-0.41 ± 0.15 ml/dl/min, week 16: -0.51 ± 0.21 ml/dl/min).

Conclusion: The α-adrenergic tone is preserved in diabetic CAN. However, the sympathetic response to LBNP is blunted, but seems to improve after 16 weeks treatment with *rosiglitazone*.

Introduction

Autonomic neuropathy is a frequent complication of longstanding diabetes and can result in dysfunction of several organ systems[1]. Cardiovascular autonomic neuropathy (CAN) is probably the best studied entity of diabetic autonomic neuropathy. When assessed by noninvasive (Ewing) tests[2], the prevalence of CAN was over 20% in a referral population of type 2 diabetic patients in Europe[3]. The clinical impact of CAN is high as it is associated with major cardiovascular events[4]. In the ACCORD cardiovascular outcome study, the presence of CAN strongly predicted all-cause (hazard ratio 2.14) and cardiovascular disease (CVD) mortality (hazard ratio 2.62) independently of baseline CVD, diabetes duration, multiple traditional CVD risk factors and medication[5].

CAN is characterized by two main abnormalities: cardiac denervation and decreased efferent sympathetic nervous system response to orthostatic stress. Cardiac denervation results in resting tachycardia and exercise intolerance. The decreased efferent sympathetic nervous system response results in decreased vasoconstriction in the peripheral vascular bed leading to orthostatic hypotension[5]. Although clinically important, CAN is a frequently overlooked complication of diabetes mellitus, and as a consequence many questions are unresolved. In this article we focus on two issues.

First, opposing results have been reported on the basal vascular sympathetic tone in patients with diabetes in general[6, 7]. Baseline vascular sympathetic tone is relevant for understanding the potential consequences of CAN.

Second, a blunted response to orthostatic stress is characteristic for autonomic neuropathy and trials have shown that tight glycaemic control induced by pancreas transplantation[8] or multifactorial intervention[9] is capable to improve autonomic neuropathy. Whether specific glucose lowering compounds can restore the blunted response to orthostatic stress is unknown. There is some evidence that PPAR-γ agonists may restore the autonomic imbalance in diabetes[10,11].

Recently, we studied *rosiglitazone*-induced vascular leakage in the context of autonomic neuropathy in patients with type 2 diabetes on insulin treatment[12]. In a substudy, we assessed the responsiveness of the sympathetic nervous system in patients with and without autonomic neuropathy. This enabled us to investigate

whether baseline sympathetic tone (α-adrenergic tone) in patients with clinical diabetic autonomic neuropathy is decreased and whether *rosiglitazone* restores the expected blunted response to orthostatic stress.

Methods

Protocol

The study was a randomized, placebo-controlled, double-blind, single-centre, parallel study with 4 weeks of single-blind run-in (NCT00422955)[12]. At screening, the Ewing score was determined in patients with type 2 diabetes to quantify cardiac autonomic neuropathy. The participants were divided into two groups, with either Ewing scores ≥2.5 (CAN) or <2.5 (no CAN). Details regarding general study protocol, study population and experimental day have been reported previously[12]. In short: the study population consisted of 40 insulin treated subjects with type 2 diabetes, who were treated with *rosiglitazone* or placebo for 16 weeks. Based on Ewing scores, 40% of the study population had autonomic neuropathy. At baseline and at the end of treatment sympathetic responsiveness was measured. The study was approved by the ethics committee and all participants gave written consent. The data presented here result from a substudy and have not been included in the earlier publication.

Experimental procedures

The procedures started at the clinical research center at 8.00 am with the subject in supine position after an overnight fast without taking insulin or oral blood glucose lowering pharmacotherapy in the morning. A venous catheter and subsequently an arterial catheter were inserted. After 30 minutes of equilibration time, baseline forearm blood flow (FBF) was measured simultaneously in the experimental and control arm by mercury-in-silastic strain-gauge venous occlusion plethysmography[13]. The experimental forearm was subsequently used to assess the maximal forearm vasodilator response to intra-artrial infusion of the α-adrenergic receptor antagonist *phentolamine* into the brachial artery. This vasodilator response is considered to reflect the baseline α-adrenergic tone of the forearm vascular bed[14]. *Phentolamine* infusion was started in a dose of 15 µg/min per 100 ml of forearm volume during 10 minutes (dose based on pilot

experiments, data not shown). FBF of the contra-lateral arm was used as a time-control value so that systemic effects could be observed and vasoactive effects of intra arterial *phentolamine* could be expressed as a quotient of the experimental and control arm[15]. FBF was expressed in ml/minute per 100 ml of forearm tissue. In addition, forearm vascular resistance (FVR) was calculated. These data are not shown but always resemble the results derived by using FBF. Arterial and venous blood samples were drawn for measurement of catecholamines levels. Subsequently, an equilibration period was included to allow FBF to return towards baseline levels. Then, the sympathetic nervous system was stimulated with orthostatic stress applied by Lower Body Negative Pressure (LBNP) (25 mmHg)[16] for 15 minutes. Before and after LBNP, FBF was measured to quantify the forearm vasoconstrictor response to sympathetic stimulation (LBNP). Arterial and venous blood sampling was repeated to quantify the catecholamine response to LBNP.

Analytical methods

The samples were collected in pre-cooled 5ml lithium-heparin tubes on melting ice. Immediately after collection, the blood was centrifuged (10 minutes at 1500g, 4ºC) and plasma was stored at -70ºC. Noradrenaline and adrenaline were analyzed with high performance liquid chromatography (HPLC) with fluorometric detection[17].

Circulating noradrenaline is a function of the amount of adrenergic neurotransmitter that is released from nerve terminals with a small contribution of the adrenal medulla, clearance in the sympathetic cleft and in the plasma compartment[18]. Noradrenaline appearance rate can be calculated from arterial (a) and venous (v) noradrenaline concentrations and reflects the changes in noradrenaline release in the sympathetic cleft[19], especially during LBNP[16]. Noradrenaline appearance rate can be estimated by infusion of titriated noradrenaline to assess local noradrenaline clearance. Under the assumption that the extraction of noradrenaline is similar to that of adrenaline, we used the extraction of adrenaline as a substitute for noradrenaline extraction[20]. We calculated forearm noradrenaline (NA) appearance rate (AR) as follows:

AR=NA spillover/ 1-FEA

NAspillover = (1-Ht) x FBF x ([NA]$_v$-[NA]$_a$+[NA]$_a$ x FEA)

FEA=[A]$_a$-[A]$_v$/[A]$_a$

FEA: fractional extraction of adrenaline. Ht: haematocrit. A: adrenaline

Statistical analysis

The groups were balanced for gender and Ewing score and all patients with paired-observations were included in the analyses. The difference between both Ewing score groups and treatment was estimated with t-tests for independent samples or nonparametric tests where applicable. The effect of sympathetic stimuli in the total population was estimated with paired t-tests or where applicable nonparametric tests. All statistical tests were two-sided and the overall Type I error was 5%. Treatment effects are presented as mean ± standard error. All statistical analyses were performed using the SPSS software package.

Results

Characteristics of study participants

A total of 40 insulin-treated patients with diabetes participated in the study. Sixteen participants were classified with Ewing scores ≥ 2.5, while 24 participants had a Ewing score <2.5. Participants classified having CAN appeared to have a higher BMI (Table 1).

In 36 participants we were able to assess baseline activity of the sympathetic nervous system (Figure 1). In total 39 participants (placebo: 21; *rosiglitazone*: 18) finished the treatment period of the study. Some reported mild side effects, including oedema. One patient in the *rosiglitazone* group was withdrawn after 8 weeks because of atrial fibrillation and heart failure. Drug adherence was measured by tablet counting and appeared to be excellent[12].

Table 1: Baseline characteristics, mean (SD).

	Total Population				No CAN	CAN		
	Rosiglitazone	Placebo	No CAN	CAN	Rosiglitazone	Placebo	Rosiglitazone	Placebo
	n = 19	n = 21	n = 24	n = 16	n = 11	n = 13	n = 8	n = 8
Age (years)	58 (8)	59 (10)	59 (8)	58 (10)	58 (9)	60 (8)	59 (6)	57 (13)
Male (%)	58	57	63	50	64	62	50	50
BMI (kg/m²)	29 (4)	29 (6)	28 (4)	31 (6)^a	28 (4)	27 (5)	30 (3)	32 (8)
Waist (cm)	101 (10)	101 (17)	97 (13)	105 (14)	98 (10)	97 (15)	104 (9)	107 (19)
SBP (mmHg)	132 (15)	132 (15)	132(12)	132 (18)	131 (13)	133 (13)	133(19)	131 (19)
DBP (mmHg)	80 (6)	80 (7)	80 (6)	80 (7)	80 (4)	80 (8)	79 (8)	80 (6)
Heart rate (1/min)	73 (10)	75 (11)	73 (12)	76 (7)	70 (10)	75 (13)	77 (7)	75 (8)
Duration of DM (years)	12.8 (7.8)	14.1 (5.7)	13.1 (6.9)	14.0 (6.5)	11.2(8.0)	14.6 (5.8)	14.9 (7.4)	13.1 (5.9)
HbA1c (%)	7.7 (1.4)	8.1 (1.1)	7.7 (1.2)	8.3 (1.3)	7.5(1.3)	7.8 (1.0)	8.0 (1.4)	8.6 (1.1)
Insulin dose (U/day)	60 (40)	64 (33)	58 (28)	69 (45)	57 (24)	58 (32)	64 (56)	74 (34)
Ewing score	2.1 (1.3)	1.7 (0.9)	1.2(0.6)	3.0 (0.8)^b	1.2 (0.6)	1.1 (0.6)	3.3 (0.9)^c	2.7(0.5)^d

CAN   Cardiovascular Autonomic Neuropathy
BP: Diastolic Blood Pressure
SBP: Systolic Blood Pressure
^{a} P < 0.05 vs. no CAN
^{b} P < 0.001 vs. no CAN
^{c} P < 0.05 vs. *Rosiglitazone*, no CAN
^{d} P < 0.05 vs. Placebo, no CAN

Figure 1: Enrolment of study participants.

* The response to *phentolamine* was not measured due to mastectomy with lymph node dissection
in their history (n = 3) and because of a backache-induced haemodynamic collapse just before
phentolamine-infusion (n = 1).

**The response to LBNP was not measured due to a mild haemodynamic collapse (n = 3) during LBNP
and because of a backache-induced haemodynamic collapse just before LBNP (n = 1).

***During *rosiglitazone*-treatment the response to *phentolamine* could not be measured in one
additional subject due to cannulation failure.

****During placebo-treatment the response to LBNP could not be measured in one additional subject
again due to backache-induced haemodynamic collapse during LBNP.
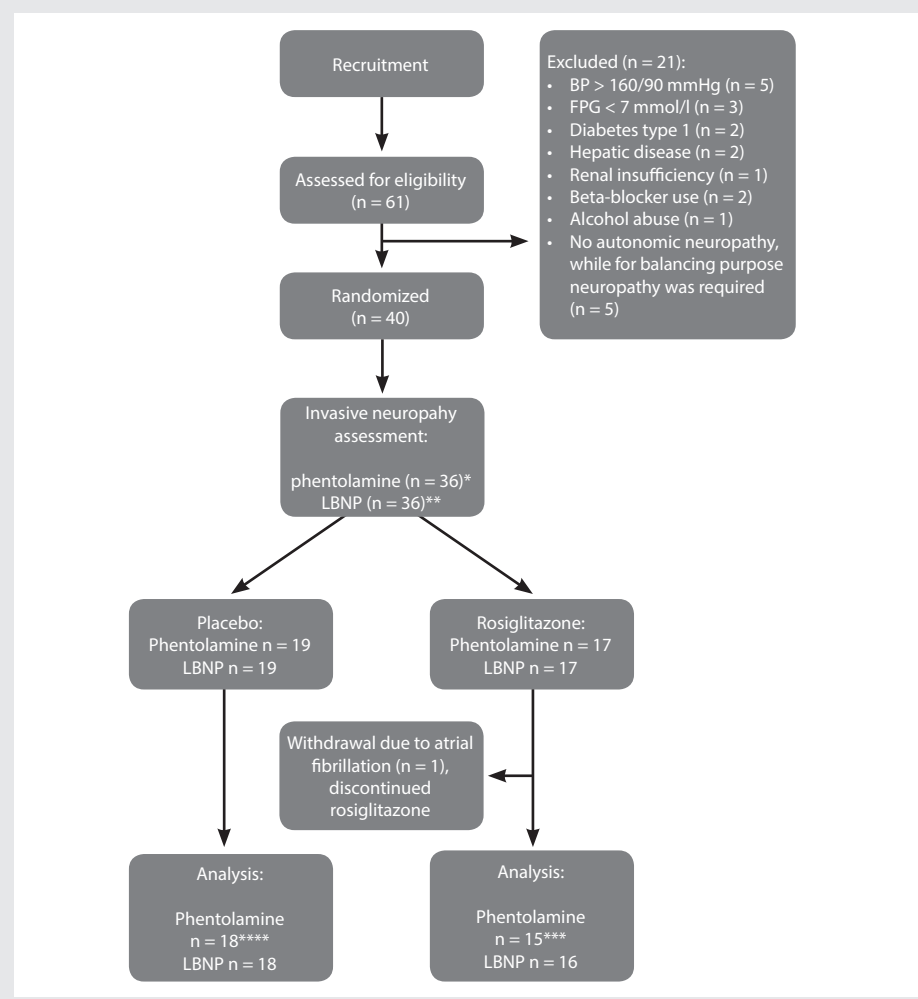
Baseline sympathetic nervous system activity

At baseline, sympathetic nervous system activity, expressed as noradrenaline
appearance rate, was significantly higher in patients with CAN (see Figure 2. Left
top panel). In response to *phentolamine*, FBF ratio (experimental arm/control
arm), representing baseline α-adrenergic tone, increased significantly (from
1.02 ± 0.05, to 3.60 ± 0.37, $P < 0.001$). While numerically higher, the vasodilator
response to intra-arterial infusion of *phentolamine* was not significantly different
in patients with and without CAN either when expressed in relative or in absolute
numbers (ratio FBF experimental/ control arm: CAN 3.87 ± 0.38; no CAN 3.25
± 0.33; absolute increase in flow in the experimental arm: CAN: 6.7 ± 1.6 ml/dl/
min; no CAN: 5.4 ± 0.7 ml/dl/min; Table 2; Figure 2).

Table 2: Comparison of invasive autonomic function tests between subpopulations of autonomic
function

Parameter	No CAN	CAN	*P*-value
Baseline Noradrenaline Appearance rate (pmol/min/dl forearm tissue)	6.83 ± 2.66	6.90 ± 0.78	**0.008**
Phentolamine FBF ratio (E/C)	3.25 ± 0.33	3.87 ± 0.38	0.23
Phentolamine FBF (absolute increase)	5.38 ± 0.72	6.69 ± 1.57	0.46
LBNP Δ Noradrenaline Appearance rate (pmol/min/dl forearm tissue)	6.05 ± 1.39	2.17 ± 1.33	0.07
LBNP Δ DBP (mmHg)	2.2 ± 0.7	-1.4 ± 1.6	**0.03**
LBNP Δ FBF (ml/dl/min)	-0.89 ± 0.20	-0.37 ± 0.20	0.08

CAN: Cardiovascular Autonomic Neuropathy
DBP: Diastolic Blood Pressure
E/C: experimental arm/control arm
FBF: Forearm Blood Flow
LBNP: Lower Body Negative Pressure

Sympathetic response to LBNP

As expected, sympathetic nervous system stimulation by LBNP induced a
significant increase in the global sympathetic response (noradrenaline appearance
rate increased from 7.0 ± 1.8 to 11.6 ± 2.4 pmol/min/dl forearm tissue; $P < 0.001$)

and a significant decrease in the forearm blood flow (FBF in the experimental arm before LBNP: 2.81 ± 0.24 ml/dl/min; after LBNP: 2.13 ± 0.16 ml/dl/min, *P* < 0.001). LBNP did not change diastolic blood pressure.

In the subset of patients with CAN, the sympathetic response to LBNP was diminished as documented by a reversal of the diastolic blood pressure response. Furthermore, we observed a trend towards a blunted response in forearm vasoconstriction and noradrenaline appearance rate (Table 2; Figure 2).

Effects of *rosiglitazone*

Compared to placebo, the forearm vasoconstrictor response to LBNP was stronger during *rosiglitazone* treatment (LBNP-induced change in FBF (*P* = 0.02): placebo week 0: −0.95 ± 0.25 ml/dl/min, week 16: -0.25 ± 0.14 ml/dl/min; *rosiglitazone* week 0: -0.41 ± 0.15 ml/dl/min, week 16: -0.51 ± 0.21 ml/dl/min), but did not affect baseline activity of the sympathetic nervous system, neither at the hormonal nor at the functional level of the forearm vascular bed (Figure 3).

Discussion

There are two main findings in this study. First, in patients with cardiovascular autonomic neuropathy, the baseline activity of the sympathetic nervous system is preserved while the response to orthostatic stress is diminished. Second, sympathetic responsiveness in this group was improved by *rosiglitazone* treatment.

The first conclusion is based on the preserved response to *phentolamine* and on the fact that the baseline noradrenaline appearance rate is not reduced, but even increased in patients with CAN. The finding of an increased baseline activity is a new finding in the vascular bed, but to some extend in accordance with findings of Pop-Busui et al. who reported an increase in cardiac sympathetic tone in the early phase of CAN[21].

Figure 2: Invasive autonomic neuropathy assessment with *phentolamine*-infusion disclosing the α-adrenergic tone and with lower body negative pressure (LBNP) measuring sympathetic responsiveness.
A) Baseline noradrenaline (NA) appearance rate (AR) in patients with cardiovascular autonomic neuropathy (CAN) is higher than in patients without CAN.
B) *Phentolamine*-infusion induced a clear increase in forearm blood flow (FBF) in the total population. There was no decreased response in subjects with CAN.
C) Patients with CAN had an impaired increase in noradrenaline appearance rate during LBNP compared to those without CAN.
D) Change in diastolic blood pressure as a response to LBNP in the total population. There was no change in the total population, but a clear difference between patients with CAN and those without CAN.
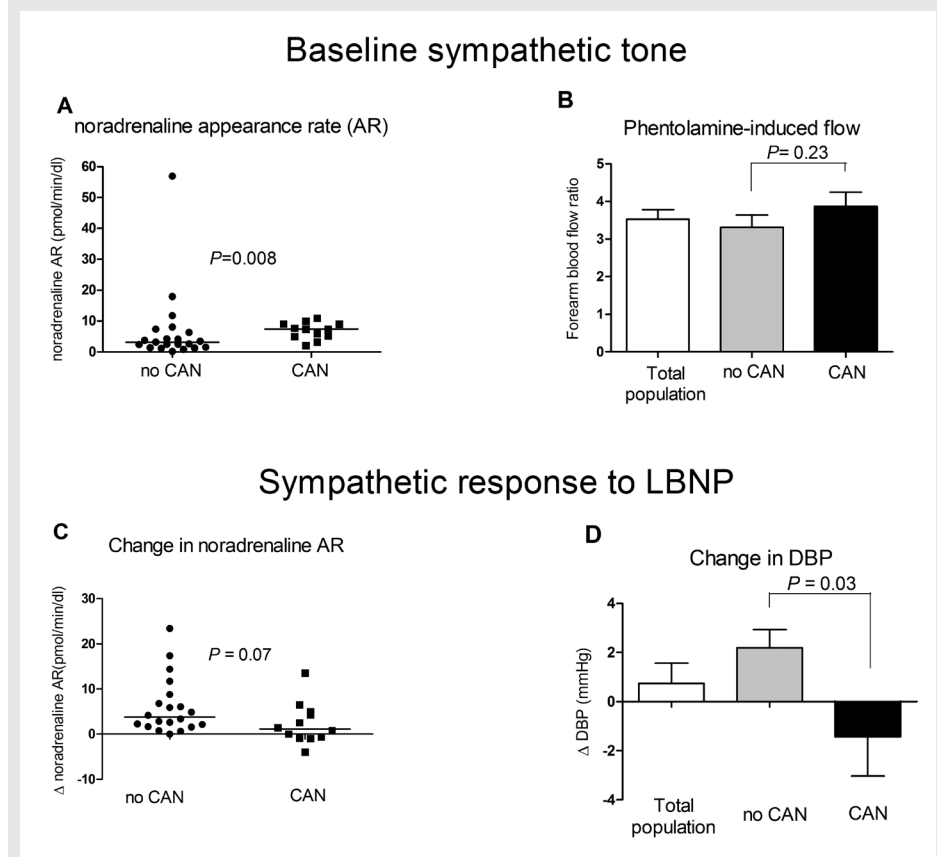
Earlier reports from Hogikyan et al.[7] suggest that the response to *phentolamine* infusion in patients with type 2 diabetic without clinical signs of autonomic neuropathy is higher compared to non diabetic subjects which is consistent with an increase in α-adrenergic tone in patients with diabetes. As we only studied patients with diabetes, our results cannot be compared to this study. The finding of a preserved α-adrenergic tone in autonomic neuropathy seems to be paradoxical, but is in line with earlier observations from our group found in a population of individuals with autonomic failure due to spinal cord-injury[22]. A number of mechanisms may explain such a preserved α-adrenergic tone, including upregulation of α-adrenergic receptors.

The combination of a preserved baseline noradrenaline appearance rates and α-adrenergic tone and an impaired response to LBNP, suggests that at baseline, the sympathetic nervous system is intact, but at the cost of an increased basal α-adrenergic tone. In response to sympathetic stress, however, the responsiveness is decreased.

Figure 3:
A) The influence of *rosiglitazone* compared to placebo on the *phentolamine*-induced change in forearm blood flow.
B) Individual forearm blood flow (FBF) response during LBNP (Lower Body Negative Pressure) before and after treatment with either *rosiglitazone* or placebo. Mean and standard error at each time point.
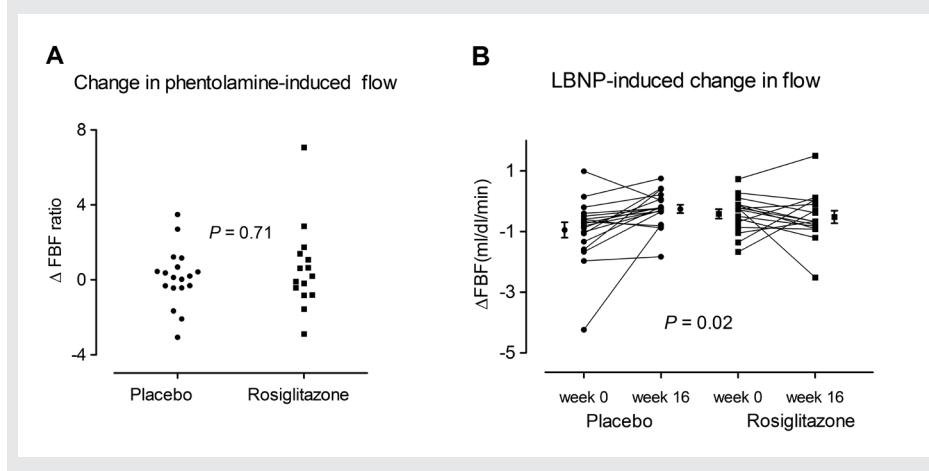
The second main finding of our study is the suggestion that *rosiglitazone* compared to placebo enhances the forearm vasoconstrictor response to LBNP. This effect may theoretically be explained by improved glycaemic control. Indeed *rosiglitazone* decreased HbA_{1c} compared to placebo, but there was no significant correlation between the change in HbA_{1c} and the vasoconstrictor response to LBNP. Alternatively, *rosiglitazone* may affect CAN by influencing factors that are crucial in the pathophysiology of autonomic dysfunction such as reduction in leptin and/or TNF-α[23,24]. The observed effect could be a class effect as also from *pioglitazone* a beneficial effect has been reported on cardiac autonomic regulation as quantified by heart rate variability[25].

A limitation of our study is that the sympathetic nervous system endpoints in the present study were not primary endpoints and hence the study was not powered on these. However, they were originally prespecified secondary endpoints. We used a Ewing score without correction for age as there is no internationally accepted standard age correction for the Ewing score. In addition, we used a binominal division of the Ewing score instead of the original division.

Our study also has strengths, it provides a detailed quantification of sympathetic nervous system responsiveness in a clinically relevant population and tests the effect of a glucose lowering therapy.

In conclusion, in a population of insulin treated patients with type 2 diabetes and cardiac autonomic neuropathy, α-adrenergic tone is preserved, probably at the cost of maximal compensatory mechanisms with the consequence that the sympathetic responsiveness to an orthostatic stimulus fails. *Rosiglitazone* may have a beneficial effect on sympathetic responsiveness both in patients with and without autonomic neuropathy, which seems to be independent from improvements in glycaemic control.

Acknowledgments

References

1. Vinik AI, Maser RE, Mitchell BD, Freeman R. Diabetic autonomic neuropathy. *Diabetes Care* 2003; 26:1553-1579.

2. Ewing DJ. Cardiovascular reflexes and autonomic neuropathy. *Clin Sci Mol Med* 1978; 55:321-327.

3. Ziegler D, Gries FA, Spuler M, Lessmann F. The epidemiology of diabetic neuropathy. Diabetic Cardiovascular Autonomic Neuropathy Multicenter Study Group. *J Diabetes Complications* 1992; 6:49-57.

4. Gerritsen J, Dekker JM, TenVoorde BJ, Kostense PJ, Heine RJ, Bouter LM, Heethaar RM, Stehouwer CD. Impaired autonomic function is associated with increased mortality, especially in subjects with diabetes, hypertension, or a history of cardiovascular disease: the Hoorn Study. *Diabetes Care* 2001; 24:1793-1798.

5. Pop-Busui R. What Do We Know and We Do Not Know About Cardiovascular Autonomic Neuropathy in Diabetes. *J Cardiovasc Transl* 2012; 5:463-478.

6. Vervoort G, Wetzels JF, Lutterman JA, van Doorn LG, Berden JH, Smits P. Elevated skeletal muscle blood flow in noncomplicated type 1 diabetes mellitus - Role of nitric oxide and sympathetic tone. *Hypertension* 1999; 34:1080-1085.

7. Hogikyan RV, Galecki AT, Halter JB, Supiano MA. Heightened norepinephrine-mediated vasoconstriction in type 2 diabetes. *Metabolism* 1999; 48:1536-1541.

8. Navarro X, Sutherland DE, Kennedy WR. Long-term effects of pancreatic transplantation on diabetic neuropathy. *Ann Neurol* 1997; 42:727-736.

9. Gaede P, Vedel P, Larsen N, Jensen GV, Parving HH, Pedersen O. Multifactorial intervention and cardiovascular disease in patients with type 2 diabetes. *N Engl J Med* 2003; 348:383-393.

10. Petrofsky J, Lee S, Cuneo-Libarona M, Apodaca P. The effect of rosiglitazone on orthostatic tolerance during heat exposure in individuals with type II diabetes. *Diabetes Technol Ther* 2007; 9:377-386.

11. Nagai Y, Sugiyama Y, Nakatsumi Y, Abe T, Nomura G. Rapid amelioration of muscle sympathetic nerve activity by pioglitazone in an obese Type 2 diabetic patient. *Diabet Med* 2003; 20:868-869.

12. Rennings AJ, Smits P, Stewart MW, Tack CJ. Autonomic neuropathy predisposes to rosiglitazone-induced vascular leakage in insulin-treated patients with type 2 diabetes: a randomised, controlled trial on thiazolidinedione-induced vascular leakage. *Diabetologia* 2010; 53:1856-1866.

13. Whitney RJ. The measurement of volume changes in human limbs. *J Physiol* 1953 ; 121:1-27.

14. Egan B, Panis R, Hinderliter A, Schork N, Julius S. Mechanism of increased alpha adrenergic vasoconstriction in human essential hypertension. *J Clin Invest* 1987; 80:812-817.

15. Tack CJ, Smits P, Willemsen JJ, Lenders JW, Thien T, Lutterman JA. Effects of insulin on vascular tone and sympathetic nervous system in NIDDM. *Diabetes* 1996; 45:15-22.

16. Rongen GA, Lenders JW, Smits P, Floras JS. Comparison of two indices for forearm noradrenaline release in humans. *Clin Sci (Lond)* 2000; 99:363-369.

17. Willemsen JJ, Ross HA, Jacobs MC, Lenders JW, Thien T, Swinkels LM, Benraad TJ. Highly sensitive and specific HPLC with fluorometric detection for determination of plasma epinephrine and norepinephrine applied to kinetic studies in humans. *Clin Chem* 1995; 41:1455-1460.

18. Esler M, Jennings G, Korner P, Willett I, Dudley F, Hasking G, Anderson W, Lambert G. Assessment of Human Sympathetic Nervous-System Activity from Measurements of Norepinephrine Turnover. *Hypertension* 1988; 11:3-20.

19. Chang PC, Kriek E, Vanderkrogt JA, Vanbrummelen P. Does Regional Norepinephrine Spillover Represent Local Sympathetic Activity. *Hypertension* 1991; 18:56-66.

20. Riksen NP, Bosselaar M, Bakker SJ, Heine RJ, Rongen GA, Tack CJ, Smits P. Acute elevation of plasma non-esterified fatty acids increases pulse wave velocity and induces peripheral vasodilation in humans in vivo. *Clin Sci (Lond)* 2007; 113:33-40.

21. Pop-Busui R, Kirkwood I, Schmid H, Marinescu V, Schroeder J, Larkin D, Yamada E, Raffel DM, Stevens MJ. Sympathetic dysfunction in type 1 diabetes: association with impaired myocardial blood flow reserve and diastolic dysfunction. *J Am Coll Cardiol* 2004; 44:2368-2374.

22. Kooijman M, Rongen GA, Smits P, Hopman MT. Preserved alpha-adrenergic tone in the leg vascular bed of spinal cord-injured individuals. *Circulation* 2003; 108:2361-2367.

23. Vinik AI, Maser RE, Ziegler D. Autonomic imbalance: prophet of doom or scope for hope? *Diabet Med* 2011; 28:643-651.

24. Miyazaki Y, DeFronzo RA. Rosiglitazone and pioglitazone similarly improve insulin sensitivity and secretion, glucose tolerance and adipocytokines in type 2 diabetic patients. *Diabetes Obes Metab* 2008; 10:1204-1211.

25. Gianiorio FE, Casu M, Patrone V, Egan CG, Murialdo G. Effect of pioglitazone on cardiac sympathovagal modulation in patients with type 2 diabetes. *Acta diabetologica* 2011; 48:283-290.

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Rosiglitazone reduces ischaemia-reperfusion injury in patients with the metabolic syndrome

Abstract

Aims: Cardiovascular disease is the leading cause of death in patients with type 2 diabetes due to both a high event rate and a worse outcome. *Rosiglitazone* and *pioglitazone* are registered for the treatment of hyperglycaemia. Animal data suggest that these drugs can protect against ischaemia-reperfusion injury by improving insulin responsiveness. A meta-analysis of clinical trials suggests that *rosiglitazone* increases the incidence of myocardial infarction. However, no human data on a possible benefit on infarct size are available. Therefore, we investigated whether *rosiglitazone* reduces ischaemia-reperfusion injury in humans with insulin resistance.

Methods and results: This is a randomized, double-blind, placebo-controlled crossover study with a washout period of 6 weeks comparing 8 weeks of treatment with *rosiglitazone* 4mg bd with placebo. We included 10 evaluable subjects with the metabolic syndrome. At the end of each treatment period, volunteers were subjected to 10 minutes of forearm ischaemia, combined with standardized intermittent handgripping. At reperfusion, 500MBq ^{99}mTc-Annexin A5 was administered intravenously in the control arm. Annexin A5 uptake in the thenar muscle was quantified 1 and 4 hours post-reperfusion using a gamma camera to quantify ischaemia-reperfusion injury.

Results: *Rosiglitazone* reduced annexin targeting from 8.4% (median; range 0.6-49%) to 4.7% (0.7-20%) at 1hour after reperfusion ($P = 0.037$) and from 7.4% (0.5-50%) to 4.8% (-0.1-13%) at 4 hours of reperfusion ($P = 0.06$). There was no correlation between changes in glycaemic regulation and the changes in annexin targeting.

Conclusion: We present the first human in vivo data on the beneficial effects of *rosiglitazone* on ischaemia-reperfusion injury.

Introduction

Cardiovascular disease is one of the leading causes of death in patients with diabetes[1]. This increased cardiovascular risk is the result of both an increased incidence of events[2], and a worse outcome of these events[3]. In type 2 diabetes, there is evidence that ischaemic preconditioning is reduced[4] and ischaemia-reperfusion (I/R) injury exaggerated[5]. As such, a pharmacological intervention to reduce I/R injury would be a valuable strategy to improve survival in diabetes.

The thiazolidinedione derivative *rosiglitazone* activates the peroxisome proliferator-activated receptor-γ (PPAR-γ) thereby sensitizing insulin effects in type 2 diabetes. Recent data suggest that the therapeutic effects of PPAR-γ ligands reach beyond their use as insulin sensitizers[6]. In the present study we will focus on the effect of *rosiglitazone* on I/R injury.

Beneficial effects of *rosiglitazone* on I/R injury were observed in in vitro and in vivo preclinical studies, both after acute[7] or chronic[8] use. With respect to *rosiglitazone's* insulin sensitizing effects, it is well known that insulin itself has anti-apoptotic properties in myocardial ischaemia-reperfusion[9]. Therefore, the protective effects of *rosiglitazone* on I/R injury may be coupled to its insulin sensitizer properties. Indeed, in one study, the protective effect of *rosiglitazone* was more pronounced in insulin-resistant animals and was positively correlated with the improvement in insulin sensitization[10].

Despite this mechanistic preclinical evidence for a beneficial effect of *rosiglitazone* on I/R injury, several epidemiological meta-analyses[11,12] showed an increase in myocardial events in patients treated with *rosiglitazone*, challenging whether the improvements in I/R injury could be translated to humans. In addition, it is obvious that an experimental approach to investigate ischaemia-reperfusion injury in the human heart in vivo is limited by ethical and methodological restrictions. Therefore, we developed and validated a human in vivo model to quantify I/R injury in forearm skeletal muscle using annexin-A5 scintigraphy[13]. This forearm ischaemia-reperfusion model detects the loss of membrane asymmetry which results from increased phosphatidylserine (PS) exposure on the outer membrane leaflet. Phosphatidylserine exposition occurs shortly after an ischaemic insult. In addition to its diagnostic properties to detect early, reversible, signs of cell injury[14,15], annexin A5 may be a potential

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therapeutic tool to reduce injury[16]. By labeling recombinant annexin-A5 with Tc-99m, it is possible to visualize PS exposure by gamma camera imaging. We have validated this model thoroughly by showing that well-known protective strategies like ischaemic preconditioning and adenosine are also protective in this model[13].

In the present study, we used this human in vivo model of I/R injury to assess the effect of *rosiglitazone* treatment during 8 weeks on I/R injury in subjects with the metabolic syndrome, who by definition are characterized by insulin resistance.

Methods

Subjects
A group of 13 non-diabetic subjects, characterized with the metabolic syndrome[17], aged between 30 and 70 years, was selected. Subjects were not eligible for inclusion if fasting glucose was higher than 7.0 mmol/L or if they used hypoglycaemic agents, if they had exposure to a PPAR-γ agonist during the last 4 months or a documented significant hypersensitivity to a PPAR-γ agonist, if they were participant in another study, or if they were premenopausal. Additional exclusion criteria were clinical significant liver disease, anaemia, angina or heart failure, abnormal renal function, alcohol or drug abuse, physical inability to perform the exercise protocol and administration of any radiotracer for research purposes during the previous 5 years. Study participants were selected by advertisement, and gave written informed consent. Subjects who participated in this study received a financial compensation. The study complies with the Declaration of Helsinki and was performed according to Good Clinical Practice guidelines. Furthermore, this study was approved by the hospital ethics committee and registered at clinical trials.gov (NCT00405015).

Study design
This was a randomized, placebo-controlled, double-blind, single-centre, crossover study with 6 weeks of wash out comparing placebo (GlaxoSmithKline, Brentford, United Kingdom) with *rosiglitazone* 4 mg (GlaxoSmithKline) twice daily during 8 weeks, which was chosen to be able to detect both acute and chronic effects of *rosiglitazone*. At randomization eligible subjects received a specific treatment sequence, either *rosiglitazone*-placebo or placebo-*rosiglitazone*. Block randomization and blinding was performed at the department of Clinical Pharmacy. At the end of each period, the primary end-point experiments were performed in the morning after a 24-hour abstinence from caffeine containing beverages and an overnight fast. All interventions and the scintigraphic imaging were performed at the Clinical Research Center Nijmegen (CRCN).

One hour after ingestion of the last trial medication, injury in response to ischaemic hand gripping was measured using previously described experimental procedures[13,18]. Immediately on reperfusion recombinant human annexin-A5 labeled with Tc-99m (0.1mg; 400MBq) was administered intravenously. Both hands were imaged at 1 and 4 hours of reperfusion by using a gamma camera (Orbiter, Siemens Healthcare, Hoffman Estates, IL, USA) connected to a Hermes Gold image processing system (Hermes, Stockholm, Sweden). Recombinant human annexin-A5 was obtained from Theseus Imaging Corporation (Houston, Texas) and labeled with Tc-99m as described in detail previously[18].

During all visits adverse events and pill compliance were recorded. In addition, physical examination was performed and safety chemical, haematological, and glycaemic profiles were determined. Participants were strictly advised to maintain their diet and not to change lifestyle throughout the study.

Statistical analysis
A predefined region of interest was identified for each hand representing the thenar muscle. Within this region of interest radioactivity was expressed as counts per pixel. Annexin-A5 targeting after ischaemic exercise was calculated as the percentage difference in radioactivity between the experimental and control hand. To allow detection of at least 40% reduction in annexin targeting by *rosiglitazone* treatment with confidence on 5% level and power of 90% we calculated the sample size to be 9 subjects. A group of 13 subjects was selected to allow for some dropouts. Statistical analysis was performed with SPSS software. Descriptive statistics are presented as mean ±SD or median with the range for variables which were not distributed normally (Shapiro Wilk test <0.05). For the primary analysis we compared *rosiglitazone* with placebo using a distribution free analysis for paired observations (Wilcoxon) between the scintigraphic measurements at one and four hours after the ischaemic insult. The HOMA-index was used to

assess insulin resistance and calculated according to Matthews formula[19]. For vital signs, safety parameters and parameters of glucose regulation the change during a treatment period was calculated. Treatment effect of *rosiglitazone* was defined as the difference between the changes in each period.

Results

Subjects' enrolment and flow through the study are represented in Figure 1. The final statistical analysis on the primary endpoint was performed on 10 subjects in whom a complete data set was obtained. Indeed, the selected subjects showed characteristics of the metabolic syndrome with insulin resistance as shown in Table 1.

Adherence to the study drug was excellent during both treatment regimens (placebo: 98%, range 92-100%; *rosiglitazone*: 95%, range 88-100%). The subjects reported slightly more adverse events during *rosiglitazone* treatment (total 19 events, 75% of subjects) in comparison with placebo (total: 13 events, 75% of subjects). All adverse events were mild and included headache, common cold, nausea and oedema. Two subjects reported oedema while using *rosiglitazone*, one during placebo treatment.

Ischaemia-reperfusion injury

Rosiglitazone reduced annexin-A5 targeting at 1 hour after the ischaemic exercise by 43% (median 8.4% (range 0.6-49%) versus 4.7% (range 0.7-20%); $P = 0.037$). At 4 hours after reperfusion, targeting was reduced by 35% (median: 7.4% (range 0.5-50%) versus 4.8% (range -0.1-13%); $P = 0.059$) (Figure 2). There was no difference in workload (product of force (kg) and duration of exercise (seconds)) between the two ischaemic-exercise tests (*rosiglitazone*: 4049 ± 1981, placebo: 3693 ± 1450 kg·sec, $P = 0.323$). Annexin targeting during placebo treatment did not differ whether performed in period 1 or 2 (period 1: median 11% (range (range 0.5-22%); period 2: median 8.3 % (range 1.3-49%); $P = 0.91$).

Figure 1: Enrolment of study participants.
Flow of subjects through the study.
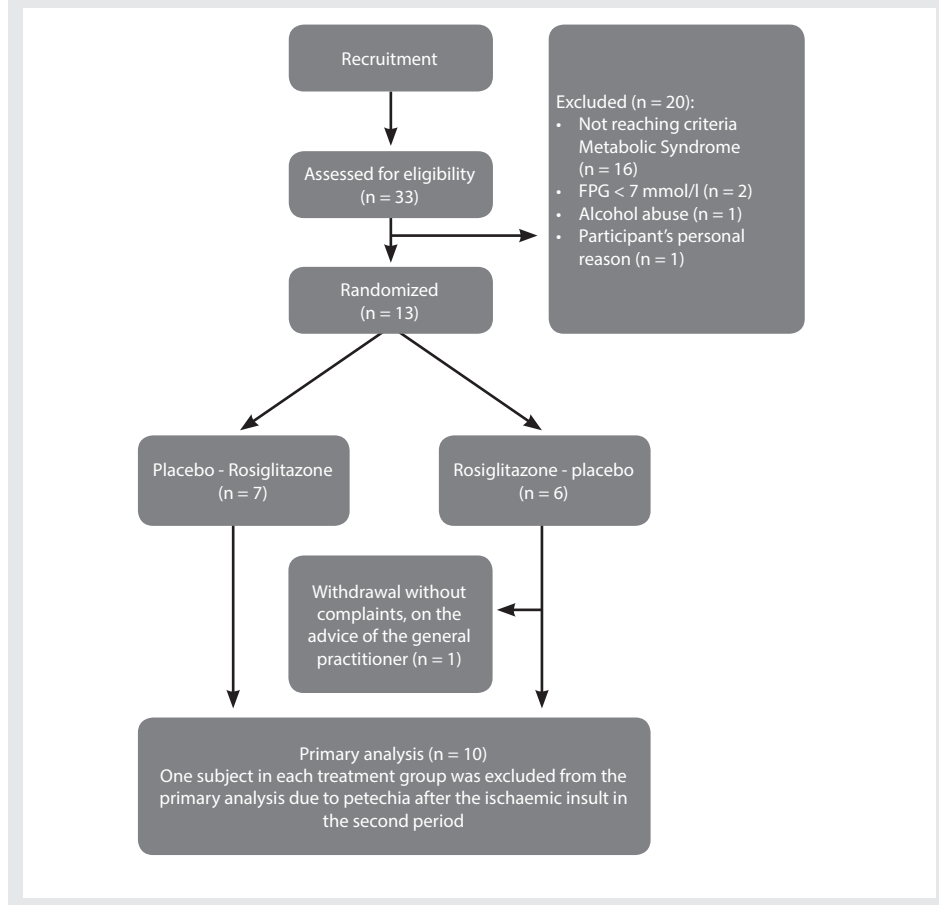
Clinical effects of *rosiglitazone*

As expected *rosiglitazone* decreased haematocrit (Table 2) compared to placebo (*rosiglitazone*: -0.02 ± 0.01; placebo: 0.001 ± 0.02; $P = 0.03$). In addition, *rosiglitazone* tended to decrease diastolic blood pressure, fasting glucose and waist circumference, but none was statistically significant. *Rosiglitazone* did not change systolic blood pressure, body mass, Homeostasis Model Assessment (HOMA) index and plasma insulin (Table 3).

Table 1: Screening characteristics of 10 evaluable subjects

Screening characteristics	Mean (SD) N=10
Age (year)	56 (7)
Male (%)	60
BMI (kg/m^2)	30.7 (4.6)
Systolic blood pressure (mmHg)	136 (17)
Diastolic blood pressure (mmHg)	84 (8)
Waist circumference (cm)	108 (12)
Plasma Triglycerides (mmol/L)	2.12 (0.59)
HDL-cholesterol (mmol/L)	1.25 (0.22)
Fasting plasma glucose (mmol/L)	5.2 (0.5)
HOMA-index	4.10 (2.14)
Medication	
Statin	50%
SSRI	20%

BMI: Body Mass Index
HDL: High Density Lipoprotein
HOMA: Homeostasis Model Assessment
SSRI: Selective Serotonin Reuptake Inhibitors

Table 2: Therapeutic effect of *rosiglitazone* on vital signs en safety parameters.

Vital signs and safety parameters	Baseline Mean (SD)	Rosiglitazone Mean (SE)
Body mass (kg)	90 (17)	0.61 (0.74)
Systolic blood pressure (mmHg)	130 (12)	-2.7 (4.4)
Diastolic blood pressure (mmHg)	82 (5)	-5.0 (2.0)
Waist circumference (cm)	104 (12)	0.9 (1.6)
ALAT (U/L)	33 (19)	-1.2 (3.6)
Haematocrit (1/L)	0.40 (0.03)	-0.02 (0.01)

The treatment effect of *rosiglitazone* was defined as the difference between the change during *rosiglitazone* minus the change during placebo.
ALAT: Alanine aminotransferase

Table 3: Therapeutic effect of *rosiglitazone* on parameters of glycaemic regulation.

Parameters of glycaemic regulation	Baseline Mean (SD)	Rosiglitazone Mean (SE)
Fasting plasma glucose (mmol/L)	5.5 (0.5)	-0.3 (0.17)
Plasma insulin (mU/L)	16 (7)	-1.3 (2.5)
HOMA index	4.10 (2.14)	-0.52 (0.66)

The treatment effect of rosiglitazone was defined as the difference between the change during the *rosiglitazone*-period minus the change during the placebo-period.
HOMA: Homeostasis Model Assessment

Figure 2: Therapeutic effect of *rosiglitazone* on annexin targeting.
Annexin-A5 targeting (expressed as percentage difference between experimental and control hand) in the thenar muscle at 1h (A) and 4h (B) after reperfusion at the end of either the *rosiglitazone* (□) or placebo (■) period).
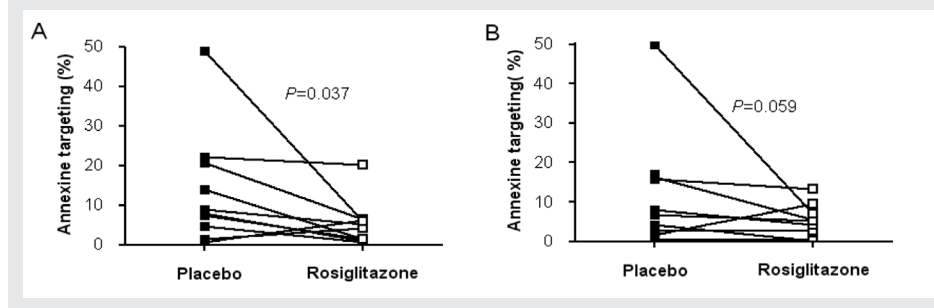
Discussion

The main finding of this study is that *rosiglitazone* is able to reduce annexin targeting after ischaemic exercise. As annexin targeting is considered to be an early marker of I/R injury, this study supports the view that the previously observed protective influence of *rosiglitazone* in animal models also holds for human in vivo conditions.

This study was designed to translate preclinical animal data to humans. In comparison with the animal studies the daily dose in the present study was lower than was used in the rat (8mg/day versus up to 3 mg/kg/dag). The oral route of administration was also used by Yue et al. in the rat. In contrast to the animal study the endpoint in the present study was measured in skeletal muscle of the forearm and not in the heart for ethical reasons. Despite these differences in design, our results provide evidence in humans for the concept as developed in animals that *rosiglitazone* protects against the deleterious sequelae of ischaemia and reperfusion. The observed *rosiglitazone*-related reduction in annexin targeting was 43%. Differences in ischaemic challenge cannot explain this observations since workload did not differ between placebo and *rosiglitazone* treated individuals. In addition, our data are in line with the effect of other interventions in previous human studies with this model. Ischaemic preconditioning, and treatment with *rosuvastatin, dipyridamole*, or *adenosine* resulted in reductions

of annexin targeting of around 50%[13, 20,21]. Since half of the participants of the present study were already on statin treatment, the additional protective effect of *rosiglitazone* is remarkable, and may suggest that the mechanism of action of the protective effects of *rosiglitazone* and statins do not interfere with each other.

After the meta-analysis performed by Nissen et al.[11] an intense scientific dispute started whether thiazolidinediones and particularly *rosiglitazone*, can protect against cardiovascular disease or are associated with an increased risk of ischaemic cardiovascular events accumulating with the recent publication of the final results of the RECORD study[22,23]. Our results are not favouring any side in this dispute as we did not investigate the frequency of an ischaemic event but the size of injury after a standardized ischaemic insult. Extrapolating our observation to clinical practice would suggest that *rosiglitazone* reduces the infarct size at the moment of an event. Interestingly, this could be one of the mechanisms behind the finding that *rosiglitazone* did not increase mortality in conjunction with the elevation in myocardial ischaemic events seen in the meta-analyses by Nissen et al. and Singh et al. In addition, the RECORD study showed numerically less cardiovascular deaths in the group treated with *rosiglitazone*.

The main therapeutic objective of *rosiglitazone* treatment in type 2 diabetes is insulin sensitization. It is well known that insulin itself has anti-apoptotic properties in myocardial ischaemia-reperfusion[9] and in one animal study the improvement in insulin sensitivity was associated with the beneficial effect on I/R injury[10]. However, with the current data we cannot proof this association. Our study was not powered for the endpoint "insulin sensitivity" and we did not measure this endpoint with the gold standard, a hyperinsulinaemia euglycaemic clamp procedure. Nevertheless, our data suggest that *rosiglitazone* has direct effects on I/R injury. Potential alternative mechanisms not directly affecting insulin sensitivity, could be upregulation of haem-oxygenase or NO-synthase. Recently, the protective effect of haem-oxygenase 1 upregulation in recurrent myocardial injury was shown[24]. Furthermore, expression of haem-oxygenase 1 in human vascular cells was shown to be regulated by PPAR-γ[25]. With respect to NO-synthase, *pioglitazone* did not induce upregulation of NO-synthase in an animal I/R injury model[26]. In line with this preclinical study we were not able to show increased insulin-induced NO-related vasodilation after treatment with *rosiglitazone* in a previous clinical study[27].

The reason we did not find significant changes in diastolic blood pressure, waist circumference and body weight was that this study was underpowered for these endpoints and the duration of the intervention was probably too short for significant changes in body composition. The *rosiglitazone*-related reduction of haematocrit is a well known side effect of *rosiglitazone* therapy and supports adequate compliance to their study treatment.

Inherent drawbacks of the crossover design are carry-over effects. In this study there were two potential candidates for inducing carry-over: treatment with *rosiglitazone* and the ischaemic exercise test with annexin-A5 injection. We did not observe any difference in annexin targeting during placebo treatment in both periods making a carry-over unlikely.

In this study we did not include subjects with diabetes to overcome confounding by a *rosiglitazone*-mediated improvement of glycaemic control. Since the beneficial effect of *rosiglitazone* on I/R injury was supposed to be more pronounced in insulin-resistant than in healthy subjects we decided to include non-diabetic subjects with the metabolic syndrome without glucose intolerance. Insulin resistance is a mainstay of this syndrome and the clinical criteria for the metabolic syndrome are better defined than for insulin resistance. Indeed, the HOMA-index of these subjects at baseline indicated that they were insulin-resistant. In addition, we included only male and postmenopausal female subjects because potential formation of antibodies against annexin-A5 could in theory interfere with placental function.

In conclusion, we present the first human in vivo data on the beneficial effects of *rosiglitazone* on ischaemia-reperfusion injury.

Funding

References

1. Grundy SM, Benjamin IJ, Burke GL, Chait A, Eckel RH, Howard BV, Mitch W, Smith SC, Jr., Sowers JR. Diabetes and cardiovascular disease: a statement for healthcare professionals from the American Heart Association. *Circulation*; 100:1134-1146.

2. Kannel WB, McGee DL. Diabetes and cardiovascular risk factors: the Framingham study. *Circulation* 1979; 59:8-13.

3. Aronson D, Rayfield EJ, Chesebro JH. Mechanisms determining course and outcome of diabetic patients who have had acute myocardial infarction. *Ann Intern Med* 1997; 126:296-306.

4. Lee TM, Chou TF. Impairment of myocardial protection in type 2 diabetic patients. *J Clin Endocrinol Metab* 2003; 88:531-537.

5. Forrat R, Sebbag L, Wiernsperger N, Guidollet J, Renaud S, De LM. Acute myocardial infarction in dogs with experimental diabetes. *Cardiovasc Res* 1993; 27(11):1908-1912.

6. Abdelrahman M, Sivarajah A, Thiemermann C. Beneficial effects of PPAR-gamma ligands in ischaemie-reperfusion injury, inflammation and shock. *Cardiovasc Res* 2005; 65:772-781.

7. Wayman NS, Hattori Y, McDonald MC, Mota-Filipe H, Cuzzocrea S, Pisano B, Chatterjee PK, Thiemermann C. Ligands of the peroxisome proliferator-activated receptors (PPAR-gamma and PPAR-alpha) reduce myocardial infarct size. *FASEB J* 2002; 16:1027-1040.

8. Khandoudi N, Delerive P, Berrebi-Bertrand I, Buckingham RE, Staels B, Bril A. Rosiglitazone, a peroxisome proliferator-activated receptor-gamma, inhibits the Jun NH(2)-terminal kinase/activating protein 1 pathway and protects the heart from ischaemie/reperfusion injury. *Diabetes* 2002; 51:1507-1514.

9. Gao F, Gao E, Yue TL, Ohlstein EH, Lopez BL, Christopher TA, Ma XL. Nitric oxide mediates the antiapoptotic effect of insulin in myocardial ischaemie-reperfusion: the roles of PI3-kinase, Akt, and endothelial nitric oxide synthase phosphorylation. *Circulation* 2002; 105:1497-1502.

10. Yue TL, Bao W, Gu JL, Cui J, Tao L, Ma XL, Ohlstein EH, Jucker BJ. Rosiglitazone treatment in Zucker diabetic Fatty rats is associated with ameliorated cardiac insulin resistance and protection from ischaemie/reperfusion-induced myocardial injury. *Diabetes* 2005; 54:554-562.

11. Nissen SE, Wolski K. Effect of rosiglitazone on the risk of myocardial infarction and death from cardiovascular causes. *N Engl J Med* 2007; 356:2457-2471.

12. Singh S, Loke YK, Furberg CD. Long-term risk of cardiovascular events with rosiglitazone: a meta-analysis. *JAMA* 2007; 298:1189-1195.

13. Rongen GA, Oyen WJ, Ramakers BP, Riksen NP, Boerman OC, Steinmetz N, Smits P. Annexin A5 scintigraphy of forearm as a novel in vivo model of skeletal muscle preconditioning in humans. *Circulation* 2005; 111:173-178.

14. Hammill AK, Uhr JW, Scheuermann RH. Annexin V staining due to loss of membrane asymmetry can be reversible and precede commitment to apoptotic death. *Exp Cell Res* 1999; 251:16-21.

15. Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* 1994; 84:1415-1420.

16. Teoh NC, Ito Y, Field J, Bethea NW, Amr D, McCuskey MK, Farell GC, Allison AC. Diannexin, a novel annexin V homodimer, provides prolonged protection against hepatic ischaemie-reperfusion injury in mice. *Gastroenterology* 2007; 133:632-646.

17. Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, Gordon DJ, Krauss RM, Savage PJ, Smith SC, Jr., Spertus JA, Costa F. Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. *Circulation* 2005; 112:2735-2752.

18. Riksen NP, Zhou Z, Oyen WJ, Jaspers R, Ramakers BP, Brouwer RM, Boerman OC, Steinmetz N, Smits P, Rongen GA. Caffeine prevents protection in two human models of ischemic preconditioning. *J Am Coll Cardiol* 2006; 48:700-707.

19. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985; 28(7):412-419.

20. Riksen NP, Oyen WJ, Ramakers BP, Van den Broek PH, Engbersen R, Boerman OC, Smits P, Rongen GA. Oral therapy with dipyridamole limits ischaemie-reperfusion injury in humans. *Clin Pharmacol Ther* 2005; 78:52-59.

21. Meijer P, Oyen WJ, Dekker D, Van den Broek PH, Wouters CW, Boerman OC, Scheffer GJ, Smits P, Rongen GA. Rosuvastatin Increases Extracellular Adenosine Formation in Humans In Vivo. A New Perspective on Cardiovascular Protection. *Arterioscler Thromb Vasc Biol* 2009; 29:963-968.

22. Home PD, Pocock SJ, Beck-Nielsen H, Curtis PS, Gomis R, Hanefeld M, Jones NP, Komajada M, McMurray JJ. Rosiglitazone evaluated for cardiovascular outcomes in oral agent combination therapy for type 2 diabetes (RECORD): a multicentre, randomised, open-label trial. *Lancet* 2009; 373:2125-2135.

23. Retnakaran R, Zinman B. Thiazolidinediones and clinical outcomes in type 2 diabetes. *Lancet* 2009; 373:2088-2090.

24. Pachori AS, Melo LG, Zhang L, Solomon SD, Dzau VJ. Chronic recurrent myocardial ischemic injury is significantly attenuated by pre-emptive adeno-associated virus heme oxygenase-1 gene delivery. *J Am Coll Cardiol* 2006; 47:635-643.

25. Kronke G, Kadl A, Ikonomu E, Bluml S, Furnkranz A, Sarembock IJ, Bochkov VN, Exner M, Binder BN, Leitinger N. Expression of heme oxygenase-1 in human vascular cells is regulated by peroxisome proliferator-activated receptors. *Arterioscler Thromb Vasc Biol* 2007; 27:1276-1282.

26. Ye Y, Lin Y, Atar S, Huang MH, Perez-Polo JR, Uretsky BF, Birnbaum Y. Myocardial protection by pioglitazone, atorvastatin, and their combination: mechanisms and possible interactions. *Am J Physiol Heart Circ Physiol* 2006; 291:H1158-H1169.

27. Rennings AJ, Smits P, Stewart MW, Tack CJ. Fluid retention and vascular effects of rosiglitazone in obese, insulin-resistant, nondiabetic subjects. *Diabetes Care* 2006; 29:581-587.

Appendix: published version in the European Heart Journal

In animals, thiazolidinediones reduce ischaemia-reperfusion injury. A clinical meta-analysis raised suspicion that *rosiglitazone* increases the *incidence* of myocardial infarction (Nissen et al. N Engl J Med 2007). However, human data on a possible benefit on infarct *size* (i.e. ischaemia-reperfusion injury) are not available. Therefore, we investigated the effect of *rosiglitazone* on ischaemia-reperfusion injury in 10 insulin-resistant participants without hyperglycaemia. We used a thoroughly validated human *in vivo* model to quantify ischaemia-reperfusion injury in skeletal muscle by annexin-A5-scintigraphy (Rongen et al. Circulation 2005). At the end of each treatment period (*rosiglitazone* 4mg b.d. *vs.* placebo), the participants were subjected to 10 minutes of forearm ischaemia, combined with standardized intermittent handgripping. At reperfusion, 500MBq ^{99m}Tc-annexin-A5 was administered intravenously. Annexin-uptake (counts per pixel) was measured in thenar muscle one hour post-reperfusion using a gamma camera. Ischaemia-reperfusion injury was quantified as the percentage difference in uptake between experimental and control side (annexin-targeting). *Rosiglitazone* reduced annexin-targeting from 8.4% (median; range 0.6-49%) to 4.7% (0.7-20%) ($P = 0.037$). We present the first human *in vivo* data on the beneficial effects of *rosiglitazone* on ischaemia-reperfusion injury. This observation puts the disputed elevation in myocardial ischaemic events during *rosiglitazone* treatment in perspective.
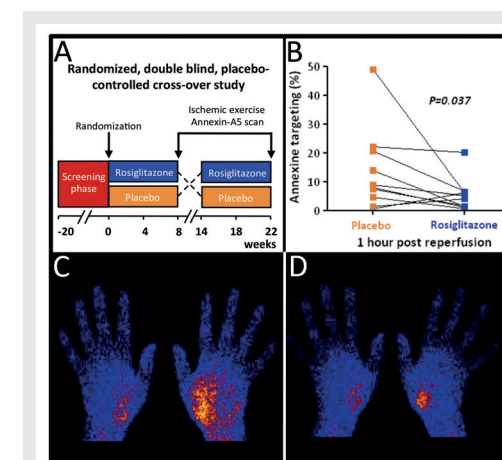
Panel A: Study design.
Panel B: Individual plots of the effects of *rosiglitazone* on ^{99}mTc-annexin-targeting in insulin-resistant subjects.
Panel C: Typical ^{99}mTc-annexin-uptake one hour after reperfusion at end of placebo period. Left: control hand; right: post-ischaemic hand. Counts increase from blue to yellow.
Panel D: Same patient, but at the end of *rosiglitazone* treatment period.

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Summary, general discussion and perspectives

This thesis addresses important effects of the thiazolidinedione derivatives: negative effects on fluid retention and oedema formation, but also positive effects of thiazolidinediones on autonomic neuropathy and on ischaemic preconditioning. This chapter contains four parts. In the first part, the main findings of the performed studies are summarized. In part two an extensive updated literature review on the topic of thiazolidinedione-related fluid accumulation is presented, culminating in a proposed scheme, in which the results of the present studies are intertwined to data from the literature. During the years of this PhD project *rosiglitazone* became the subject of discussion, because of doubt around its cardiovascular safety. Therefore, *rosiglitazone*'s presumed cardiovascular unsafety is discussed in part three. Finally, future perspectives of PPAR-γ agonists and the importance of the presented studies with respect to newer hypoglycaemic agents are briefly outlined.

Summary

Insulin induces vasodilation and increases capillary permeability. To determine whether thiazolidinedione-related fluid retention and oedema formation is based on amplification of these vascular effects of insulin ("insulin sensitization"), a randomized double-blind crossover study comparing *rosiglitazone* 4 mg b.i.d. and placebo was performed[1]. The results are described in **chapter 2**. *Rosiglitazone* treatment increased insulin sensitivity, as measured by the hyperinsulinaemia euglycaemic clamp, but neither affected insulin-mediated vasodilation, nor the nitric oxide-dependent part of insulin-mediated vasodilation, nor vascular permeability. Nevertheless, thiazolidinediones may induce vasodilation as suggested by the fact that *rosiglitazone* caused a fall in diastolic blood pressure.

As expected, *rosiglitazone* induced a rise in plasma volume of 255 ml/1.73 m². The increase in foot volume during *rosiglitazone* treatment was correlated with the change in insulin-induced glucose uptake (insulin sensitivity). This implies that the stronger the effect on insulin sensitivity the more oedema was formed. These findings suggest that thiazolidinediones augment capillary recruitment, either structurally or functionally. This observation on capillary recruitment was recently confirmed by Gealekman et al. who found an increase in adipose capillary density during treatment with thiazolidinediones[2]. In summary, our

study suggests that the effects of thiazolidinediones are independent of insulin-induced vasodilation and insulin-induced increases in capillary permeability.

Assuming that vasodilation is at least one of the pathways leading to thiazolidinedione-related fluid retention, and with the notion that changes in vascular resistance would normally be counterbalanced by the autonomic nervous system, we explored whether autonomic neuropathy is a risk factor for oedema formation. This hypothesis was tested in a randomized double-blind parallel study comparing *rosiglitazone* 4 mg b.i.d. and placebo for 16 weeks in insulin-treated patients with type 2 diabetes with and without autonomic neuropathy[3]. As described in **chapter 3**, autonomic neuropathy indeed appeared to be a risk factor for thiazolidinedione-induced increases in vascular permeability, which may potentially lead to oedema formation. In contrast, autonomic neuropathy did not increase *rosiglitazone*-related fluid retention. We conclude that autonomic neuropathy should be considered a risk factor for thiazolidinedione-related oedema but not for fluid retention.

As described in **chapter 1**, thiazolidinedione-related fluid accumulation can be explained by primary renal sodium retention. The study described in **chapter 4** was based on this renal hypothesis and aimed at determining whether thiazolidinediones induce resistance to the natriuretic response of loop diuretics. This hypothesis was tested in a double-blind placebo-controlled crossover study[4]. The natriuretic response to *amiloride* and *furosemide* was measured, to detect *rosiglitazone*-induced upregulation of ENaC or furosemide resistance. We calculated *furosemide* excretion rate-response curves (analogous to dose-response curves) for the individual subjects to compare *furosemide*'s natriuretic response during *rosiglitazone* and placebo in detail. We also measured the amount of ENaC excreted in exosomes in the urine as an assessment of renal ENaC expression. We did not find an increased natriuretic response to *amiloride* during *rosiglitazone* treatment and we did not find a higher amount of ENaC in the urine which argues against upregulation of ENaC both on a functional and molecular level. In addition, the *furosemide* dose response curves were not influenced by *rosiglitazone* treatment. We provide a number of explanations for the apparent discrepancy with the previous clinical observations of *furosemide*-resistant oedema[5]. Finally, the finding of normal pharmacodynamic responses to

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furosemide combined with the previously observed *furosemide*-resistant oedema in clinical settings, may imply that thiazolidinedione-induced vasodilation is the key mechanism of thiazolidinedione-related oedema, analogously to oedema associated with calcium entry blocker treatment, in particular *nifedipine*[6,7], a drug which combines natriuresis and vasodilation. In conclusion, thiazolidinediones do not upregulate ENaC and do not induce resistance to loop diuretics and the clinical consequence of these findings is that thiazolidinedione-related fluid retention can be treated with diuretics.

It is clinically relevant to monitor for thiazolidinedione-related fluid accumulation. In **chapter 5** we have described the relationship between changes in haematocrit and changes in plasma volume during *rosiglitazone* treatment, based on the studies described in **chapter 2** and **3** where we measured plasma volume using a gold standard technique as well as haematocrit. In contrast to most views, we found no correlation whatsoever between change in haematocrit and change in plasma volume during chronic treatment with *rosiglitazone* neither in subjects with insulin resistance nor in patients with diabetes. From a pathophysiological point of view the nonrelationship between haematocrit and plasma volume changes suggests that thiazolidinediones, at least *rosiglitazone*, have additional effects on top of plasma volume expansion, such as lowering of total erythrocyte volume. The clinical implication of this uncoupling is that monitoring change in haematocrit cannot be used to detect fluid overload in an individual patient on chronic thiazolidinedione treatment.

An important topic in this thesis is diabetic autonomic neuropathy. In **chapter 6**, we report on α-adrenergic tone and α-adrenergic responsiveness in insulin-treated patients with type 2 diabetes with and without autonomic neuropathy. We found that in patients with cardiac autonomic neuropathy the α-adrenergic tone is preserved, while adrenergic responsiveness is diminished. The preservation of α-adrenergic tone in patients with cardiac autonomic neuropathy may be rather counter-intuitive, but is in line with previous findings from our group obtained in spinal cord-injured individuals[8]. Moreover, we found that *rosiglitazone* enhanced the forearm vasoconstrictor response to lower body negative pressure, unrelated to the improvement in glycaemic control.

It had been hypothesized that thiazolidinediones were beneficial for ischaemic cardiovascular outcomes and that one of the hypothesized beneficial mechanisms was protection against ischaemia-reperfusion injury. **Chapter 7** describes the results of a randomized double-blind placebo-controlled crossover study on the influence of *rosiglitazone* treatment on ischaemia-reperfusion injury, as measured with annexin A5 scintigraphy[9]. Indeed, *rosiglitazone* reduced annexin targeting after ischaemic exercise, suggesting a protective effect on ischaemia-reperfusion injury in human in vivo conditions.

Mechanisms of action of thiazolidinedione-related fluid accumulation

The previous section provides a summary of our findings on the mechanism of action, risk factors and treatment of thiazolidinedione-related fluid retention and oedema formation. In this part, these findings are put in perspective and integrated with the literature on this issue. This review follows the division by the potential mechanisms of action of fluid accumulation: cardiac, renal and vascular hypothesis.

Cardiac hypothesis

Several clinical trials have studied echocardiographic changes in cardiac function during thiazolidinedione treatment. Compared to *glibenclamide*, *rosiglitazone* did not impair cardiac function in type 2 diabetic patients without heart failure [10]. Neither *pioglitazone*[11,12] nor *rosiglitazone*[13] did reduce cardiac function in patients with pre-existing heart failure. Narang et al. found no differences in cardiac function as measured by cardiac Magnetic Resonance Imaging (MRI) between subjects, with and without *rosiglitazone*-related oedema[14]. In one study, *pioglitazone* increased left ventricular end-diastolic volume and left–atrial end-systolic volumes, together with a borderline significant increase in ejection fraction[15]. On the other hand *pioglitazone* has been shown to improve left ventricular diastolic function in subjects with essential hypertension[16]. Taken together these results suggest that thiazolidinediones do not negatively influence cardiac function.

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Despite this, thiazolidinediones increase the risk of heart failure, as shown both in observational[17] and prospective clinical trials[18]. The clinical trials were performed in a wide range of populations: pharmacotherapy-naïve type 2 diabetic patients[21], patients inadequately controlled on sulfonylurea or *metformin*[22], patients without pre-existing evidence for cardiovascular disease or diabetes[20], and patients with established cardiovascular disease[19]. In the last study the relative risk of heart failure events was 2.6. Additionally, an excess in heart failure deaths was found. Independent risk factors for thiazolidinedione-associated heart failure were age, microalbuminuria, Body Mass Index (BMI) and systolic blood pressure[22]. Thiazolidinedione-associated heart failure is suggested to be a milder form of heart failure as one retrospective cohort study reported lower mortality rates for patients with heart failure prescribed thiazolidinediones despite an increase in re-admissions for heart failure[23].

Following the introduction of *rosiglitazone* and *pioglitazone,* several case reports on weight gain and pulmonary oedema have been described[5,24-37]. These reports provide a common profile of the subject prone to develop pulmonary oedema and the setting: roughly, thiazolidinediones can induce a sub-immediate (start of the adverse event, weeks to months after initiation of the drug), gradual volume overload, without haemodynamic compromise in patients on *insulin* treatment with either known renal failure or diastolic dysfunction[38].

Actually, all glucose-lowering therapies[39,40], but also poor glycaemic control[41] may increase the risk for heart failure. Of course, this notion complicates the exploration of the relation between thiazolidinediones and heart failure.

We did not investigate the cardiac hypothesis. Literature reports show that thiazolidinedione treatment is associated with a higher incidence of symptomatic heart failure with in general a milder clinical course, while thiazolidinediones have no negative influence on cardiac function and structure. An accepted explanation for this paradox is that heart failure becomes symptomatic due to thiazolidinedione-related fluid overload[14]. There are cases of new onset heart failure in heart failure naïve patients and overt fluid overload after thiazolidinedione derivatives, but these are rare.

Renal hypothesis

The fluid retaining properties of thiazolidinediones are found in many studies[1,42]. Fluid accumulation in the body is always the consequence of either primary or secondary sodium retention in the kidney. In this paragraph, the literature on primary renal sodium retention is reviewed. Thiazolidinedione-induced primary renal sodium retention could involve insulin-dependent en insulin-independent pathways.

Insulin has proven antinatriuretic properties both in diabetic patients[43] and in subjects without diabetes[44]. As thiazolidinediones are insulin sensitizers, at least with regard to glucose regulation, one may hypothesize that thiazolidinediones also amplify the renal actions of insulin. The mechanism of insulin's antinatriuretic effect seems to be a modulation of tubular sodium transport[45] but the exact anatomic location is not fully clear[45,46]. Insulin binds to the basolateral renal cortical membrane and has the highest binding density in the distal convolute and the thick ascending limb[47].

Insulin-independent pathways of thiazolidinedione-related sodium retention could theoretically be related to alterations in renal haemodynamics or to modification of the tubular sodium transport. Preclinical[48] and human studies[49] have found neither changes in glomerular filtration rate nor in renal blood flow during thiazolidinedione treatment, despite clear sodium retention, together supporting an effect of thiazolidinediones on tubular sodium reabsorption. PPAR-γ is expressed in several parts of the kidney, especially in the medullary collecting duct, but also in the proximal tubules, glomeruli and microvasculature[50] and therefore both the proximal tubules and the distal nephron could be involved in thiazolidinedione-induced tubular sodium retention. Although several reports favour modification of proximal salt handling, such as reduction of lithium clearance[49], increments in protein levels of subunits of sodium channels and aquaporins from proximal sites[51], and elevation of bicarbonate-coupled proximal sodium transport[52], many studies have suggested that thiazolidinedione-related renal sodium retention is caused by epithelial sodium channel (ENaC) upregulation in the distal nephron. Key publications in this field were Zhang et al[53] and Guan et al.[54] demonstrating upregulation of ENaC by *rosiglitazone* and *pioglitazone*. Both groups showed that increases in weight, plasma volume, and urinary sodium excretion during thiazolidinedione treatment did not occur in PPAR-γ knockout mice. Guan et al. also showed that

amiloride was able to prevent weight and water increase in wild-type mice. On the other hand, neither Chen et al.[55] using *amiloride* for direct ENaC inhibition, nor Vallon et al.[56] using a collecting-duct specific gene inactivation of ENaC, were able to reduce thiazolidinedione-induced oedema. Furthermore, Chen did not find evidence for changes in renal expression of ENaC in vivo, and, in another report, PPAR-γ agonists failed to enhance sodium transport in collecting duct cell lines[57]. Our human in vivo study[4] provided in vivo data on the importance of ENaC in thiazolidinedione-induced sodium retention in the human situation. As we observed neither functional nor molecular effects of *rosiglitazone* on renal ENaC, we in a way rehabilitated the view as hypothesized by Vallon that instead of ENaC, the *amiloride*-sensitive non-selective cation-channels in the medullary collecting duct may be critical in thiazolidinedione-induced sodium retention.

In summary, there is evidence that thiazolidinediones increase renal sodium reabsorption through direct pathways, but the exact mechanism and the site in the kidney and the exact transporter remains unclear. Moreover, the precise contribution of thiazolidinedione-related primary renal sodium reabsorption to body fluid retention and weight gain is probably limited, because a primary renal mechanism in itself is not compatible with the robust blood pressure lowering actions of thiazolidinediones[58]. Therefore, direct thiazolidinedione-induced renal sodium retention is probably an additional mechanism.

Vascular hypothesis

The key mechanism in the vascular hypothesis is arterial vasodilation resulting in both blood pressure reduction and oedema formation. In this hypothesis, renal sodium retention is a secondary phenomenon. Direct evidence comes from a study by Shargorodsky et al.[59] showing lower systemic vascular resistance in subjects using *rosiglitazone*. In line with these findings, we found a non-significant reduction in systemic vascular resistance during treatment with *rosiglitazone*[1]. Zanchi et al.[49] observed an increased plasma renin activity, and a reduced urinary sodium excretion and *lithium* clearance during *pioglitazone* treatment, supporting secondary sodium retention. Basu et al.[42] found higher day time heart rate during *pioglitazone* treatment. The results of Zanchi and Basu may point to sympathetically driven activation of the renin-angiotensin-aldosterone system (RAAS). In line with these findings, we observed that diabetic patients with autonomic neuropathy treated with *rosiglitazone* seemed to be protected against fluid overload[3].

It is important to note that thiazolidinedione-related oedema formation is a common adverse event (up to 16%) and most prevalent when thiazolidinediones are combined with insulin therapy[60]. PPAR-γ expression is common in the vascular system on several levels, for instance endothelial cells[61] and monocytes/macrophages[62]. Finally, thiazolidinediones are proposed to induce oedema both directly and indirectly (insulin) through several vascular mechanisms. In the next paragraph we summarize these mechanisms, starting with insulin-dependent pathways.

Insulin has vasodilator actions[63] which are diminished in case of insulin resistance[1,64]. Several mechanisms to explain insulin-induced vasodilation have been proposed and proven, such as sympathetic activation[63, 65, 66], nitric oxide (NO) release[67,68], the activation of calcium-dependent potassium channels[69], and the stimulation of Na^+-K^+ ATP- ase[70]. An argument for the role of the sympathetic nervous system in insulin-mediated oedema formation is the beneficial response to therapy with *ephedrine*, an α-adrenergic receptor agonist[71]. Despite the strong evidence of insulin's vasodilator actions, restoration of these vascular actions by thiazolidinediones is speculative. In our own study[1] we did not find proof for any effect of *rosiglitazone* on insulin-induced vasodilation or on the contribution of nitric oxide. In addition, there was also no evidence for upregulation of prostacycline mediated, calcium-activated potassium channel mediated or β-adrenergic mediated vasodilation (unpublished data). The absence of an effect on insulin-induced vasodilation are in agreement with findings with *troglitazone* in a previous report of our group[72]. In some in vivo studies, insulin improved microvascular perfusion without evidence of increased total forearm blood flow, which is consistent with (functional) capillary recruitment[73,74] and was first observed in animals by Rattigan et al.[75]. Recently, two reports showing a reduction in muscle insulin-induced glucose uptake in animals with a structural[76] or functional[77] reduction in capillary recruitment confirmed the concept of reduced capillary recruitment as a cause of muscle insulin resistance[78,79]. In line with this concept it may be hypothesized that thiazolidinediones restore insulin's ability to stimulate capillary recruitment. Circumstantial evidence for this view comes from our finding of a clear correlation between change in foot volume and glucose disposal during *rosiglitazone* treatment[1]. Recently, another group observed that *rosiglitazone* increased adipose capillary density[2].

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In addition to insulin's vasodilator actions, insulin also increases capillary permeability[80]. Several mechanisms are proposed, including micropinocytosis[81] and activation of vascular endothelial growth factor (VEGF) production[82,83]. In a situation of insulin-related oedema, leakage of albumin can lead to secondary hypoalbuminemia, which could potentiate oedema formation by reduction of the plasma oncotic pressure[84]. Notwithstanding insulin's actions on the capillary membrane, we could not find evidence for the hypothetical restoration of insulin's capillary permeability during treatment with *rosiglitazone*[1].

Direct effects of thiazolidinediones have been described at several levels of the vascular system. With regard to vasodilation, Calnek et al. has observed increased NO production in endothelial cells after exposure to PPAR-γ ligands[85]. In vivo studies, either preclinical[86] or clinical[87,88] have shown increased nitric oxide production during treatment with thiazolidinediones, but these studies do not prove that this effect is independent of insulin. Other proposed direct mechanisms to explain thiazolidinedione-induced vasodilation are decreased sympathetic activity[89], reduced endothelin-1 secretion[61], and anti-inflammatory effects reflected by lower levels of either plasma C-reactive protein[90] or tissue necrosis factor (TNF)-α[91].

Apart from haemodynamic actions, Idris et al. showed that *rosiglitazone* increases capillary permeability for macromolecules[92] in human endothelial cells. Some studies suggest that thiazolidinediones increase capillary permeability by elevation of VEGF[93,94]. Conversely, several in vitro studies did show repression of VEGF production by thiazolidinediones[95,96]. Another suggested explanation for thiazolidinedione-associated increase in permeability is activation of protein kinase C[97]. Interestingly, in this study protein kinase C inhibition was able to prevent thiazolidinedione-induced oedema formation. Despite this mechanistic substantiation for thiazolidinedione-induced enhanced capillary permeability, our clinical study with *insulin*-treated diabetic patients found no effect of *rosiglitazone* itself on capillary permeability[3], which was in line with an animal study[98]. Only in the subgroup of patients with autonomic neuropathy we found *rosiglitazone* to induce vascular leakage[3].

In summary, thiazolidinedione-induced vasodilation can explain the high incidence of oedema in combination with blood pressure reduction. There are data compatible with reflex activation of the sympathetic nervous and renin- angiotensin-aldosterone system (RAAS) inducing renal sodium retention. Our own data could not confirm the hypothetical restoration of insulin-induced vasodilation or permeability by thiazolidinediones. Still, insulin sensitizing effects on capillary recruitment are likely to contribute to thiazolidinedione-related oedema.

Integrated framework of thiazolidinedione-related fluid accumulation

After having reviewed the literature and our own findings, we construct in this section our current view on the pathogenesis of thiazolidinedione-related fluid retention and oedema formation.

We think that the cause of thiazolidinedione-induced oedema formation, fluid retention and heart failure is multifactorial. This is a logical consequence of the notion that these clinical situations have some overlap but are not exchangeable. Figure 1 shows our hypothetical framework. The driving force for oedema formation, which is the most prevalent clinical outcome of thiazolidinedione-related fluid accumulation, is of vascular origin. Either capillary recruitment, increments in vascular permeability or direct arterial vasodilation, could play a role. We refer arterial vasodilation sometimes as microvascular imbalance, since vasodilation will change the equilibrium of the Starling forces in favour of oedema formation. Primary renal sodium/fluid retention may contribute to some extent, but this is an additional mechanism. The fall in systemic blood pressure following arterial vasodilation will increase sympathetic tone and activate RAAS, which in turn induces secondary renal sodium/fluid retention. Sodium retention will sequentially increase plasma volume and microvascular perfusion pressure, fueling oedema formation. In a subset of patients, sodium retention and the sequential rise in plasma volume will unmask asymptomatic heart failure, that will subsequently increase sodium retention in the kidney and oedema formation.

We also include genetic predisposition for fluid retention in this scheme. About 8-20% of the population carries a polymorphism in the PPAR-γ protein (Pro12Ala) that reduces its ligand-induced activity[99]. This polymorphism reduces the risk of thiazolidinedione-induced oedema[100] by pharmacodynamic and not physiological mechanisms. We did not include the finding that concomitant use of *fenofibrate*, a PPAR-α agonist, could protect against fluid retention[101], as this was only found in one small study, while several other studies with dual PPAR-α/γ agonists[102,103] did not show a reduction in oedema formation.

Figure 1: Proposed mechanisms leading to oedema formation, fluid retention and chronic heart failure during thiazolidinedione treatment. The shaded text in this Figure marks where this thesis adds new information.

The part of the scheme above the two braces represents the literature review of the effects of thiazolidinediones on renal and vascular processes. Thicker lines above the braces indicate more influence on fluid retention and oedema formation (at least in our view). The part under the braces visualizes the physiological consequences of the various factors that lead to the clinical conditions of fluid retention, oedema formation and heart failure.

RAAS: renin-angiotensin-aldosterone system

*: Chapter 4; **: Chapter 2; †: Chapter 3; ‡: Chapter 5
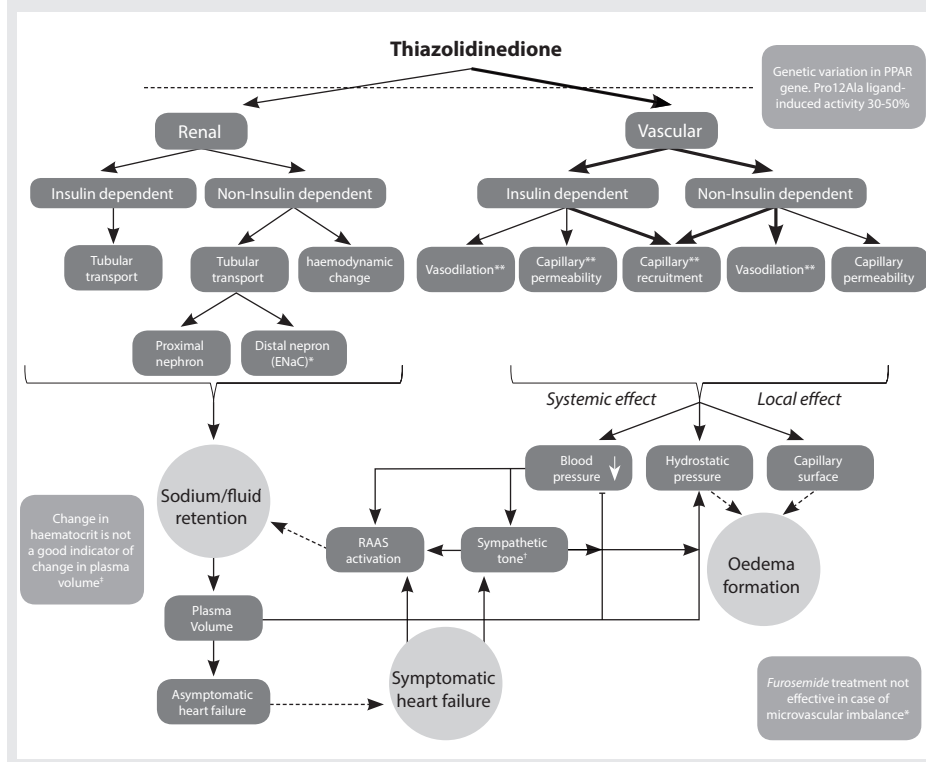
Implications for research and clinical care

At the time *rosiglitazone* and *pioglitazone* were approved for diabetes treatment it was expected that fluid accumulation was the Achilles heel of these drugs. Nowadays, there are two important arguments against this view. First, the primary pathway for fluid accumulation is from microvascular origin, leading to

benign oedema formation. Second, other unwanted effects were finally the reason for restriction of prescription, namely doubt about cardiac safety and increased risk of bladder cancer in case of *rosiglitazone*[104] and *pioglitazone*[105] respectively. Regarding the mechanism of thiazolidinedione-related fluid accumulation Figure 1 provides a good starting point to explain most clinical situations. We believe it is no longer appropriate to search for one pathway covering all cases of fluid accumulation. Indeed, this multifactorial scheme is the result of intensive research over the last decade. Obviously there remain areas of uncertainty, for instance, why some patients are more susceptible to develop serious pulmonary oedema. Perhaps, these cases can be explained by idiosyncratic drug-patient interactions.

The recommendations for clinicians given in the 2003 consensus statement[106] of the American Heart Association in conjunction with the American Diabetes Association are still applicable, but based on the studies described in this thesis, three additional recommendations can be added. First, autonomic neuropathy is a risk factor for oedema formation[3]. Second, monitoring haematocrit in the individual patient on thiazolidinedione treatment cannot detect fluid accumulation. Lastly, thiazolidinedione-treatment does not decrease the natriuretic response to loop diuretics and hence these agents can be used along with tapering of the thiazolidinedione dose to treat plasma volume overload[4].

While the exact pathogenesis and therapeutic approach towards thiazolidinedione-related fluid accumulation has not been solved, the studies described in this thesis have contributed to the knowledge regarding this clinical problem.

Rosiglitazone and cardiovascular safety

At the time of their introduction, the thiazolidinediones seemed promising regarding cardiovascular outcomes as they had beneficial effects on risk factors such as lipid profile, blood pressure, inflammatory markers and microalbuminuria. Meanwhile, cardiovascular outcome studies have provided more information. *Pioglitazone* seemed to redeem the expectations on cardiovascular benefit in the Proactive trial[14], and, although here the primary outcome parameter was not met, secondary outcomes were significantly better in patients treated with

pioglitazone instead of placebo. These results were confirmed in a meta-analysis a few years later[107]. With *rosiglitazone*, concerns regarding its cardiac safety, however, have eventual lead to withdrawal from the European market. In this paragraph the scientific proof for the cardiac (un)safety of *rosiglitazone* will be summarized.

The effect of *rosiglitazone* on cardiovascular outcomes has been debated extensively after the release of a meta-analysis by Nissen et al. in 2007[104]. Following this publication, many reports have appeared on this topic: one randomized controlled trial (RCT), several meta-analyses, epidemiological studies, and post-hoc nonrandomized evaluations of RCTs. RECORD[22] was the only RCT that has investigated *rosiglitazone*'s safety on cardiovascular outcomes. While it met the non-inferior criteria for the combined cardiovascular endpoint, it could not proof that *rosiglitazone* did not increase myocardial infarction. The RECORD study also received severe criticisms regarding its open-label design and concerns about endpoint reporting[108].

The published meta-analyses show diverging results[109]. In 2007 Nissen et al.[104] reported that *rosiglitazone* treatment was associated with a statistically significant increased risk for acute myocardial infarction (Hazard Ratio 1.43, p = 0.03) and a nonsignificant increased risk for cardiovascular death. Subsequently, Singh et al.[110] reported similar results. In contrast, 3 groups of independent researchers did not find a statistically significant increased risk for myocardial infarction with the use of *rosiglitazone*[111-113]. In 2010 a second wave of meta-analyses were published. The Federal Drug Agency (FDA), GlaxoSmithKline (GSK) and Nissen[114] updated their initial analyses. The updated FDA[115] and GSK[115] analyses were based on the same data sets, but used different statistical methods and reported a statistically significant increase in myocardial infarction (MI) of 80%, and a non-significant increase in MI of 41%, respectively.

Many epidemiological studies have tried to solve the dispute[116]. The largest study included more than 200,000 Medicare beneficiaries over 65 years of age and compared *rosiglitazone* with *pioglitazone*[117]. The results in this study were rather typical for the rest of the epidemiological data. *Rosiglitazone* yielded small increases in risk for chronic heart failure (25%) and death (14%) but did not increase the risk for acute myocardial infarction. In brief, the epidemiological studies showed weak associations, but are limited to their inherent limitations of potential confounding and bias[118].

Fueled by the scientific dispute, post-hoc analyses were performed on the data of 3 RCTs[119-121]. These analyses did not disclose an increase in risk for ischaemic cardiovascular disease in patients using *rosiglitazone*, but the post-hoc nature with its nonrandomized comparisons diluted their scientific strength.

Recently, an independent reevaluation of the results of the RECORD study reported no increase in cardiovascular events, myocardial infarction or death during *rosiglitazone* treatment compared with *metformin*/sulfonylurea[122]. As a consequence of this report the FDA relaxed some market restrictions and the scientific debate seemed to tilt again[123].

In summary, when all scientific evidence is taken together, there is no definite proof that *rosiglitazone* harms, but there is sufficient evidence to remain concerned about *rosiglitazone*'s cardiovascular safety. The fact that *rosiglitazone* does not have a unique health advantage over *pioglitazone*, justifies restriction of its use.

Future perspectives

The thiazolidinediones combine adverse effects with unique properties, and interestingly, they are now off patent. In Europe, *rosiglitazone* has been withdrawn from the market, while in the USA and other parts of the world its use is restricted. Despite extended warning labels, due to non-cardiac health concerns, for instance increase in risk of bladder cancer[105,124], *pioglitazone* appears in many international guidelines[125,126] and is still widely used around the world with a market share of 10.8% in 2011[127]. Strategies to optimize the risk/benefit ratio by using low dose thiazolidinedione in combination with *metformin* have received limited attention[128]. Thiazolidinediones, especially *pioglitazone*, are also used for the treatment of non-alcoholic steatohepatitis (NASH)[129], polycystic ovary syndrome (PCO)[130] and lipodystrophy in human immunodeficiency virus (HIV) infected patients[131].

Newer PPAR-γ agonists may reach the diabetes market the coming years. They can be divided into the following four groups, thiazolidinediones (*rivoglitazone*), dual PPAR-α/γ agonists (*aleglitazar*), partial PPAR-γ agonists (*balaglitazone*) and the selective PPAR-γ modulators (SPPARM)(*INT 131*). *Rivoglitazone*[132],

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and *aleglitazar*[133] appear fairly comparable with *pioglitazone* with respect to glycaemic control and oedema formation. In view of the previous members of these classes[134-136], expectations for these compounds need to be tempered. Recently, further development of *aleglitazar* has been interrupted due to side effects occurring in a cardiovascular outcome study (ALECARDIO). These side effects related to bone fractures, heart failure and gastrointestinal bleeding, mainly side effects already known from this class[137]. *Balaglitazone* and *INT 131* are from a pharmacological point of view very interesting. Being a partial agonist, *balaglitazone* is supposed to have less adverse effects, which was shown in a phase III challenge against *pioglitazone*[138]. *INT 131* is designed to be selectively a full agonist for the beneficial insulin sensitizing properties of PPAR-γ. Indeed, in a phase II trial, *INT 131* showed beneficial glycaemic action without the typical thiazolidinediones off-target adverse effects[139]. If one of these drugs reaches the market and will have a role in diabetes management, knowledge of the mechanism of *rosiglitazone*-related fluid retention condensed in this thesis will contribute to monitoring potential fluid related side effects.

However, whether one of these new PPAR-γ agonists will actually reach the diabetes market is questionable as following the introduction of the thiazolidinediones other alternatives have become available. These are the incretins, represented by the Di Peptidyl Peptidase-4 inhibitors (DPP-4) and Glucagon-Like-Peptide-1 agonists (GLP-1)[140], and the Sodium-Glucose co-transporter-2 inhibitors (SGLT2)[141], and are all approved for the treatment of type 2 diabetes. As a consequence of the discussion about *rosiglitazone*'s cardiovascular safety, these new agents are monitored carefully for cardiovascular outcomes and as part of their registration, cardiovascular outcome safety trials are ongoing. Recently, the results of a cardiovascular outcome study with the DPP-4 inhibitor *saxagliptin*[40] have been reported. While the study showed that use of *saxagliptin* was not associated with any increase in cardiovascular events (but neither with a decrease), surprisingly and unexpectedly, the risk for hospitalization because of heart failure was increased[40]. The explanation for this side effect is unclear.

So, it is quite remarkable that while we started our studies to solve the mechanism of action of thiazolidinedione-related fluid accumulation, the observation of increased risk of heart failure during treatment with a new hypoglycaemic agent in a way completes the circle of this thesis.

References

1. Rennings AJ, Smits P, Stewart MW, Tack CJ. Fluid retention and vascular effects of rosiglitazone in obese, insulin-resistant, nondiabetic subjects. *Diabetes Care* 2006; 29:581-587.

2. Gealekman O, Guseva N, Gurav K, Gusev A, Hartigan C, Thompson M, Malkani S, Corvera S. Effect of rosiglitazone on capillary density and angiogenesis in adipose tissue of normoglycaemic humans in a randomised controlled trial. *Diabetologia* 2012; 55:2794-2799.

3. Rennings AJ, Smits P, Stewart MW, Tack CJ. Autonomic neuropathy predisposes to rosiglitazone-induced vascular leakage in insulin-treated patients with type 2 diabetes: a randomised, controlled trial on thiazolidinedione-induced vascular leakage. *Diabetologia* 2010; 53:1856-1866.

4. Rennings AJ, Russel FG, Li Y, Deen PM, Masereeuw R, Tack CJ, Smits P. Preserved response to diuretics in rosiglitazone-treated subjects with insulin resistance: a randomized double-blind placebo-controlled crossover study. *Clin Pharmacol Ther* 2011; 89:587-594.

5. Niemeyer NV, Janney LM. Thiazolidinedione-induced oedema. *Pharmacotherapy* 2002; 22:924-929.

6. Wetzels JF, Wiltink PG, Hoitsma AJ, Huysmans FT, Koene RA. Diuretic and natriuretic effects of nifedipine in healthy persons. *Br J Clin Pharmacol* 1988; 25:547-553.

7. van der Heijden AG, Huysmans FT, van Hamersvelt HW. Foot volume increase on nifedipine is not prevented by pretreatment with diuretics. *J Hypertens* 2004; 22:425-430.

8. Kooijman M, Rongen GA, Smits P, Hopman MTE. Preserved alpha-adrenergic tone in the leg vascular bed of spinal cord-injured individuals. *Circulation* 2003; 108:2361-2367.

9. Rennings AJ, Meijer P, van Uden DJ, Tack CJ, Smits P, Boerman OC, Oyen WJ, Rongen GA. Rosiglitazone reduces ischaemia-reperfusion injury in patients with the metabolic syndrome. *Eur Heart J* 2010; 31:983.

10. Sutton MS, Rendell M, Dandona P, Dole JF, Murphy K, Patwardhan R, Patel J, Freed M. A comparison of the effects of rosiglitazone and glyburide on cardiovascular function and glycemic control in patients with type 2 diabetes. *Diabetes Care* 2002; 25:2058-2064.

11. Giles TD, Miller AB, Elkayam U, Bhattacharya M, Perez A. Pioglitazone and heart failure: Results from a controlled study in patients with type 2 diabetes mellitus and systolic dysfunction. *J Card Fail* 2008; 14:445-452.

12. Giles TD, Elkayam U, Bhattacharya M, Perez A, Miller AB. Comparison of pioglitazone vs glyburide in early heart failure: insights from a randomized controlled study of patients with type 2 diabetes and mild cardiac disease. *Congest Heart Fail* 2010; 16:111-117.

13. Dargie HJ, Hildebrandt PR, Riegger GA, McMurray JJ, McMorn SO, Roberts JN, Zambanini A, Wilding JP. A randomized, placebo-controlled trial assessing the effects of rosiglitazone on echocardiographic function and cardiac status in type 2 diabetic patients with New York Heart Association Functional Class I or II Heart Failure. *J Am Coll Cardiol* 2007; 49:1696-1704.

14. Narang N, Armstead SI, Stream A, Abdullah SM, See R, Snell PG, McGavock J, Ayers CR, Gore MO, Khera A, de Lemos JA, McGuire DK. Assessment of cardiac structure and function in patients without and with peripheral oedema during rosiglitazone treatment. *Diab Vasc Dis Res* 2011; 8:101-108.

15. Dorkhan M, Dencker M, Stagmo M, Groop L. Effect of pioglitazone versus insulin glargine on cardiac size, function, and measures of fluid retention in patients with type 2 diabetes. *Cardiovasc Diabetol* 2009; 8:15.

16. Horio T, Suzuki M, Suzuki K, Takamisawa I, Hiuge A, Kamide K, Takiuchi S, Iwashima Y, Kihara S, Funahashi T, Yoshimasa Y, Kawano Y. Pioglitazone improves left ventricular diastolic function in patients with essential hypertension. *Am J Hypertens* 2005; 18:949-957.

17. Delea TE, Edelsberg JS, Hagiwara M, Oster G, Phillips LS. Use of thiazolidinediones and risk of heart failure in people with type 2 diabetes: a retrospective cohort study. *Diabetes Care* 2003; 26:2983-2989.

18. Singh S, Loke YK, Furberg CD. Thiazolidinediones and heart failure: a teleo-analysis. *Diabetes Care* 2007; 30:2148-2153.

19. Kahn SE, Haffner SM, Heise MA, Herman WH, Holman RR, Jones NP, Kravitz BG, Lachin JM, O'Neill MC, Zinman B, Viberti G, Group AS. Glycemic durability of rosiglitazone, metformin, or glyburide monotherapy. *N Eng J Med* 2006; 355:2427-2443.

20. Home PD, Pocock SJ, Beck-Nielsen H, Curtis PS, Gomis R, Hanefeld M, Jones NP, Komajda M, McMurray JJ, Team RS. Rosiglitazone evaluated for cardiovascular outcomes in oral agent combination therapy for type 2 diabetes (RECORD): a multicentre, randomised, open-label trial. *Lancet* 2009; 373:2125-2135.

21. Investigators DT, Gerstein HC, Yusuf S, Bosch J, Pogue J, Sheridan P, Dinccag N, Hanefeld M, Hoogwerf B, Laakso M, Mohan V, Shaw J, Zinman B, Holman RR. Effect of rosiglitazone on the frequency of diabetes in patients with impaired glucose tolerance or impaired fasting glucose: a randomised controlled trial. *Lancet* 2006; 368:1096-1105.

22. Dormandy JA, Charbonnel B, Eckland DJ, Erdmann E, Massi-Benedetti M, Moules IK, Skene AM, Tan MH, Lefebvre PJ, Murray GD, Standl E, Wilcox RG, Wilhelmsen L, Betteridge J, Birkeland K, Golay A, Heine RJ, Koranyi L, Laakso M, Mokan M, Norkus A, Pirags V, Podar T, Scheen A, Scherbaum W, Schernthaner G, Schmitz O, Skrha J, Smith U, Taton J, investigators PR. Secondary prevention of macrovascular events in patients with type 2 diabetes in the PROactive Study (PROspective pioglitAzone Clinical Trial In macroVascular Events): a randomised controlled trial. *Lancet* 2005; 366:1279-1289.

23. Masoudi FA, Inzucchi SE, Wang Y, Havranek EP, Foody JM, Krumholz HM. Thiazolidinediones, metformin, and outcomes in older patients with diabetes and heart failure: an observational study. *Circulation* 2005; 111:583-590.

24. Thomas ML, Lloyd SJ. Pulmonary oedema associated with rosiglitazone and troglitazone. *Ann Pharmacother* 2001; 35:123-124.

25. McMorran M, Vu D. Rosiglitazone (Avandia): hepatic, cardiac and hematological reactions. *CMAJ* 2001; 165:82-83, 86-87.

26. Page RLP, Gozansky WS, Ruscin JM. Possible heart failure exacerbation associated with rosiglitazone: Case report and literature review. *Pharmacotherapy* 2003; 23:945-954.

27. Wang F, Aleksunes LM, Reagan LA, Vergara CM. Management of rosiglitazone-induced oedema: two case reports and a review of the literature. *Diabetes Technol Ther* 2002; 4:505-514.

28. Tang WH, Francis GS, Hoogwerf BJ, Young JB. Fluid retention after initiation of thiazolidinedione therapy in diabetic patients with established chronic heart failure. *J Am Coll Cardiol* 2003; 41:1394-1398.

29. Bell DSH. Unilateral oedema due to a thiazolidinedione. *Diabetes care* 2003; 26:2700-2700.

30. Kermani A, Garg A. Thiazolidinedione-associated congestive heart failure and pulmonary oedema. *Mayo Clin Proc* 2003; 78:1088-1091.

31. Jamieson A, Abousleiman Y. Thiazolidinedione-associated congestive heart failure and pulmonary oedema. *Mayo Clin Proc* 2004; 79:571-571.

32. Srivastava PM, Calafiore P, MacIsaac RJ, Hare DL, Jerums G, Burrell LM. Thiazolidinediones and congestive heart failure - exacerbation or new onset of left ventricular dysfunction? *Diabetic Med* 2004; 21:945-950.

33. Singh N. Rosiglitazone and heart failure: Long-term vigilance. *J Cardiovasc Pharmacol Ther* 2004; 9:21-25.

34. Cheng JWM, Frank L, Garrett SD, Lu Y, Sanoski CA, White CM. Key articles and guidelines in pharmacotherapeutic management of arrhythmias. *Pharmacotherapy* 2004; 24:248-279.

8

8

35. Marceille JR, Goins JA, Soni R, Biery JC, Lee TA. Chronic heart failure-related interventions after starting rosiglitazone in patients receiving insulin. *Pharmacotherapy* 2004; 24:1317-1322.

36. Cheng AY, Fantus IG. Thiazolidinedione-induced congestive heart failure. Ann *Pharmacotherapy* 2004; 38:817-820.

37. Shah M, Kolandaivelu A, Fearon WE. Pioglitazone-induced heart failure despite normal left ventricular function. *Am J Med* 2004; 117:973-974.

38. Tang WH, Maroo A. PPARgamma agonists: safety issues in heart failure. *Diabetes Obes Metab* 2007; 9:447-454.

39. Maru S, Koch GG, Stender M, Clark D, Gibowski L, Petri H, White AD, Simpon RJ. Antidiabetic drugs and heart failure risk in patients with type 2 diabetes in the U.K primary care setting. *Diabetes Care* 2005; 28:20-26.

40. Scirica BM, Bhatt DL, Braunwald E, Steg PG, Davidson J, Hirshberg B, Ohman P, Frederich R, Wiviott SD, Hoffman EB, Cavender MA, Udell JA, Desai NR, Mozenson O, McGuire DK, Ray KK, Leiter LA, Raz I, SAVOR-TIMI 53 Steering Committee and Investigators. Saxagliptin and Cardiovascular Outcomes in Patients with Type 2 Diabetes Mellitus. *N Eng J Med* 2013; 369: 1317-1326.

41. Iribarren C, Karter AJ, Go AS, Ferrara A, Liu JY, Sidney S, Selby JV. Glycemic control and heart failure among adult patients with diabetes. *Circulation* 2001; 103:2668-2673.

42. Basu A, Jensen MD, McCann F, Mukhopadhyay D, Joyner MJ, Rizza RA. Effects of pioglitazone versus glipizide on body fat distribution, body water content, and hemodynamics in type 2 diabetes. *Diabetes Care* 2006; 29:510-514.

43. Gans RO, Bilo HJ, Nauta JJ, Heine RJ, Donker AJ. Acute hyperinsulinemia induces sodium retention and a blood pressure decline in diabetes mellitus. *Hypertension* 1992; 20:199-209.

44. Stenvinkel P, Bolinder J, Alvestrand A. Effects of insulin on renal haemodynamics and the proximal and distal tubular sodium handling in healthy subjects. *Diabetologia* 1992; 35:1042-1048.

45. Herlitz H, Widgren B, Urbanavicius V, Attvall S, Persson B. Stimulatory effect of insulin on tubular sodium reabsorption in normotensive subjects with a positive family history of hypertension. *Nephrol Dial Transplant* 1996; 11:47-54.

46. Trevisan R, Fioretto P, Semplicini A, Opocher G, Mantero F, Rocco S, Remuzzi G, Morocutti A, Zanette G, Donadon V, Perico N, Giorato C, Nosadini R. Role of Insulin and Atrial-Natriuretic-Peptide in Sodium Retention in Insulin-Treated Iddm Patients during Isotonic Volume Expansion. *Diabetes* 1990; 39:289-298.

47. Nakamura R, Emmanouel DS, Katz AI. Insulin Binding-Sites in Various Segments of the Rabbit Nephron. *J Clin Invest* 1983; 72:388-392.

48. Yang BC, Clifton LG, McNulty JA, Chen LH, Brown KK, Baer PG. Effects of a PPAR gamma agonist, GI262570, on renal filtration fraction and nitric oxide level in conscious rats. *J Cardiovasc Pharmacol* 2003: 42:436-441.

49. Zanchi A, Chiolero A, Maillard M, Nussberger J, Brunner HR, Burnier M. Effects of the peroxisomal proliferator-activated receptor-gamma agonist pioglitazone on renal and hormonal responses to salt in healthy men. *J Clin Endocr Metab* 2004; 89:1140-1145.

50. Guan Y, Zhang Y, Davis L, Breyer MD. Expression of peroxisome proliferator-activated receptors in urinary tract of rabbits and humans. *Am J Physiol* 1997; 273:F1013-1022.

51. Song J, Knepper MA, Hu X, Verbalis JG, Ecelbarger CA. Rosiglitazone activates renal sodium- and water-reabsorptive pathways and lowers blood pressure in normal rats. *J Pharmacol Exp Ther* 2004; 308:426-433.

52. Endo Y, Suzuki M, Yamada H, Horita S, Kunimi M, Yamazaki O, Shirai A, Nakamura M, Iso ON, Li Y, Hara M, Tsukamoto K, Moriyama N, Kudo A, Kawakami H, Yamauchi T, Kubota N, Kadowaki T, Kume H, Enomoto Y, Homma Y, Seki G, Fujita T. Thiazolidinediones enhance sodium-coupled bicarbonate absorption from renal proximal tubules via PPARgamma-dependent nongenomic signaling. *Cell Metab* 2011; 13:550-561.

53. Zhang H, Zhang AH, Kohan DE, Nelson RD, Gonzalez FJ, Yang TX. Collecting duct-specific deletion of peroxisome proliferator-activated receptor gamma blocks thiazolidinedione-induced fluid retention. *Proc Natl Acad Sci USA* 2005; 102:9406-9411.

54. Guan Y, Hao C, Cha DR, Rao R, Lu W, Kohan DE, Magnuson MA, Redha R, Zhang Y, Breyer MD. Thiazolidinediones expand body fluid volume through PPARgamma stimulation of ENaC-mediated renal salt absorption. *Nat Med* 2005; 11:861-866.

55. Chen L, Yang B, McNulty JA, Clifton LG, Binz JG, Grimes AM, Strum JC, Harrington WW, Chen Z, Balon TW, Stimpson SA, Brown KK. GI262570, a peroxisome proliferator-activated receptor {gamma} agonist, changes electrolytes and water reabsorption from the distal nephron in rats. *J Pharmacol Exp Ther* 2005; 312:718-725.

56. Vallon V, Hummler E, Rieg T, Pochynyuk O, Bugaj V, Schroth J, Dechenes G, Rossier B, Cunard R, Stockand J. Thiazolidinedione-induced fluid retention is independent of collecting duct alphaENaC activity. *J Am Soc Nephrol* 2009; 20:721-729.

57. Nofziger C, Chen L, Shane MA, Smith CD, Brown KK, Blazer-Yost BL. PPARgamma agonists do not directly enhance basal or insulin-stimulated Na(+) transport via the epithelial Na(+) channel. *Pflugers Archiv* 2005; 451:445-453.

58. Qayyum R, Adomaityte J. A meta-analysis of the effect of thiazolidinediones on blood

pressure. *J Clin Hypertens (Greenwich)* 2006; 8:19-28.

59. Shargorodsky M, Wainstein J, Gavish D, Leibovitz E, Matas Z, Zimlichman R. Treatment with rosiglitazone reduces hyperinsulinemia and improves arterial elasticity in patients with type 2 diabetes mellitus. *Am J Hypertens* 2003; 16:617-622.

60. Raskin P, Rendell M, Riddle MC, Dole JF, Freed MI, Rosenstock J, Rosiglitazone Clinical Trials Study G. A randomized trial of rosiglitazone therapy in patients with inadequately controlled insulin-treated type 2 diabetes. *Diabetes Care* 2001; 24:1226-1232.

61. Satoh H, Tsukamoto K, Hashimoto Y, Hashimoto N, Togo M, Hara M, Maekawa H, Isoo N, Kimura S, Watanabe T. Thiazolidinediones suppress endothelin-1 secretion from bovine vascular endothelial cells: a new possible role of PPARgamma on vascular endothelial function. *Biochem Biophys Res Commun* 1999; 254:757-763.

62. Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature* 1998; 391:79-82.

63. Creager MA, Liang CS, Coffman JD. Beta adrenergic-mediated vasodilator response to insulin in the human forearm. *J Pharmacol Exp Ther* 1985; 235:709-714.

64. Laakso M, Edelman SV, Brechtel G, Baron AD. Decreased effect of insulin to stimulate skeletal muscle blood flow in obese man. A novel mechanism for insulin resistance. *J Clin Invest 1990;* 85:1844-1852.

65. Anderson EA, Hoffman RP, Balon TW, Sinkey CA, Mark AL. Hyperinsulinemia produces both sympathetic neural activation and vasodilation in normal humans. *J Clin Invest* 1991; 87:2246-2252.

66. Berne C, Fagius J, Pollare T, Hjemdahl P. The sympathetic response to euglycaemic hyperinsulinaemia. Evidence from microelectrode nerve recordings in healthy subjects. *Diabetologia* 1992; 35:873-879.

67. Scherrer U, Randin D, Vollenweider P, Vollenweider L, Nicod P. Nitric oxide release accounts for insulin's vascular effects in humans. *J Clin Invest* 94:2511-2515, 1994.

68. Steinberg HO, Brechtel G, Johnson A, Fineberg N, Baron AD. Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release. *J Clin Invest* 1994; 94:1172-1179.

69. Iida S, Taguchi H, Watanabe N, Kushiro T, Kanmatsuse K. Insulin-induced relaxation of rat mesenteric artery is mediated by Ca2+-activated K+ channels. *Eur J Pharmacol 2001;* 411:155-160.

70. Ferrannini E, Taddei S, Santoro D, Natali A, Boni C, Del Chiaro D, Buzzigoli G. Independent stimulation of glucose metabolism and Na+-K+ exchange by insulin in the human forearm. *Am J Physiol* 1988; 255:E953-958.

71. Hopkins DF, Cotton SJ, Williams G. Effective treatment of insulin-induced oedema using ephedrine. *Diabetes Care* 1993; 16:1026-1028.

72. Tack CJJ, Ong MKE, Lutterman JA, Smits P. Insulin-induced vasodilatation and endothelial function in obesity/insulin resistance. Effects of troglitazone. *Diabetologia* 1998; 41:569-576.

73. Coggins M, Lindner J, Rattigan S, Jahn L, Fasy E, Kaul S, Barrett E. Physiologic hyperinsulinemia enhances human skeletal muscle perfusion by capillary recruitment. *Diabetes* 2001; 50:2682-2690.

74. Zhang L, Vincent MA, Richards SM, Clerk LH, Rattigan S, Clark MG, Barrett EJ. Insulin sensitivity of muscle capillary recruitment in vivo. *Diabetes* 2004; 53:447-453.

75. Rattigan S, Clark MG, Barrett EJ. Haemodynamic actions of insulin in rat skeletal muscle: evidence for capillary recruitment. *Diabetes* 1997; 46:1381-1388.

76. Bonner JS, Lantier L, Hasenour CM, James FD, Bracy DP, Wasserman DH. Muscle-specific vascular endothelial growth factor deletion induces muscle capillary rarefaction creating muscle insulin resistance. *Diabetes* 2013; 62:572-580.

77. Kubota T, Kubota N, Kumagai H, Yamaguchi S, Kozono H, Takahashi T, Inoue M, Itoh S, Takamoto I, Sasako T, Kumagai K, Kawai T, Hashimoto S, Kobayashi T, Sato M, Tokuyama K, Nishimura S, Tsunoda M, Ide T, Murakami K, Yamazaki T, Ezaki O, Kawamura K, Masuda H, Moroi M, Sugi K, Oike Y, Shimokawa H, Yanagihara N, Tsutsui M, Terauchi Y, Tobe K, Nagai R, Kamata K, Inoue K, Kodama T, Ueki K, Kadowaki T. Impaired insulin signaling in endothelial cells reduces insulin-induced glucose uptake by skeletal muscle. *Cell Metab* 2011; 13:294-307.

78. Rattigan S, Richards SM, Keske MA. Microvascular contributions to insulin resistance. *Diabetes* 2013; 62:343-345.

79. Muris DM, Houben AJ, Schram MT, Stehouwer CD. Microvascular dysfunction: an emerging pathway in the pathogenesis of obesity-related insulin resistance. *Rev Endocr Metab Disord* 2013; 14:29-38.

80. Gundersen HJ, Christensen NJ. Intravenous insulin causing loss of intravascular water and albumin and increased adrenergic nervous activity in diabetics. *Diabetes* 1977; 26:551-557.

81. Osterby R, Gundersen HJ, Christensen NJ. The acute effect of insulin on capillary endothelial cells. *Diabetes* 1978; 27:745-749.

82. Poulaki V, Qin W, Joussen AM, Hurlbut P, Wiegand SJ, Rudge J, Yancopoulos GD, Adamis AP. Acute intensive insulin therapy exacerbates diabetic blood-retinal barrier breakdown via hypoxia-inducible factor-1alpha and VEGF. *J Clin Invest* 2002; 109:805-815.

83. Doronzo G, Russo I, Mattiello L, Anfossi G, Bosia A, Trovati M. Insulin activates vascular endothelial growth factor in vascular smooth muscle cells: influence of nitric oxide and of insulin resistance. *Eur J Clin Invest* 2004; 34:664-673.

84. Evans DJ, Pritchard-Jones K, Trotman-Dickenson B. Insulin oedema. *Postgrad Med J* 1986; 62:665-668.

85. Calnek DS, Mazzella L, Roser S, Roman J, Hart CM. Peroxisome proliferator-activated receptor gamma ligands increase release of nitric oxide from endothelial cells. *Arterioscler Thromb Vasc Biol* 2003; 23:52-57.

86. St-Pierre P, Bouffard L, Maheux P. Rosiglitazone increases extravasation of macromolecules and endothelial nitric oxide synthase in skeletal muscles of the fructose-fed rat model. *Biochem Pharmacol* 2004; 67:1997-2004.

87. Pistrosch F, Passauer J, Fischer S, Fuecker K, Hanefeld M, Gross P. In type 2 diabetes, rosiglitazone therapy for insulin resistance ameliorates endothelial dysfunction independent of glycaemic control . *Diabetes Care* 2004; 27:484-490.

88. Vinik AI, Stansberry KB, Barlow PM. Rosiglitazone treatment increases nitric oxide production in human peripheral skin: a controlled clinical trial in patients with type 2 diabetes mellitus. *J Diabetes Complications* 2003; 17:279-285.

89. Yosefy C, Magen E, Kiselevich A, Priluk R, London D, Volchek L, Viskoper RJ, Jr. Rosiglitazone improves, while Glibenclamide worsens blood pressure control in treated hypertensive diabetic and dyslipidemic subjects via modulation of insulin resistance and sympathetic activity. *J Cardiovasc Pharmacol 2004;* 44:215-222.

90. Sidhu JS, Cowan D, Kaski JC. The effects of rosiglitazone, a peroxisome proliferator-activated receptor-gamma agonist, on markers of endothelial cell activation, C-reactive protein, and fibrinogen levels in non-diabetic coronary artery disease patients. *J Am Coll Cardiol* 2003; 42:1757-1763.

91. Marx N, Froehlich J, Siam L, Ittner J, Wierse G, Schmidt A, Scharnagl H, Hombach V, Koenig W. Antidiabetic PPAR gamma-activator rosiglitazone reduces MMP-9 serum levels in type 2 diabetic patients with coronary artery disease. *Arterioscler Thromb Vasc Biol* 2003*;* 23:283-288.

92. Idris I, Gray S, Donnelly R. Rosiglitazone and pulmonary oedema: an acute dose-dependent effect on human endothelial cell permeability. *Diabetologia* 2003; 46:288-290.

93. Emoto M, Anno T, Sato Y, Tanabe K, Okuya S, Tanizawa Y, Matsutani A, Oka Y. Troglitazone treatment increases plasma vascular endothelial growth factor in diabetic patients and its mRNA in 3T3-L1 adipocytes. *Diabetes* 2001; 50:1166-1170.

94. Yamakawa K, Hosoi M, Koyama H, Tanaka S, Fukumoto S, Morii H, Nishizawa Y. Peroxisome proliferator-activated receptor-gamma agonists increase vascular endothelial growth factor expression in human vascular smooth muscle cells. *Biochem Biophys Res Commun* 2000; 271:571-574.

95. Peeters LL, Vigne JL, Tee MK, Zhao D, Waite LL, Taylor RN. PPAR gamma represses VEGF expression in human endometrial cells: implications for uterine angiogenesis. *Angiogenesis* 2005; 8:373-379.

96. Sander TL, Noll L, Klinkner DB, Weihrauch D, He BJ, Kaul S, Zangwill SD, Tweddell JS, Pritchard KA, Jr., Oldham KT. Rosiglitazone antagonizes vascular endothelial growth factor signaling and nuclear factor of activated T cells activation in cardiac valve endothelium. *Endothelium* 2006; 13:181-190.

97. Sotiropoulos KB, Clermont A, Yasuda Y, Rask-Madsen C, Mastumoto M, Takahashi J, Della Vecchia K, Kondo T, Aiello LP, King GL. Adipose-specific effect of rosiglitazone on vascular permeability and protein kinase C activation: novel mechanism for PPARgamma agonist's effects on oedema and weight gain. *FASEB J* 2006; 20:1203-1205.

98. Cosson E, Cohen-Boulakia F, Tarhzaoui K, Dabire H, Leger G, Charnaux N, Lestrade R, Behar A, Valensi P. Capillary endothelial but not lymphatic function is restored under rosiglitazone in Zucker diabetic fatty rats. *Microvasc Res* 2009; 77:220-225.

99. Deeb SS, Fajas L, Nemoto M, Pihlajamaki J, Mykkanen L, Kuusisto J, Laakso M, Fujimoto W, Auwerx J. A Pro12Ala substitution in PPARgamma2 associated with decreased receptor activity, lower body mass index and improved insulin sensitivity. *Nat Genet* 1998; 20:284-287.

100. Hansen L, Ekstrom CT, Tabanera YPR, Anant M, Wassermann K, Reinhardt RR. The Pro12Ala variant of the PPARG gene is a risk factor for peroxisome proliferator-activated receptor-gamma/alpha agonist-induced oedema in type 2 diabetic patients. *J Clin Endocrinol Metab* 2006; 91:3446-3450.

101. Boden G, Homko C, Mozzoli M, Zhang M, Kresge K, Cheung P. Combined use of rosiglitazone and fenofibrate in patients with type 2 diabetes: prevention of fluid retention. *Diabetes* 2007; 56:248-255.

102. Saad MF, Greco S, Osei K, Lewin AJ, Edwards C, Nunez M, Reinhardt RR, Ragaglitazar Dose-Ranging Study G. Ragaglitazar improves glycemic control and lipid profile in type 2 diabetic subjects: a 12-week, double-blind, placebo-controlled dose-ranging study with an open pioglitazone arm. *Diabetes Care* 2004; 27:1324-1329.

103. Herz M, Gaspari F, Perico N, Viberti G, Urbanowska T, Rabbia M, Wieczorek Kirk D. Effects of high dose aleglitazar on renal function in patients with type 2 diabetes. *Int J Cardiol* 2011; 151:136-142.

8

8

104. Nissen SE, Wolski K. Effect of rosiglitazone on the risk of myocardial infarction and death from cardiovascular causes. *N Eng J Med* 2007; 356:2457-2471.

105. Lewis JD, Ferrara A, Peng T, Hedderson M, Bilker WB, Quesenberry CP, Jr., Vaughn DJ, Nessel L, Selby J, Strom BL. Risk of bladder cancer among diabetic patients treated with pioglitazone: interim report of a longitudinal cohort study. *Diabetes Care* 2011; 34:916-922.

106. Nesto RW, Bell D, Bonow RO, Fonseca V, Grundy SM, Horton ES, Le Winter M, Porte D, Semenkovich CF, Smith S, Young LH, Kahn R, American Heart A, American Diabetes A 2003 Thiazolidinedione use, fluid retention, and congestive heart failure: a consensus statement from the American Heart Association and American Diabetes Association. October 7, 2003. *Circulation* 2003; 108:2941-2948.

107. Lincoff AM, Wolski K, Nicholls SJ, Nissen SE. Pioglitazone and risk of cardiovascular events in patients with type 2 diabetes mellitus: a meta-analysis of randomized trials. *JAMA* 2007; 298:1180-1188.

108. Winterstein AG. Rosiglitazone and the risk of adverse cardiovascular outcomes. *Clin Pharmacol Ther* 2011; 89:776-778.

109. Bourg CA, Phillips BB. Rosiglitazone, myocardial ischemic risk, and recent regulatory actions. *Ann Pharmacother* 2012; 46:282-289.

110. Singh S, Loke YK, Furberg CD. Long-term risk of cardiovascular events with rosiglitazone: a meta-analysis. *JAMA* 2007; 298:1189-1195.

111. Diamond GA, Bax L, Kaul S. Uncertain effects of rosiglitazone on the risk for myocardial infarction and cardiovascular death. *Ann Intern Med* 2007; 147:578-581.

112. Shuster JJ, Jones LS, Salmon DA. Fixed vs random effects meta-analysis in rare event studies: the rosiglitazone link with myocardial infarction and cardiac death. *Stat Med* 2007; 26:4375-4385.

113. Richter B, Bandeira-Echtler E, Bergerhoff K, Clar C, Ebrahim SH. Rosiglitazone for type 2 diabetes mellitus. *Cochrane Database Syst Rev* 2007.

114. Nissen SE, Wolski K. Rosiglitazone revisited: an updated meta-analysis of risk for myocardial infarction and cardiovascular mortality. *Arch Intern Med* 2010; 170:1191-1201.

115. FDA 2010 Briefing document Advisory committee meeting http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/EndocrinologicandMetabolicDrugsAdvisoryCommittee/UCM218493.pdf.

116. Kaul S, Bolger AF, Herrington D, Giugliano RP, Eckel RH, American Heart A, American College Of Cardiology F. Thiazolidinedione drugs and cardiovascular risks: a science advisory from the American Heart Association and American College Of Cardiology Foundation. *J Am Coll Cardiol* 2010; 55:1885-1894.

117. Graham DJ, Ouellet-Hellstrom R, MaCurdy TE, Ali F, Sholley C, Worrall C, Kelman JA. Risk of acute myocardial infarction, stroke, heart failure, and death in elderly Medicare patients treated with rosiglitazone or pioglitazone. *JAMA* 2010; 304:411-418.

118. Kaul S, Diamond GA. Is there clear and convincing evidence of cardiovascular risk with rosiglitazone? *Clin Pharmacol Ther* 2011; 89:773-776.

119. Action to Control Cardiovascular Risk in Diabetes Study G, Gerstein HC, Miller ME, Byington RP, Goff DC, Jr., Bigger JT, Buse JB, Cushman WC, Genuth S, Ismail-Beigi F, Grimm RH, Jr., Probstfield JL, Simons-Morton DG, Friedewald WT. Effects of intensive glucose lowering in type 2 diabetes. *N Eng J Med* 2008; 358:2545-2559.

120. Duckworth W, Abraira C, Moritz T, Reda D, Emanuele N, Reaven PD, Zieve FJ, Marks J, Davis SN, Hayward R, Warren SR, Goldman S, McCarren M, Vitek ME, Henderson WG, Huang GD, VADT Investigators. Glycaemic control and vascular complications in veterans with type 2 diabetes. *N Eng J Med* 2009; 360:129-139.

121. Group BDS, Frye RL, August P, Brooks MM, Hardison RM, Kelsey SF, MacGregor JM, Orchard TJ, Chaitman BR, Genuth SM, Goldberg SH, Hlatky MA, Jones TL, Molitch ME, Nesto RW, Sako EY, Sobel BE. A randomized trial of therapies for type 2 diabetes and coronary artery disease. *N Eng J Med* 290; 360:2503-2515.

122. Mahaffey KW, Hafley G, Dickerson S, Burns S, Tourt-Uhlig S, White J, Newby LK, Komajda M, McMurray J, Bigelow R, Home PD, Lopes RD. Results of a reevaluation of cardiovascular outcomes in the RECORD trial. *Am Heart J* 2013; 166:240-249 e241.

123. Hiatt WR, Kaul S, Smith RJ. The Cardiovascular Safety of Diabetes Drugs - Insights from the Rosiglitazone Experience. *N Eng J Med* 2013; 369(14):1285-7.

124. Loke YK, Singh S, Furberg CD. Long-term use of thiazolidinediones and fractures in type 2 diabetes: a meta-analysis. *CMAJ* 2009; 180:32-39.

125. Inzucchi SE, Bergenstal RM, Buse JB, Diamant M, Ferrannini E, Nauck M, Peters AL, Tsapas A, Wender R, Matthews DR. Management of Hyperglycemia in Type 2 Diabetes: A Patient-Centered Approach. *Diabetes Care* 2012; 35:1364-1379.

126. IDF Global guideline for type 2 diabetes. http://www.idf.org/sites/default/files/IDF-Guideline-for-Type-2-Diabetes.pdf, 2012

127. evaluate http://www.evaluategroup.com/Universal/View.aspx?type=Entity&entityType=Product&lType=modData&id=9391&componentID=1003#.

128. Zinman B, Harris SB, Neuman J, Gerstein HC, Retnakaran RR, Raboud J, Qi Y, Hanley AJ. Low-dose combination therapy with rosiglitazone and metformin to prevent type 2 diabetes mellitus (CANOE trial): a double-blind randomised controlled

8

8

study. *Lancet* 2010; 376:103-111.

129. Musso G, Cassader M, Rosina F, Gambino R. Impact of current treatments on liver disease, glucose metabolism and cardiovascular risk in non-alcoholic fatty liver disease (NAFLD): a systematic review and meta-analysis of randomised trials. *Diabetologia* 2012; 55:885-904.

130. Li XJ, Yu YX, Liu CQ, Zhang W, Zhang HJ, Yan B, Wang LY, Yang SY, Zhang SH. Metformin vs thiazolidinediones for treatment of clinical, hormonal and metabolic characteristics of polycystic ovary syndrome: a meta-analysis. *Clin Endocrinol* 2011; 74:332-339.

131. Raboud JM, Diong C, Carr A, Grinspoon S, Mulligan K, Sutinen J, Rozenbaum W, Cavalcanti RB, Wand H, Costagliola D, Walmsley S, Wo GLMA. A Meta-Analysis of Six Placebo-Controlled Trials of Thiazolidinedione Therapy for HIV Lipoatrophy. *Hiv Clin Trials* 2010; 11:39-50.

132. Chou HS, Truitt KE, Moberly JB, Merante D, Choi Y, Mun Y, Pfutzner A. A 26-week, placebo- and pioglitazone-controlled monotherapy study of rivoglitazone in subjects with type 2 diabetes mellitus. *Diabetes Obes Metab* 2012; 14:1000-1009.

133. Henry RR, Lincoff AM, Mudaliar S, Rabbia M, Chognot C, Herz M. Effect of the dual peroxisome proliferator-activated receptor-alpha/gamma agonist aleglitazar on risk of cardiovascular disease in patients with type 2 diabetes (SYNCHRONY): a phase II, randomised, dose-ranging study. *Lancet* 2009; 374:126-135.

134. Long GG, Reynolds VL, Lopez-Martinez A, Ryan TE, White SL, Eldridge SR. Urothelial carcinogenesis in the urinary bladder of rats treated with naveglitazar, a gamma-dominant PPAR alpha/gamma agonist: lack of evidence for urolithiasis as an inciting event. *Toxicol Pathol* 2008; 36:218-231.

135. Nissen SE, Wolski K, Topol EJ. Effect of muraglitazar on death and major adverse cardiovascular events in patients with type 2 diabetes mellitus. *JAMA* 2005; 294:2581-2586.

136. Wilding JP, Gause-Nilsson I, Persson A, Group GS. Tesaglitazar, as add-on therapy to sulphonylurea, dose-dependently improves glucose and lipid abnormalities in patients with type 2 diabetes. *Diab Vasc Dis Res* 2007; 4:194-203.

137. Pollack http://www.nytimes.com/2013/07/11/business/roche-abandons-new-diabetes-drug.html?_r=1&. *New York times* 2013

138. Henriksen K, Byrjalsen I, Qvist P, Beck-Nielsen H, Hansen G, Riis BJ, Perrild H, Svendsen OL, Gram J, Karsdal MA, Christiansen C, Investigators BT. Efficacy and safety of the PPAR gamma partial agonist balaglitazone compared with pioglitazone and placebo: a phase III, randomized, parallel-group study in patients with type 2 diabetes on stable insulin therapy. *Diabetes Metab Res Rev* 2011; 27:392-401.

139. Depaoli AM, Dunn F, Fredrickson J, Higgins L. INT131: A Selective PPAR gamma Modulator (SPPARM) Significantly Lowers Glucose without Typical Thiazolidinedione (TZD) Side Effects in Patients with Type 2 Diabetes. *Diabetes* 2009; 58:A31-A32.

140. Nauck MA, Vilsboll T, Gallwitz B, Garber A, Madsbad S. Incretin-based therapies: viewpoints on the way to consensus. *Diabetes Care* 2009; 32 Suppl 2:S223-231.

141. Kurosaki E, Ogasawara H. Ipragliflozin and other sodium-glucose cotransporter-2 (SGLT2) inhibitors in the treatment of type 2 diabetes: preclinical and clinical data. *Pharmacol Ther* 2013; 139:51-59.

142. Monami M, Dicembrini I, Mannucci E. Dipeptidyl peptidase-4 inhibitors and heart failure: A meta-analysis of randomized clinical trials. *Nutr Metab Cardiovasc Dis* 2014; 24: 689-697.

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Samenvatting voor geïnteresseerden

Inleiding

De meeste geïnteresseerde vrienden, familieleden, collegae en kennissen zullen de teksten uit dit proefschrift niet gemakkelijk kunnen verteren. Het is namelijk in jargon geschreven, borduurt voort op een specialistisch denkkader en is nu eenmaal geen 'pageturner'. Ook na omzetting naar Jip en Janneke taal zal het geen bestseller worden. Toch zullen we de komende bladzijden een poging doen om de kennis uit dit proefschrift toegankelijker te verwoorden en in perspectief te plaatsen. U wordt uitgenodigd ons te volgen.

Diabetes mellitus type 2 (ouderdomssuikerziekte) is een grote bedreiging voor de volksgezondheid. Iemand heeft suikerziekte als de suiker(glucose)concentratie in het bloed te hoog is. Normaal gesproken zorgt het hormoon insuline voor een daling van de bloedsuikerspiegel. Suikerziekte ontstaat als de productie van insuline te kort schiet of als het lichaam onvoldoende reageert op dit hormoon (resistentie). Ondanks verscheidene beschikbare medicamenteuze behandelingen is de ziekte progressief en leidt het tot invaliditeit, zoals door zenuwschade, en vroegtijdige dood, vaak door hart- en vaatziekte. Een fraaie omschrijving omtrent de aard van suikerziekte komt uit een Amerikaans rapport van het National Institute of Health: "Diabetes doodt niet snel of doelgericht, maar door een trage opeenstapeling van verborgen aanvallen die het lichaam schaadt. Daardoor wordt iemand beroofd van de mogelijkheid te kunnen zien, denken, lopen en vrijen". Het zal duidelijk zijn dat er daarom gezocht werd en wordt naar een behandeling die niet alleen de bloedsuikerspiegel doet dalen, maar ook de schade op hart, bloedvaten en zenuwen vermindert of voorkomt. Een nieuwe klasse van geneesmiddelen diende zich twee decennia geleden aan. Het betrof de klasse van de PPAR-γ agonisten en dan met name een subgroep hiervan, de thiazolidinedionederivaten (TZD's). *Rosiglitazon* en *pioglitazon* zijn vertegenwoordigers van deze subgroep. Het werkingsmechanisme wordt gekenmerkt door versterking van het effect van insuline, waardoor de bloedsuikerwaarde daalt. Daarnaast bestond het idee dat deze middelen ook bijkomende voordelen konden hebben, zoals het voorkomen of verminderen van schade aan zenuw, hart en bloedvaten. Daarentegen kleefden er direct ook nadelen aan de TZD's. Mensen die een TZD gebruikten hielden namelijk vocht vast, met als gevolg dikke benen, maar soms zelfs ook kortademigheid door vochtophoping in de longen. Kortom, indien de oorzaak van de vochtophoping achterhaald en behandeld zou kunnen worden, dan zou een ideaal middel ontstaan voor de behandeling van ouderdomssuikerziekte. Hier startte onze zoektocht. Het eerste deel van dit proefschrift richt zich op de oorzaak, risicofactoren en mogelijke behandeling van vochtophoping tijdens gebruik van *rosiglitazon*. Hoofdstuk zes en zeven richten zich op de mogelijke positieve effecten van *rosiglitazon* op zenuwenschade en op schade veroorzaakt door zuurstofgebrek, zoals bijvoorbeeld bij een infarct.

Oorzaken vochtophoping en mogelijk aangrijpingspunt *rosiglitazon*

Grofweg kan de bron voor het vasthouden van vocht liggen hetzij in het hart, de nier of de bloedvaten. Als het hart niet goed werkt, ontstaat er ophoping van bloed (file) in de wegen (aders) naar het hart. De aders kunnen de hoeveelheid bloed niet verwerken, door de toegenomen druk treedt lekkage van vocht op naar de omringende weefsels. Onder invloed van de zwaartekracht is dat meestal het beste te zien in de benen. Anderzijds kan de nier ook de bron zijn van vochtophoping. Als de nier niet goed werkt en zout met water gaat vasthouden, wordt de druk in de bloedvaten ook opgevoerd en gelijk aan de beschrijving bij het hart zal er vocht uit de bloedvaten gaan lekken. Tot slot kan vochtophoping ook ontstaan als de kleine bloedvaatjes, de haarvaten, lek raken. Bij elke passage door de haarvaten moet er vocht, zout en voedingsstoffen afgegeven worden aan de weefsels. Als door het uitzetten van de aanvoerende bloedvaten de druk in het haarvat stijgt of als het haarvatfilter op zich lek is, neemt de stroom naar het weefsel toe. Bij aanvang van het proefschrift was reeds duidelijk dat de hartfunctie niet duidelijk slechter wordt tijdens behandeling met *rosiglitazon*. Kortom, op dat moment waren de belangrijkste verdachten voor het vasthouden van vocht onder invloed van een TZD de nier en de bloedvaten.

Onderzoeksgereedschap (meetmethoden)

Dit proefschrift bevat een aantal onderzoeken waarbij *rosiglitazon* wordt vergeleken met een neppil (placebo). De onderzoeken zijn geblindeerd, zodat zowel de onderzoeker als de deelnemer niet weet wat hij slikt, en voorkomen wordt, dat je waarneemt wat je denkt te moeten waarnemen. Het effect van *rosiglitazon* treedt vrij langzaam in, zodat de testen pas na enkele weken kunnen plaatsvinden. In de tussentijd wordt het lichaam regelmatig doorgemeten, waardoor een uitspraak gedaan kan worden omtrent de hoeveelheid extra vocht in het lichaam en waar het zich bevindt. Hieronder volgt een korte beschrijving van de gebruikte meetmethodieken.

a). *Voetvolume meting*. De voet wordt op een gestandaardiseerde manier ondergedompeld in een bak met water die weer op een weegschaal rust. Op deze manier geeft de weegschaal niet het gewicht van de voet aan, maar het gewicht van de hoeveelheid water dat verplaatst is door de voet (Wet van Archimedes). Een indruk van deze methode kunt u krijgen op de plaatjes van hoofdstuk 1.

b). *Elektrische bio-impedantie meting*. Hierbij wordt door middel van elektrische stroompjes de hoeveelheid water in het lichaam berekend. Onderliggend principe is dat water elektrische stroom beter geleidt dan vet. Een apparaat meet de stroomgeleiding van het lichaam en doet de berekening.

c). *Plasmavolume meting*. De hoeveelheid water in het bloed van een deelnemer wordt met een verdunningsmethode bepaald. Radioactief eiwit wordt ingespoten en zal zich verdelen over het totale bloedvolume. Hierna wordt een bloedmonster afgenomen, waarin de hoeveelheid radioactiviteit wordt bepaald. De gemeten waarde zal lager liggen dan de ingespoten hoeveelheid, door verdunning in het bloed. Op basis van deze verdunning kan de hoeveelheid bloed worden berekend.

De volgende twee testen werden verricht om meer inzicht in de functie van bloedvaten te verkrijgen. d). *Permeabiliteit van de bloedvaten*. De doorlaatbaarheid van haarvaten kan gemeten worden door na inspuiting van het genoemde radioactieve eiwit gedurende een uur bloed af te nemen. De hoeveelheid radioactiviteit in het bloed wordt alsmaar minder door lekkage van het eiwit naar de weefsels toe. De verdwijningssnelheid is dan een maat voor lekkage uit de bloedvaten.

e). *Vaatverwijding*, bepaling door middel van *plethysmografie*. Hierbij wordt een arm kortdurend afgeklemd zodat er wel bloed in kan, maar niet uit. Het gevolg zal zijn dat de arm een beetje uitzet. Dit uitzetten kan nauwkeurig gemeten worden met een elektrische armband die mee uitrekt. Door uitrekking verandert de elektrische weerstand van de armband, en die wordt gemeten. Als deze veranderingen snel gaan, moet er in korte tijd veel bloed in de arm stromen en dat kan alleen als de bloedvaten wijdt open staan.

f). *Meting van de insuline gevoeligheid*. De effecten van insuline kunnen nauwkeurig gemeten worden door een hoge dosis insuline toe te dienen. Het gevolg zal zijn dat de bloedsuikerwaarde daalt (denk aan het slot van de film 'Zwartboek'). Door tegelijkertijd glucose (suiker) toe te dienen in een dusdanige hoeveelheid dat de suikerspiegel gelijk blijft, verkrijgen we een maat voor de werkzaamheid van insuline (insuline gevoeligheid van het lichaam). Als er weinig suiker nodig is om de bloedwaarde gelijk te houden, is het lichaam insulineresistent. Deze test wordt een *clamp* genoemd.

Overzicht van de beschreven onderzoeken

In het **tweede hoofdstuk** nemen we aan dat de oorzaak van vochtophoping tijdens behandeling met *rosiglitazon* gelegen is in bloedvaten. Eerder beschreven we al dat onder invloed van insuline de bloedsuikerwaarde daalt, maar daarnaast heeft insuline ook allerlei effecten op de bloedvaten. We vragen ons dan ook af of *rosiglitazon* niet alleen het effect van insuline op de bloedsuikerwaarde versterkt, maar ook de effecten van insuline op de bloedvaten. Daartoe lieten we mensen, waarbij insuline minder goed werkt (insulineresistentie), een periode *rosiglitazon* en een periode placebo gebruiken. Tijdens een experiment aan het einde van die periodes maten we de effecten van insuline tijdens gestandaardiseerde omstandigheden. Wat bleek, *rosiglitazon* versterkt de werking van insuline met betrekking tot de bloedsuikerwaarde, maar *rosiglitazon* versterkt niet de werking van insuline op de bloedvaten, zoals verwijding van de aanvoerende bloedvaten of lekkage van de haarvaten.

Ook in het **derde hoofdstuk** gaan we er van uit dat het wijder worden van de aanvoerende bloedvaten in ieder geval één van de routes is waardoor *rosiglitazon*

vochtophoping geeft. Normaal gesproken treedt in het lichaam een tegenreactie op van het onwillekeurige zenuwstelsel als je iets aan de vaatomvang verandert. Laat dat nu net een onderdeel zijn, dat wordt aangetast bij suikerziekte. Daarom onderzochten we in dit hoofdstuk of schade aan het onwillekeurige zenuwstelsel een risicofactor is voor het vasthouden van vocht tijdens behandeling met *rosiglitazon*. Gedurende 16 weken gebruikten mensen met suikerziekte, die tevens insuline spoten, een neppil of *rosiglitazon*. Deze personen hadden we verdeeld in mensen met en zonder zenuwschade. En inderdaad bleken mensen met zenuwschade meer lekkage van de bloedvaten te hebben tijdens behandeling met *rosiglitazon* dan mensen zonder zenuwschade. Door bloedvatverwijding en vochtlekkage zal de bloeddruk dalen, wat een signaal is voor de nier om vocht vast te gaan houden, waardoor het bloedvolume kan toenemen. Echter in ons onderzoek leidde de toename in vochtlekkage bij mensen met zenuwschade die behandeld werden met *rosiglitazon* niet tot een toename van het bloedvolume. We concluderen dat zenuwschade een risicofactor is voor *rosiglitazon*-gerelateerde vochtlekkage, maar niet voor extra vochtstapeling door de nier.

In **hoofdstuk vier** nemen we de nier onder de loep als mogelijk aangrijpingspunt voor de vochtstapeling tijdens behandeling met TZD's. We vragen ons af of behandeling met een TZD leidt tot verminderde werking van plaspillen zoals eerder gesuggereerd werd in de literatuur. Dit is van belang voor de behandeling van vochtstapeling. Eigenlijk net als in hoofdstuk twee kregen vrijwilligers een periode placebo en een periode *rosiglitazon*. Aan het eind van iedere periode werd het zout afdrijvende vermogen van de plaspil *furosemide* bepaald. Deze plaspil zou in combinatie met een TZD zijn werkzaamheid verliezen, doordat een specifieke zoutpomp in de nier (ENaC) harder zou werken. Wij vonden echter dat *furosemide* even effectief was *tijdens* behandeling met *rosiglitazon* als tijdens behandeling met placebo. Verder onderzochten we in de urine het zoutgehalte en de aanwezige kapotte niercellen. Hierbij vonden we geen aanwijzingen voor verhoogde activiteit van de zoutpomp ENaC of voor een toename van het aantal pompjes (ENaC) in de nier. Concluderend, TZD's leiden niet tot stimulering van ENaC of vermindering van de werking van plaspillen. De klinische gevolgtrekking uit deze bevinding is dat TZD-gerelateerde vochtophoping kan worden behandeld met plaspillen.

Ter voorkoming van verdere complicaties is het van belang om bij patiënten die behandeld worden met TZD's te letten op tekenen van vochtophoping. Vaak wordt daartoe het hematocriet, de verhouding van bloedcellen en vocht in het bloed (plasmavolume), vervolgd. In **hoofdstuk vijf** hebben we de relatie beschreven tussen veranderingen in hematocriet en plasmavolume tijdens behandeling met *rosiglitazon*. Hiervoor hebben we gebruik gemaakt van de gegevens van de onderzoeken beschreven in **hoofdstukken twee** en **drie**. In tegenstelling tot de gangbare mening, vonden we geen enkel verband tussen de verandering in hematocriet en verandering in plasmavolume tijdens chronische behandeling met *rosiglitazon* noch bij patiënten met insulineresistentie, noch bij patiënten met diabetes mellitus. De arts leert hiervan dat het bepalen van hematocriet bij het vervolgen van een individuele patiënt, geen meerwaarde heeft voor het vroegtijdig opsporen van vochtophoping tijdens behandeling met TZD's.

Een belangrijk onderwerp in dit proefschrift is schade aan het onwillekeurige zenuwstelsel. In **hoofdstuk zes** rapporteren we over functiemetingen van het onwillekeurig zenuwstelsel in patiënten met ouderdomssuikerziekte, met en zonder zenuwschade. Wij vonden dat bij patiënten met zenuwschade de functie in rust nog gespaard is, maar dat de functie tijdens piekbelasting duidelijk verminderd is ten opzichte van patiënten zonder schade. Bovendien vonden we enkele aanwijzingen dat *rosiglitazon* de functie van het beschadigde zenuwstelsel gunstig beïnvloedt. Dit effect trad op, onafhankelijk van het effect op de bloedsuiker.

Ten tijde van de start van de in dit proefschrift beschreven onderzoeken bestond de overtuiging dat TZD's gunstig zouden zijn om schade aan hart en bloedvaten te voorkomen. Eén van de hypotheses volgens welk mechanisme dit positieve effect zou verlopen, was bescherming tegen schade door zuurstoftekort. **Hoofdstuk zeven** beschrijft de resultaten van een onderzoek naar de invloed van de behandeling met *rosiglitazon* op deze schade, gemeten aan de hand van aankleuring op foto's gemaakt na toediening van een radioactief isotoop. Deelnemers moesten een zware knijpoefening verrichten terwijl de arm werd afgekneld, waardoor er dus zuurstofschuld kon ontstaan. Inderdaad bleken

deelnemers tijdens behandeling met *rosiglitazon* minder schade te hebben ten opzichte van behandeling met placebo. Dit suggereert inderdaad dat TZD's beschermende effecten hebben op schade door zuurstofschuld.

Vochtophoping in kernwoorden en de nieuwe inzichten van dit proefschrift voor de klinische praktijk

Wetenschappelijk perspectief: Vochtophoping in zijn algemeenheid kent verschillende uitingsvormen in de kliniek. Het verschijnsel dat we in de klinische praktijk het frequentst tegenkomen tijdens behandeling met een TZD is vochtophoping in de huid (oedeem). Als we de literatuur op een rij zetten en daar de bevindingen uit dit proefschrift aan toevoegen, dan moeten we concluderen dat de drijvende kracht achter deze ophoping een disbalans in de bloedvaten is. Van de eerder genoemde hoofdverdachten vallen het hart en de nier dus af. De precieze aard van de disbalans in de bloedvaten is overigens niet duidelijk. Zowel toegenomen vaatverwijding, als ook toegenomen lekkage of toename van het aantal actieve haarvaten kunnen een rol spelen bij TZD-gerelateerde vochtophoping.

Klinisch perspectief: Drie nieuwe aandachtspunten met betrekking tot risicofactor-screening, vroegtijdige signalering en behandeling van TZD-gerelateerde vochtophoping geeft dit proefschrift de clinicus mee. Allereerst blijkt de patiënt met schade aan het onwillekeurige zenuwstelsel gevoeliger voor het krijgen van oedeem. Ten tweede lijkt het vervolgen van het hematocriet in de individuele patiënt die wordt behandeld met een TZD niet bijdragend aan het vroegtijdig signaleren van ernstige vochtophoping. Ten slotte, mocht er onverhoopt sprake zijn van vochtophoping, dan zijn plaspillen even effectief in het afdrijven van zout en vocht als bij mensen die niet met een TZD behandeld worden.

Het liep anders

Op het moment van registratie werd van de TZD's verwacht dat zij de kans op hart- en vaatziekten zouden reduceren. In de praktijk kon *pioglitazon* deze belofte een beetje inlossen, maar *rosiglitazon* zeker niet. In 2007 was de conclusie van een meta-analyse (dat is de overkoepelende conclusie van meerdere onderzoeken tezamen) dat *rosiglitazon* juist de kans op een hartinfarct vergroot. Tussen 2007 en 2013 verschenen er talloze publicaties over dit onderwerp met tegenstrijdige conclusies waarbij de wetenschappelijke wereld werd opgedeeld in twee kampen: '*Rosiglitazon* is bewezen slecht voor hart en bloedvaten' en '*Rosiglitazon* is niet bewezen slecht voor hart en bloedvaten'. In de tussentijd werd het middel in Europa van de markt gehaald en mocht het in de VS alleen onder zware restricties in de handel blijven. Samengevat, alle wetenschappelijke bewijzen samengenomen, is er geen sluitend bewijs dat *rosiglitazon* schaadt, maar er is voldoende bewijs voor bezorgdheid omtrent *rosiglitazon*'s veiligheid voor hart en bloedvaten. Het feit dat *rosiglitazon* geen uniek gezondheidsvoordeel heeft ten opzichte van *pioglitazon*, rechtvaardigt beperking van het gebruik. Echter ook het gebruik van *pioglitazon* is aan banden gelegd vanwege een grotere kans op blaaskanker en botbreuken die je bij beide TZD's ziet. Het oorspronkelijke idee dat vochtophoping de Achilleshiel van de TZD's zou zijn werd niet bewaarheid. Enerzijds blijkt het oedeem meestal goedaardig. Anderzijds zijn er ernstigere bijwerkingen bijgekomen, zoals een toegenomen kans op blaaskanker, botbreuken en dus specifiek voor *rosiglitazon* een mogelijk toegenomen kans op een hartinfarct.

Toekomst PPAR-γ agonisten en dit proefschrift

Ondanks de bijwerkingen worden TZD's nog steeds gebruikt, aan de ene kant vanwege de unieke eigenschappen, aan de andere kant vanwege de geringe kosten nu ze uit patent zijn. In Europa is *rosiglitazon* vanwege twijfels omtrent de veiligheid van de markt gehaald, maar in de Verenigde Staten en andere delen van de wereld werd het gebruik slechts ingeperkt. Daarentegen staat het broertje *pioglitazon* nog steeds in internationale richtlijnen en wordt het wereldwijd nog regelmatig gebruikt, waarbij het marktaandeel in 2011 ongeveer tien procent bedroeg. TZD's, vooral *pioglitazon*, worden ook gebruikt voor de behandeling

van niet-alcoholische vettige leverontsteking, polycysteus eierstoksyndroom en lipodystrofie in humane immunodeficiëntievirus (HIV) geïnfecteerde patiënten.

Nieuwere PPAR-γ agonisten worden de komende jaren waarschijnlijk op de markt gebracht ter behandeling van ouderdomssuikerziekte. Slechts een enkel middel lijkt gunstige effecten te hebben op de bloedsuikerwaarde zonder de voor de thiazolidinedionen kenmerkende bijwerkingen. Als één van deze geneesmiddelen geregistreerd gaat worden voor de behandeling van ouderdomssuikerziekte zal de kennis zoals beschreven in dit proefschrift bijdragen aan het toezicht op mogelijke vocht-gerelateerde bijwerkingen.

Echter, of één van deze nieuwe PPAR-γ agonisten daadwerkelijk voor behandeling van ouderdomssuikerziekte geregistreerd gaat worden is twijfelachtig, aangezien er in het afgelopen decennium concurrentie bij is gekomen van andere geneesmiddelgroepen. Dit zijn de incretines, vertegenwoordigd door de di-peptidyl-peptidase-4-remmers (DPP-4) en de glucagon-like peptide-1-agonisten (GLP-1), en de natrium-glucose co-transporter-2 remmers (SGLT2). In ieder geval worden de namen er niet toegankelijker op. Als gevolg van de discussie over de cardiovasculaire veiligheid van *rosiglitazon*, worden deze nieuwe middelen zorgvuldig gevolgd op bijwerkingen van hart of bloedvaten. Onlangs zijn de resultaten van een dergelijk onderzoek van de DPP-4-remmer *saxagliptin* gepubliceerd. Onverwacht en vooralsnog onverklaard bleek *saxagliptin* de kans op ziekenhuisopname als gevolg van hartfalen te verhogen. Terugdenkend lijkt dit op het startpunt van het proefschrift dat nu voor u ligt. We begonnen immers deze zoektocht in een poging TZD-gerelateerde vochtophoping zoals hartfalen te verklaren. De waarneming dat een nieuw bloedsuikerverlagend middel de kans op verslechtering van hartfalen vergroot, voltooit in zekere zin de cyclus van dit proefschift.

9

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List of abbreviations used throughout this thesis

ANP: Atrial Natriuretic Peptide
AUC: Area Under the Curve
BMI: Body Mass Index
CAN: Cardiovascular Autonomic Neuropathy
DBP: Diastolic Blood Pressure
ECV: Extra Cellular Volume
ENaC: Epithelial Sodium Channel
FBF: Forearm Blood Flow
FPG: Fasting Plasma Glucose
FVR: Forearm Vascular Resistance
GIR: Glucose Infusion Rate
HbA1c: Glycosylated Haemoglobin
HDL: High Density lipoprotein
HOMA: Homeostasis Model Assessment
HPLC: High-Performance Liquid Chromatography
I/R: Ischaemie/Reperfusion
LBNP: Lower Body Negative Pressure
L-NMMA: N-MonoMethyl-L-Arginin-acetate
MCV: Mean Corpuscular Volume
NO: Nitric Oxide
PLAC: Placebo
PPAR-γ: Peroxisome Proliferator Activated Receptor-γ
PV: Plasma Volume
RAAS: Renin-Angiotensin-Aldosterone System
RSG: Rosiglitazone
SBP: Systolic Blood Pressure
TBW: Total Body Water
TERalb: Transcapillary Escape Rate of albumin
TZD: Thiazolidinedione
VEGF: Vascular Endothelial Growth Factor

Dankwoord

"In de wetenschap staan we op de schouders van *reuzen*". Dat geldt natuurlijk ook voor mij, maar daarnaast heb ik duwtjes en zetjes van een groot aantal *mensen* gekregen. Zonder deze hulp waren de onderzoeken die beschreven staan in dit proefschrift nooit tot stand gekomen. Bovenal moet ik de proefpersonen (vrijwilligers en patiënten) die bereid waren mee te werken aan mijn onderzoeken in het zonnetje zetten.

Beste proefpersonen, ik heb warme herinneringen aan de boeiende gesprekken die ik heb gevoerd met u tijdens de lange onderzoekssessies. Verhalen over het leven als student onder het regime van Mubutu, over het leven als profwielrenner in de jaren '50 of als politiek journalist in de jaren '60. Maar ook gesprekken over de passie voor de muziek van Queen en over een motoraanrijding met een koe zijn mij bijgebleven en hebben mijn promotietraject verrijkt. Als academicus moet je immers het grote verband blijven zien. Ik hoop dat de warme herinneringen wederzijds zijn, al moest u zich wel infuuslijnen, bloedafnames en een aantal stresstesten laten welgevallen. Terzijde, de botsing met de koe was in ieder geval het meest originele *adverse event* uit mijn onderzoeken.

Mijn promotores:
Professor P.(A.B.M.) Smits. Beste Paul, het was in mijn eerste opleidingsjaar (1999) dat jij indruk op mij maakte tijdens het toenmalige COIG Farmacologie als jonge hoogleraar met een gelikte presentatie en voordracht. Daarna was het niet moeilijk mij te verleiden tot dit promotietraject op jouw afdeling. Dit bleek een juiste keuze, je hebt namelijk een neus voor het creëren van een goede sfeer in de werkomgeving. Ik moet je danken voor de ruimte die je me gegeven hebt, ook na afronding van mijn interne opleiding, om het onderzoek voort te zetten. Je hebt mij oog gegeven voor *detail* en *symmetrie* in presentatie, en gevoel bijgebracht voor de grote lijn bij het opzetten van een onderzoek. Ook na je verhuizing naar de bovenste verdieping bleef de afstand overbrugbaar door je amicale en hartelijke manier van benadering en begeleiding. Terzijde, ik kijk met jaloerse blik naar je altijd opgeruimde bureau. Dat lukt mij helaas vaak niet. Ik klamp me dan maar vast aan de stelling die ik op de kalender thuis tegen kwam: "*A clean house is a sign of a wasted life*".

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Beste Niels, het was Peter Pickkers die je bij jouw komst op de afdeling Farmtox aankondigde als een groot onderzoekstalent. Niets bleek minder waar, maar belangrijker, je bent een geweldig sympathieke collega. Door de dagelijkse afblaassessies op de farmacologie raakten we al snel bevriend. Terzijde, dat is best opvallend aangezien jij amper geboren was toen Nederland werd geteisterd door een zware Siberische koude–aanval op de avond van 29 december 1978, de eerste associatie die ik had toen je jouw geboortejaar vertelde. Inmiddels zijn onze rollen veranderd en ben je zelfs mijn leidinggevende. Elkaars voetstappen herkennen we niet meer, maar dat komt meer door de vloerbedekking op de afdeling interne geneeskunde dan het feit dat ik je schoenenverzameling niet meer uit het hoofd ken. In je rol als leidinggevende blijf je geïnteresseerd in de beslommeringen van collega's, ben je niet bang ruim baan te maken voor talenten van anderen en durf je, altijd wel onderbouwd, beslissingen te nemen. Kortom een *L'Uomo Universalis*. Nu nog hopen dat je het er als paranimf ook goed vanaf brengt.

Beste Bas, ook bij mijn kennismaking met jou was ik de ervaren collega, maar dan in de kliniek. Het was ten tijde van mijn laatste klinische stage (hematologie) voor aanvang van mijn promotieonderzoek. Overigens had ik nog mijn best gedaan onder deze stage uit te komen, omdat ik altijd een beetje zeeziek wordt van het turen naar bloedcellen onder een microscoop. Gelukkig kwam ik er niet onderuit, want het werd uiteindelijk mijn leukste stage en daar speelde jij een duidelijke rol in. Ik heb genoten van je kleurrijke uitvoeringen van diverse imitaties

tussen de bedrijven door. Ons contact kreeg verdieping tijdens een spekgladde kroegentocht naar café Jos. Het was dan ook een leuke gewaarwording om je later op de Farmtox weer tegen te komen. Uiteindelijk vertoont ons carrièrepad en opleidingsportfolio veel overeenkomsten. Alleen ben jij nog nooit paranimf geweest, maar dat hiaat wordt nu gevuld. Terzijde, het is misschien verstandig om je imitaties ten uitvoer te brengen in afwezigheid van familieleden van het subject.

Afdeling Farmacologie (Farmtox):
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Publication list:

Jucker B.M., **Rennings A.J.**, Cline G.W., Shulman G.I. 13C and 31P NMR studies on the effects of increased plasma free fatty acids on intramuscular glucose metabolism in the awake rat. *J Biol Chem* 1997; 272: 10464-73.

Jucker B.M., **Rennings A.J.**, Cline G.W., Petersen K.F., Shulman G.I. In vivo NMR investigation of intramuscular glucose metabolism in conscious rats. *Am J Physiol* 1997; 273: E139-148.

Cline G.W., Jucker B.M., Trajanoski Z., **Rennings A.J.**, Shulman G.I. A novel 13C NMR method to assess intracellular glucose concentration in muscle, in vivo. *Am J Physiol* 1998; 274: E381-389.

Heeringa-Karreman M., **Rennings A.J.**, Schreuder T., Jansen M., De Boer H., Van Sorge A.A. Dikke maatjes met diazoxide. *Pharmaceutisch Weekblad* 2003; 38: 1307-1309.

Schreuder T., Karreman M., R**ennings A.**, Ruinemans-Koerts J., Jansen M., de Boer H. Diazoxide-mediated insulin suppression in obese men: a dose-response study. *Diabetes Obes Met* 2005;7(3):239-45

Rennings A.J., Smits P., Stewart M.W., Tack C.J. Fluid retention and vascular effects of rosiglitazone in obese, insulin-resistant, nondiabetic subjects. *Diabetes Care* 2006;29(3):581-587.

Rennings A.J., Stalenhoef A.F. JTT-705: is there still future for a CETP inhibitor after torcetrapib? *Expert Opin Investig Drugs* 2008 Oct;17(10):1589-1597.

Lesterhuis W.J., **Rennings A.J.**, Leenders W.P., Nooteboom A., Punt C.J., Sweep F.C., Pickkers P., Geurts-Moespot A., Van Laarhoven H.W., Van der Vlag J., Berden J.H., Postma C.T., Van der Meer J.W. Vascular endothelial growth factor in systemic capillary leak syndrome. *Am J Med* 2009 Jun;122(6):e5-7.

Rennings A.J., Meijer P., van Uden D.J., Tack C.J., Smits P., Boerman O.C., Oyen W.J., Rongen G.A. Rosiglitazone reduces ischaemia-reperfusion injury in patients with the metabolic syndrome. *Eur Heart J* 2010; Apr;31(8):983.

Rennings A.J., Smits P., Stewart M.W., Tack C.J. Autonomic neuropathy predisposes to rosiglitazone-induced vascular leakage in insulin treated patients with type 2 diabetes: A randomized-controlled trial on thiazolidinedione-induced vascular leakage. *Diabetologia 2010; Sep 53: 1856-1866*

Rennings A.J., Schouwenberg B.J., van Onzenoort H.A. Duration of clopidogrel therapy with drug-eluting stents. *N Engl J Med* 2010; 363: 489

Rennings A.J., Russel F.G., Li Y., Deen P.M., Masereeuw R., Tack C.J., Smits P. Preserved response to diuretics in rosiglitazone-treated subjects with insulin resistance. *Clin Pharmacol Ther* 2011 Apr;89(4):587-94

Werkgroep INVEST. Volwassenen met een erfelijke stofwisselingsziekte. Een sterk groeiende populatie met unieke problematiek. *Ned Tijdschr Geneeskd* 2014;158:A7745

Rennings A.J., Tack C.J., Smits P. Change in haematocrit does not reflect change in plasma volume in individuals on chronic treatment with thiazolidinediones. *Submitted*

Rennings A.J., Tack C.J., Smits P. Preserved α-adrenergic tone but blunted sympathetic response to orthostatic stress in diabetic neuropathy; effects of rosiglitazone. *Submitted*

Curriculum Vitae

Alexander Rennings werd geboren op 7 mei 1970 in Oudenbosch, een plaats in het westen van Noord-Brabant, bekend om zijn Rijke Roomse Leven en de kopie van de Sint Pieter in Rome. In het naburige Roosendaal ging hij naar het Norbertus Lyceum alwaar hij in 1988 het gymnasium diploma behaalde. In datzelfde jaar startte hij na een rondreis door Europa de studie 'medicijnen' aan de Rijksuniversiteit Leiden. Na klinische en fundamentele onderzoeksstages in de diabetologie bij respectievelijk dr. H. Brussaard (LUMC) en professor G. Shulman (Yale University) was de wetenschappelijke interesse gewekt. Toch koos hij voor klinisch werk en verhuisde hij in 1998 naar Nijmegen alwaar hij in 2004 onder de bezielende leiding van professor J. van der Meer internist werd. Gefascineerd door de klinische farmacologie en de fysiologie van water en zout in ons lichaam startte hij in 2002 op de afdeling Farmacologie-Toxicologie de onderzoeken die beschreven zijn in dit proefschrift. Zoals ook in de kliniek bleef de focus tijdens het onderzoek sterk liggen op het welbevinden van de mens. Inmiddels is hij geregistreerd als vasculair-geneeskundige (opleider: professor A. Stalenhoef) en klinisch-farmacoloog (opleider: professor P. Smits). Sinds 2011 heeft hij een dubbele aanstelling. Als internist is hij verbonden aan de Sint Maartenskliniek, alwaar hij onder andere de opleiding tot klinisch-farmacoloog coördineert. In het Radboudumc legt hij zich speciaal toe op de erfelijke aangeboren stofwisselingsziekten en haemochromatose. Als arts probeert hij het perspectief van de patiënt niet uit het oog te verliezen en wil hij zijn kennis graag aan studenten overdragen. Maar de grootste passie blijft zijn gezinsleven, waarbij hij het huis deelt met zijn echtgenote Gaby van Welsem en hun drie zonen.