Aus der Medizinischen Universitätsklinik und Poliklinik Tübingen
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# The functional Gly388Arg Polymorphism in *FGFR4* affects Glucose Tolerance, Insulin Sensitivity and Liver Fat

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# 1. Introduction

## 1.1 Diabetes Mellitus

The term diabetes mellitus describes a metabolic disorder characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism (1). In the United States, diabetes mellitus afflicts 23.6 million people of all ages which makes it actually an epidemic disease (2). Worldwide the prevalence of diabetes mellitus has risen dramatically over the past two decades, from an estimated 30 million cases in 1985 to 177 million in 2000. Based on current trends, 366 million individuals will have diabetes by the year 2030 (3). Today, diabetes accounts for almost 14 percent of US health expenditures, at least one-half of which are related to complications (2,4).

The vast majority of diabetes cases fall into two broad categories, type 1 and type 2 diabetes mellitus. Type 1 diabetes mellitus accounts for 5% to 10% of all cases and occurs at any age, but most commonly in children and young adults. In contrast, type 2 diabetes mellitus accounts for 80-90% of all diabetes cases and occurs mostly among adult obese individuals (5).

Both type 1 and type 2 diabetes mellitus, if untreated, develop chronic complications after years. Since type 2 diabetes mellitus has for many years no clinical signs and symptoms, complications may be the first clinical manifestation. During this period, it is possible to demonstrate an abnormality in carbohydrate metabolism by measurement of plasma glucose in the fasting state and after a challenge with an oral glucose load (5).

Patients with diabetes mellitus and uncontrolled hyperglycemia are at increased risk for developing microvascular complications, such as retinopathy and nephropathy, and macrovascular complications, such as coronary heart disease, stroke or peripheral artery disease (6). Peripheral neuropathy with an increased risk of foot ulcers, amputations, and Charcot joints, as well as autonomic neuropathy causing gastrointestinal, genitourinary (such as erectile dysfunction), and cardiovascular symptoms, may also occur. Particularly type 2 diabetes mellitus is often accompanied by other conditions such as hypertension, dyslipidemia and central obesity comprising the so called

metabolic syndrome. Patients with the metabolic syndrome show an increased incidence of cardiovascular disease (5,7).

The causes of type 2 diabetes mellitus are largely unknown. However, type 2 diabetes appears to be caused by a complex interaction of environmental and genetic factors. The genetic background of the disease is proved by the evidence that the risk of diabetes is significantly increased among close relatives of an affected patient (8). Recent genetic association studies showed that several novel loci are associated with the risk of diabetes, each with a 5 to 37% increase in the relative odds of diabetes per risk allele (9).

Among environmental factors, excess adiposity and especially a central distribution of adipose tissue, is the most important risk factor for the development of type 2 diabetes mellitus (10). Reduced physical activity, increased calorie intake and active smoking appear also to promote diabetes in susceptible individuals (11-13).

Each of the aforementioned risk factors affects either insulin secretion or insulin sensitivity, which are considered today to be the major pathogenic mechanisms in the development of type 2 diabetes mellitus. In fact, every patient with type 2 diabetes mellitus presents with a combination of varying degrees of insulin resistance and defective insulin secretion, and it is likely that both contribute to type 2 diabetes (14-16).

### 1.2 Insulin resistance

Insulin resistance is a condition in which higher than normal insulin concentrations are needed to achieve a normal metabolic response (17). The biological action of insulin depends on a cascade of events following the interaction of insulin with its specific receptor on the cell membrane. Binding of insulin to the receptor promotes the autophosphorylation of the receptor at a tyrosine residue. The phosphorylated receptor in turn phosphorylates other protein substrates beginning with insulin receptor substrate (IRS) proteins. Three major signaling pathways are propagated in response to the activation of the IRS: the phosphatidylinositol-3-kinase (PI3K), the mitogen activated protein (MAP) kinase and the Cbl/CAP (Casitas B lineage lymphoma /c-Cbl-assosiated

protein) pathways (18). Through activation of PIK3 cascade, insulin acts as a powerful regulator of metabolic function. Insulin regulates glucose homeostasis by reducing hepatic glucose output (decreasing gluconeogenesis and glucogenolysis) and increasing the rate of glucose uptake, primarily into sceletal muscle and adipose tissue. Insulin also profoundly affects lipid metabolism, increasing de novo lipogenesis in liver and inhibiting lipolysis in adipose tissue (19).

One or more of these pathways is blocked in states of insulin resistance. Probably the most critical factor inducing insulin resistance is obesity. Products of adipose tissue such as non-esterified-fatty acids (NEFAs), hormones and proinflammatory cytokines are overexpressed in obesity and their inhibitory effect on insulin signaling is discussed below (20-23). NEFAs may play the most important role in modulating insulin sensitivity. Increased circulating NEFA levels are observed in obesity and type 2 diabetes, and are associated with the insulin resistance observed in both of them (24,25).

Adipose tissue in obesity is populated by inflammatory cells, especially by monocytes and macrophages. Products of these cells such as tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1) play also a role in the development of insulin resistance (20,26).

Adipose tissue also secretes some peptides with hormonal function, called adipokines. Among them the most abundant is the protein adiponectin which signals via AMP kinase, a stress-activated signaling enzyme and peroxisome proliferator-activated receptor (PPAR)-a (27), implicated in various metabolic responses, including suppression of hepatic gluconeogenesis, glucose uptake in exercising skeletal muscle, fatty acid oxidation, and inhibition of lipolysis, which may explain its beneficial metabolic effects. Unlike almost all the other adipokines, however, the concentrations of the adiponectin are decreased in obesity, reducing its insulin-sensitizing effects in liver and muscles (28).

Furthermore, several other adipokines have been identified as potential regulators of insulin sensitivity such as retinol-binding-protein-4 (RBP-4), resistin, visfatin and chemerin (29). Among them, RBP-4 induces insulin

resistance by stimulating liver gluconeogenesis (23,30) and possibly, by promoting fat accumulation in liver (31).

Whereas overall obesity is typically associated with insulin resistance, insulin sensitivity also varies markedly in individuals because of differences in body fat distribution (23). Individuals with a more subcutaneous distribution of fat are more insulin sensitive than subjects who have their fat distributed predominantly viscerally. Visceral fat is less sensitive to the anti-lipolytic effect of insulin (32). Moreover, visceral adipose tissue (VAT) is metabolically more active and secretes higher amounts of adipokines. The outcome of increased lipolysis in VAT is the elevated flux of NEFAs directly into the portal vein and finally to the liver, a process that is commonly referred to as the “portal hypothesis” (33,34).

Lipids are stored not only in adipocytes but also “ectopically” in tissues such as muscle, liver, beta cells and other sites. Ectopic fat storage in the aforementioned sites, especially in the skeletal muscle and the liver causes defects in insulin signaling (35). Studies in obese mice demonstrated that fat accumulation in the liver and the skeletal muscle is associated with severe whole body insulin resistance (36,37).

Recently, several groups using magnetic resonance spectroscopy (MRS) methods to determine intramyocellular lipid content (IMCL) reported an inverse relationship between IMCL and insulin sensitivity independently of body mass index (BMI) and body fat content (38). Perseghin et al reported a close association between insulin sensitivity and the amount of IMCL, in a group of first-degree relatives of type 2 diabetic patients (39), while Krssak et al. described an inverse correlation between insulin sensitivity and IMCL in normal weight non-diabetic adults (40). Moreover, insulin resistant offsprings of type 2 diabetic patients were shown to have markedly increased IMCL compared to insulin-sensitive controls, suggesting that IMCL stores may play a role in the pathogenesis of skeletal muscle insulin resistance (41). Lipodystrophic conditions in humans lead to fat accumulation in skeletal muscle and liver and are accompanied by insulin resistance (42).

The association between hepatocellular lipid (HCL) content and insulin sensitivity seems to be more reproducible than that of IMCL content. Fat

accumulation in the liver is ubiquitously associated with insulin resistance independently of body mass index, intraabdominal and overall obesity (38,43). This is discussed in detail bellow.

### 1.3 Fatty liver

Fat triglycerides can accumulate in form of droplets in hepatocytes as a consequence of specific conditions (secondary hepatic steatosis) or as a companion of obesity and diabetes (primary hepatic steatosis). Several causes have been identified to promote fat accumulation in the liver. Examples include, but are not limited to malnutrition, rapid weight loss, lipodystrophy, use of certain drugs (e.g. glucocorticoids) and inflammatory bowel disease. For primary steatosis the terms ‘fatty liver’ or ‘non alcoholic fatty liver disease (NAFLD)’ are used to describe fat accumulation in liver in the absence of alcohol intake (less than 20 gr of alcohol per day) (44). NAFLD represents a wide spectrum of pathological conditions ranging from simple steatosis, which in general follows a benign nonprogressive clinical course, to nonalcoholic steatohepatitis (NASH) that is steatosis with inflammation, to cirrhosis, end-stage liver disease and occasionally to hepatocellular carcinoma (33).

NAFLD is diagnozed by the use of invasive and non invasive methods. Among invasive, liver biopsy is considered to be the ‘gold-standard’, because it provides important information regarding the degree of liver damage, changes in the liver architecture, as well as the severity of inflammatory activity and fibrosis. Proton magnetic resonance spectroscopy ($^{1}$H-MRS) is considered to be the most accurate non-invasive method. Fatty liver is defined by $^{1}$H-MRS as hepatic triglyceride content greater than 5.56% (55.6 mg/gr liver tissue) (45). Hepatic lipid content found in $^{1}$H-MRS correlates closely to that found in biopsy. $^{1}$H-MRS is suitable for a wide use, in contrast to liver biopsy, but it cannot detect liver inflammation or determine the stage and the grade of the disease (45).

According to epidemiological studies, fatty liver is the most frequent liver disease in westernized societies. It is estimated that fatty liver affects more than 30% of the general adult population. As mentioned, the prevalence of fatty liver increases with type 2 diabetes and obesity (46). 75% of obese and almost all

morbidly obese subjects have the disease. Furthermore, 50-75% of patients with type 2 diabetes mellitus have fatty liver (47) whereas it is an almost universal finding in obese patients with type 2 diabetes (48). Fatty liver is found even in children and is estimated that it is the most common liver disease in children 2 to 19 years old (49).

Increased liver fat content is associated with particularly hepatic but also with whole body insulin resistance. More importantly this association is independently from overall and visceral obesity which are known determinants of insulin resistance (33).

Epidemiological studies reveal that NAFLD is strongly correlated with the prevalence of metabolic syndrome and its components (44,47). Central obesity and insulin resistance contribute to the development of hepatic steatosis via increased NEFA flux to the liver. In states of insulin resistance, insulin does not suppress adipose tissue lipolysis. Visceral adipose tissue, releases NEFA directly into the liver through the portal vein ('portal hypothesis') (34). Another mechanism probably leading to the excessive accumulation of hepatic lipids is enhanced de novo lipogenesis. Hyperinsulinemia, which occurs as a result of insulin resistance, can activate sterol-regulatory-binding protein 1c (SREBP-1c), which stimulates the expression of enzymes involved in fatty acid synthesis in the liver (50,51).

Recent findings point out that insulin resistance may be a consequence rather than a cause of fatty liver. Fatty liver may secrete humoral factors affecting insulin signaling ('hepatokines') (33). One of them is called fetuin-A (also called alpha-2-Heremans Schmid glycoprotein) and is predominantly expressed in the liver, and to a lesser degree in the placenta and the tongue. Because placental expression is only relevant during pregnancy and the tongue is not an organ with endocrine activity, the liver is the only organ regulating circulating fetuin-A levels (52). Fetuin-A is a natural inhibitor of the insulin receptor, both in liver and skeletal muscle (53). In humans, levels of fetuin-A correlated negatively with insulin sensitivity and positively with liver fat (53)**.** Recently, single nucleotide polymorphisms in the fetuin-A gene were shown to be associated with type 2 diabetes (54).

Many other factors have also been proposed to promote liver fat accumulation. Hypertrophic adipose tissue leads to an increased release of proinflammatory cytokines such as IL-6 and TNF-a. Moreover, TNF-a and IL-6 suppress the production of the insulin-sensitizing adipokine adiponectin (27,55). Adiponectin is a polypeptide that is strongly inversely correlated with systemic insulin sensitivity in humans. Adiponectin increases fatty acid beta oxidation in muscle and liver. In line, treatment with thiazolidinediones, which increase circulating adiponectin, results in a decrease in liver fat content (56).

Agents regulating bile acid metabolism may be an additional factor in the regulation of fat accumulation in the liver (57). At least two distinct mechanisms were proposed to be responsible for this effect. First, bile acids by binding to the G-protein-coupled receptor TGR5, or mBAR, induce peroxisome-proliferator-activated receptor γ co-activator 1a (PGC-1a) transcription, thereby increasing mitochondrial activity and β-oxidation (57,58). Second, bile acids are the major ligands of the farnesoid X receptor (FXR). FXR is a nuclear receptor highly expressed in the liver and the intestinal epithelium (59). Evidence provided by studies in humans and rodents suggest that the bile acid-mediated activation of FXR of the ileum induces the local expression of fibroblast growth factor 19 (FGF-19, in rodents FGF-15). FGF-19 is absorbed in the bloodstream and is able to activate fibroblast growth factor receptor-4 (FGFR4) in hepatocytes. Activated FGFR4 represses cytochrome P450 7a-hydroxylase (CYP7A1) gene expression via a c-Jun N-terminal kinase (JNK) pathway (60,61). CYP7A1 is the first and rate-limiting enzyme in the biosynthesis of bile acids (61) and thus activation of FGFR4 represses bile acid synthesis.

Furthermore, liver FXR has significant effects in modulating postprandial energy metabolism and particularly lipoprotein metabolism (62). In animals, both the natural FXR agonist chenodeoxycholic acid (63) and the synthetic FXR agonist GW4064 reduce plasma triglycerides and the rate of VLDL production, (64). The reduction of triglycerides is, at least partially, attributed to the downregulation of SREBP-1c (57,63) and up regulation of PPAR-α, leading to a reduced hepatic fatty acid and triglyceride synthesis and an increased fatty acid oxidation (59). Thus, activation of FXR suppresses the bile acid synthesis and is

also suggested to increase fatty acid oxidation in the liver through mechanisms possibly involving, at least partially, FGF19 and FGFR4.

### 1.4 Fibroblast Growth Factor Receptor Family

Fibroblast growth factor receptor family accounts totally four members: the FGFR1, FGFR2, FGFR3 and FGFR4. FGFR family belongs to the protein kinase superfamily. All FGFRs are transmembrane proteins with an intrinsic tyrosine kinase action (65).

FGFR family members share common structural features. Like all receptor tyrosine kinases, FGFRs consist of an extracellular ligand-binding domain, a single transmembrane domain, and a cytoplasmatic region which is further separated to a tyrosine kinase domain and a carboxyl terminal tail (66,67).

As indicated by the extreme phenotypes of constitutive knockout models, fibroblast growth factors, their receptors and signaling cascades are involved in a diverse range of cellular processes including proliferation, apoptosis, cell survival, chemotaxis, cell adhesion, motility and differentiation, organ formation and maintenance, neuronal differentiation and survival, wound healing and angiogenesis (68). Furthermore, genetic studies in humans and mice have demonstrated that mutations leading to disruption of FGF signaling cause a variety of developmental disorders including dominant skeletal diseases, infertility, and cancer (68-70).

### 1.5 Fibroblast growth factor receptor 4 (FGFR4)

FGFR4 is encoded by the *FGFR4* gene which is located on the long (q) arm of chromosome 5 between position 35, 1 and the end (terminus) of the arm. Despite the different localization of FGFR genes, FGFR proteins share highly homologous structural elements (66). The homology is greatest between FGFR1 and FGFR2 (72% amino acid identity), slightly less between FGFR1 and FGFR3, and least pronounced between FGFR1 and FGFR4 (55% identity) (71). Thus, FGFR4 on one hand share the same structure motif with FGFRs but on the other hand displays specific structural and functional differences.

FGFR4 is the receptor of fibroblast growth factor 19. FGF19 together with FGF21 and FGF23 comprise a phylogenetic subfamily with properties that distinguish them from other FGFs. One of them is that FGF19, FGF21 and FGF23 bind poorly to heparin and heparin sulfate proteoglycans. The binding of heparin sulfate proteoglycans provides a mechanism for limiting the action of FGFs to their paracrine targets. That is why FGF19 family members have activities analogous to the classical hormones (72).

Although FGFR4 is found in several tissues, it is predominantly expressed in the liver (60,67). The main role of FGFR4 in the liver is to inhibit the conversion of cholesterol into bile acids, via suppression of the gene encoding the rate limiting enzyme in bile acids biosynthesis, the CYP7A1. Mice lacking hepatic FGFR4 exhibited an elevated expression of CYP7A1 which is followed by an enhanced excretion of bile acids by the liver and an expanded bile acid pool (73). Inversely, transgenic mice with a constitutively FGF-independent active human FGFR4, displayed decreased bile acid content and a decreased circulating bile acid pool. In these mice the expression of CYP7A1 was significant suppressed (60). Moreover, depressed levels of liver CYP7A1 are associated with elevated levels of JNK kinase. This indicates that activation of FGFR4 may trigger JNK intracellular pathways which in turn suppress the expression of CYP7A1 in the hepatocyte (74).

### 1.6 FGFR4 Gly388Arg Single Nucleotide Polymorphism (SNP)

In 2002, Bange et al. discovered a single nucleotide polymorphism (SNP) at codon 388 in the gene coding for FGFR4 which represent a change from G to A and results in a change from glycine to arginine (Gly388Arg) in the amino acid sequence of the highly conserved and normally hydrophobic transmembrane region of the FGFR4. This change was associated with higher FGFR4 gene expression in breast cancer cell lines compared to the 388G allele (75).

The FGFR4 388Arg allele is associated with a wide variety of cancer types. Furthermore, this SNP is present at significantly higher frequency in cancer patients with aggressive disease and therefore represents a gene alteration that predisposes to a poor clinical outcome (70). In particular, the FGFR4 388Arg

allele was found to be associated with a poor prognosis for positive node breast cancer, high-grade soft-tissue sarcoma, colon carcinoma, and head and neck squamous cell carcinoma (76). An impact of Gly388Arg SNP on clinical outcome was also reported in patients with lung adenocarcinoma (77). More recently, Ho et al. found that FGFR4 contributes significantly to hepatocelular carcinoma (HCC) progression and the minor 388Arg allele was associated with elevated levels of the HCC biomarker alpha fetoprotein (78).

As is described elsewhere, the main role of hepatic FGFR4 is to inhibit the conversion of cholesterol into bile acids, via suppression of the gene encoding CYP7A1, the rate limiting enzyme in bile acid biosynthesis. Therefore, CYP7A1 knockout mice were at high risk to develop hypercholesterolemia and changes in lipid and bile acid metabolism. Taking these data into account, Yu et al. proposed further investigations to prove whether alterations in FGFR4 may be a risk factor of hypercholesterolemia (60). In addition to hypercholesterolemia Huang et al. also described that FGFR4-deficient mice on a normal diet exhibited features of metabolic syndrome such as increased mass of white adipose tissue, hyperlipidemia, glucose intolerance, and insulin resistance. Clearly, however, further investigations are required to establish the linkage between features of metabolic syndrome such as lipid abnormalities or glucose intolerance and type 2 diabetes mellitus and the action of FGFR4 in the liver.

### 1.7 Aim

The frequently described coincidence of cancer and type 2 diabetes suggests a common genetic background. The Gly388Arg SNP in *FGFR4* gene was found to be associated with the prevalence and course of a variety of cancers in humans. In addition, some evidence from animal studies suggests that the same SNP may also be related to prediabetes phenotypes, such as obesity, insulin resistance and fatty liver. Aim of this thesis was to specifically address the question whether the Gly388Arg SNP in *FGFR4* could be a candidate for this common genetic background of cancer and type 2 diabetes in humans.

For this purpose 170 subjects were taking out of the Tübingen Lifestyle Intervention Program (TULIP) population, which consists of precisely

phenotyped subjects at risk for type 2 diabetes. On these individuals the Gly388Arg polymorphism was genotyped and its relationships with important determinants of type 2 diabetes, such as glucose tolerance, insulin sensitivity and ectopic fat accumulation, all measured by precise, state-of-the-art methods were investigated, both at baseline and during the intervention.

# 2. Materials and Methods

## 2.1 Subjects

Subjects were recruited from the southern part of Germany and participated in the ongoing Tübingen Lifestyle Intervention Program. This study was designed to prevent type 2 diabetes in subjects at risk. All subjects underwent measurements at baseline and after 9 months of intervention with diet and physical activity. A second follow-up visit is planed 24 months after baseline. At the time of the present analysis, a total of 170 individuals had data on all parameters of interest, at baseline and at 9-months follow-up.

### 2.1.1 Inclusion Criteria

Individuals were included in the study when they fulfilled at least one of the following criteria: a family history of type 2 diabetes, a BMI greater or equal than 27 $kg/m^2$ and previous diagnosis of impaired glucose tolerance (79) and/or gestational diabetes. All the participants had to be healthy according to a physical examination and routine laboratory tests.

### 2.1.2 Exclusion Criteria

All subjects first underwent an oral glucose tolerance test (OGTT). Participants with diabetes mellitus (type 1 or 2) were excluded. Diabetes was diagnosed according to the criteria of the WHO (1). Subjects with acute illness, pregnancy and lactation (in the last three months) were not allowed to participate.
Since liver fat was a major endpoint of this analysis, the subjects

had to have no history of liver disease and consume no more than two alcoholic drinks per day. Serum aminotransferase levels at baseline had to be lower than two times the upper limit of normal.

### 2.2 Research design

In the beginning of the study all the participants were informed in written and oral form about the procedures and goals as well as the possible risks of the study. The individuals had the possibility at all times to abandon the study. Informed written consent was obtained from each subject before participation and the local medical ethics committee of the Eberhard - Karls - University of Tübingen had approved the studies.

After baseline measurements, individuals underwent dietary counseling and had up to 10 sessions with a dietician. Counseling was aimed to reduce body weight, intake of calories and particularly intake of calories from fat and to increase intake of fibers. Diet composition was estimated with a validated computer program using 2 representative days of a 3-day diary (DGEPC 3.0, Deutsche Gesellschaft für Ernährung, Bonn, Germany). Furthermore, all subjects completed a standardized self-administered and validated questionnaire to measure physical activity. Individuals were asked to perform at least 3 hours of moderate sports per week. Aerobic endurance exercise (e.g. walking, swimming) with an only moderate increase in the heart rate was encouraged. Participants were seen by the staff on a regular basis to ensure that these recommendations were accomplished. All the tests were performed at 8.00 am after an overnight fast of 12 h.

### 2.3 Methods

#### 2.3.1 Oral Glucose Tolerance Test (OGTT)

All individuals underwent an oral glucose tolerance test (OGTT). They were instructed not to restrict carbohydrate intake in the days before the test. To be tested, subjects had to be on a stable diet, at a stable weight, with a stable level of exercise, and without acute illness or recent hospitalization. After an

overnight fast, a polyethylene intravenous catheter was inserted into an antecubital vein for blood sampling. Basal samples were obtained for the determination of glucose, insulin and free fatty acids levels. After the oral administration of 75gr glucose, which were solved in 300ml of water, venous blood samples were obtained at 30, 60, 90 and 120 minutes for the measurement of glucose, insulin and free fatty acids (FFA) levels. During the procedure all individuals were lying or sitting quietly, without any consumption of caffeine, tobacco or food. Insulin sensitivity was calculated from glucose and insulin values during OGTT, as proposed by Matsuda and De Fronzo (80).

### 2.3.2 Euglycemic Hyperinsulinemic Clamp

A subgroup of 45 subjects underwent measurements of whole body insulin sensitivity by the euglycemic hyperinsulinemic clamp at baseline and at follow-up. After a 12-hour overnight fast, between 7:00 and 8:00 a.m., an antecubital vein was cannulated for the infusion of insulin and glucose. To obtain arterialized blood samples, a dorsal hand vein of the contra lateral arm was cannulated and placed under a heating device at 45°C (81). After basal blood was drawn, subjects received a primed insulin infusion at the rate of 1.0 mU/kg/min for 2h. Blood was drawn every 5 min for determination of blood glucose using a bedside glucose analyzer (YSI 2300 STAT plus, Yellow Springs, USA), and a glucose infusion was adjusted appropriately to maintain the fasting glucose level. Extra blood specimens for the determination of glucose and insulin concentrations were performed at 80, 100 and 120 min. After the end of the procedure, the glucose infusion was gradually reduced under a strict control of blood glucose, to avoid hypoglycemic episodes.

### 2.3.3 Body composition and body fat distribution

Magnetic resonance examinations to determine total body and visceral fat were performed in the early morning after an overnight fasting period on a 1.5-T whole body imager (Magnetom Sonata; Siemens Medical Solutions, Erlangen, Germany). For determination of whole-body fat distribution, an axial T1-weighted fast spin echo technique with an echo train length of 7 was applied.

Measurement parameters: echo time [TE]/ repetition time [TR] 12 msec/490 msec, slice thickness 10 mm, 5 slices per sequence, 10 mm gap between the slices. Field of view was 450mm to 530 mm depending on the extension of the volunteer. A 256 _ 178 matrix was recorded in a measuring time of 12 seconds, allowing breath hold examinations in abdominal regions. Table shift was set to 10 cm. Volunteers were in prone position with the arms extended and data were collected from fingers to toes. The body coil was used as combined transmit/receive coil. Total examination time was between 20 and 25 minutes including one rearrangement, as total table feed of the MR-imager is limited to 110 cm. In order to guarantee identical slice positions after repositioning, volunteers were marked at the iliac crest. Complete reproducibility measurements were performed by previous studies revealed low variation coefficients for all quantified tissue compartments. Postprocessing of the images was performed on a personal computer applying a home-written segmentation program based on Matlab (Mathworks, Inc.). For this purpose two threshold values were set, the first for determination of the noise level in object free parts of the image, the second for differentiation between lean tissue and fat. The threshold value for separation of lean and fat tissue was automatically set to the nadir of bright pixels corresponding to adipose tissue. Its value could be slightly varied manually by visual inspection of the image in order to correct for smaller inconsistencies, as might arise in regions with inhomogenous signal illumination. Besides subcutaneous fat, bright fatty bone marrow of the extremities also contributes to adipose tissue and is included in the analysis. Tissue volumes were calculated by multiplying the corresponding number of segmented pixels by the inplane pixel dimensions and the slice thickness: total tissue volume (TT) including all pixels with signal intensities above the noise level, adipose tissue volume (AT) including all pixels above the second threshold value, visceral adipose tissue (VAT) and abdominal subcutaneous adipose tissue (SCAT) by manually drawing two regions of interest. As the interslice gap corresponds to the slice thickness, volumes between adjacent slices are calculated by simply doubling the volumes of the slices. In order to standardize the profiles of volunteers with different body size (ranging between

154 and 193 cm in our cohort) and bodily structure, each individual dataset was divided in three parts and interpolated to a defined number of sampling points:

1. Lower extremities (LE), ranging from the heel bones to the head of the femur. This body part originally contained 45–54 recorded slices and was interpolated to 70 sampling points for each volunteer.
2. Trunk (T), from head of femur to the head of humerus. This body part originally contained 26–41 recorded slices and was interpolated to 50 sampling points for each volunteer.
3. Head and upper extremities (UE), from the head of humerus to the wrist. This body part originally contained 25–35 slices and was interpolated to 40 sampling points for each volunteer. The error caused by this interpolation algorithm regarding the integrated volumes of TT and AT of the entire body is lower 0.5% (82).

### 2.3.4 $^{1}$H-MRS for quantification of liver fat

Liver fat was determined by localized proton magnetic resonance spectroscopy using a 1.5 T whole-body imager (Magnetom Sonata, Siemens Medical Solutions, Erlangen, Germany). For volume selection, a single-voxel stimulated echo acquisition mode (STEAM) technique was applied ([TR]=4 s, [TE]=10 ms, 32 acquisitions) and a voxel of 3×3×2 $cm^3$ was placed in the posterior part of the seventh segment of the liver. Subjects were asked to breath within the TR interval and to be in expiration during data acquisition. The liver fat was assessed quantitatively by analyzing the signal integrals of methylene and methyl resonances (between 0.7 and 1.5 ppm), using the liver water signal integral at 4.8 ppm as internal reference (83).

### 2.3.5 $^{1}$H-MRS for quantitative analysis of intramyocellular lipid content (IMCL)

The intramyocellular lipid content (IMCL) in the tibialis anterior was quantified by MRS on a 1.5-tesla whole-body system (Magnetom Vision; Siemens, Erlangen, Germany). A stimulated echo acquisition mode (STEAM) single voxel technique

was applied, with a repetition time of 2 s and an echo time of 10 ms. The water signal was suppressed using a frequency-selective prepulse. The volumes of interest with a size of 2.5 ml were positioned in areas with low content (tibialis anterior) of intermuscular fat septa visible on standard T1-weighted imaging. IMCL was quantified by the integral of methylene signals in a range between 1.3 and 1.5 ppm. As the distribution of intramuscular fat in the tibialis anterior is inhomogeneous, representative data of EMCL in the latter muscle could not be achieved using a single voxel. The creatine signal at 3.1 ppm (integration range from 3.0 to 3.2 ppm) served as internal reference for IMCL and EMCL quantification. Since the relaxation times of signals from lipids and creatine are intra- and interindividually constant and the applied echo time was short, results were not corrected for relaxation effects (41).

## 2.4 Materials

### 2.4.1 Oral Glucose Tolerance Test (OGTT)

| | | |
|---|---|---|
| Glucose solution: | Name: | Dextro®O.G-T. |
| | Manufacturer: | F. Hoffman - La Roche AG, Manheim, Germany |
| Saline solution: | Name: | NaCl – Lösung 0.9% |
| | Manufacturer: | Fresenius Kabi GmbH, Bad-Hamburg, Germany |
| | Use: | IV catheter rinsing agent |

### 2.4.2 Euglycemic Hyperinsulinemic Clamp

| | | |
|---|---|---|
| Glucose solution: | Name: | Glucosteril 20% |
| | Manufacturer: | Fresenius Kabi GmbH, Bad-Hamburg, Germany |
| Saline solution: | Name: | NaCl – Lösung 0.9% |

| | | |
|---|---|---|
| | Manufacturer: | Fresenius Kabi GmbH, Bad-Hamburg, Germany |
| | Use: | IV catheter rinsing agent |
| Insulin: | Name: | Insuman Rapid |
| | Manufacturer: | Aventis Pharma Germany GmbH, Frankfurt am Main, Germany |
| | Insulin Type: | Normal Insulin |

## 2.5 Analytical Determinations

### 2.5.1 Plasma Glucose

Glucose levels in plasma were determined direct after the blood drawing, using a bedside glucose analyzer.

| | |
|---|---|
| Name: | YSI 2300 plus |
| Manufacturer: | Yellow Springs Instruments, Yellow Springs, USA |
| Method: | Glucose-Oxidative Methods |

After glucose measurements, the blood samples were put in ice and centrifuged at 4 ℃ for 10 minutes. Then the supernatant was pi petted and frozen immediately and stored at -80℃ for further determi nations.

### 2.5.2 Plasma insulin and free fatty acids

Plasma insulin and free fatty acids were measured as follows:

| | | |
|---|---|---|
| Insulin: | Manufacturer: | Abott Laboratories, Tokio, Japan |

| | | |
|---|---|---|
| | Method: | Microparticle Enzyme Immunoassay |
| Free fatty acids: | Manufacturer: | Wako chemicals, Neuss,Germany |
| | Method: | Enzymatic method |

### 2.5.3 Genotyping

The FGFR4 gene is located on the long (q) arm of chromosome 5 between position 35.1 and the end (terminus) of the arm. More precisely, the FGFR4 gene is located from base pair 176,446,526 to base pair 176,457,732 on chromosome 5 (71).

For genotyping, DNA was isolated from whole blood using a commercial DNA isolation kit (NucleoSpin; Macherey & Nagel, Düren, Germany). The single nucleotide polymorphism (SNP, rs351855 C/T), a G to A conversion that results in the substitution of glycine by arginine at position 388 in the transmembrane domain of the FGFR4 receptor, was genotyped using TaqMan assay (Applied Biosystems, Foster City, CA, USA). The TaqMan genotyping reaction was amplified on a Gene - Amp polymerase chain reaction system 7000 (50 ℃ for 2 min, 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 15 min and 60 ℃ for 1 min) and the fluorescence was detected using an ABI Prism Sequence Detector System (Applied Biosystems). The overall genotyping success rate was 99,1% and rescreening of 3,1% of the subjects gave 100% identical results.

## 2.6 Calculations

### 2.6.1 Body Mass Index (BMI)

$$BMI = \text{Body weight} / [\text{Body height}]^2$$

where body weight in kilograms (kg) and body height in meters (m)

### 2.6.2 Insulin sensitivity from OGTT

To evaluate insulin sensitivity ($ISI_{OGTT}$) from the data obtained from the OGTT ($ISI_{OGTT}$) we used the index, proposed by Matsuda:

$$ISI_{OGTT} = \frac{10.000}{\sqrt{(FPG * FPI * \hat{G} * \hat{I})}}$$

where FPG: Fasting Plasma Glucose in mg/dl, FPI: Fasting Plasma Insulin in µU/ml, $\hat{G}$: mean plasma glucose concentration and $\hat{I}$: mean plasma insulin concentration during the OGTT. [$\hat{G}$ = mean (BG 0, BG 30, BG 60, BG 90, BG120) and $\hat{I}$ = mean ( Ins 0, Ins 30, Ins 60, Ins 90, Ins 120) ]. 10,000 simply represent a constant that allows one to obtain numbers ranging from 0-12. Square-root conversion was used to correct the nonlinear distribution of values.
This index represents a composite of both hepatic and peripheral tissue sensitivity to insulin and correlates strongly with the direct measure of insulin sensitivity derived from the euglycemic insulin clamp (80).

### 2.6.3 Homeostasis Model Assessment of Insulin Resistance (HOMA IR)

Homeostasis model assessment of insulin resistance (HOMA IR) was also used to estimate insulin sensitivity from OGTT, as proposed by Matthews et al. (84):

HOMA-IR = (FPI x FPG) / 22.5

where insulin in µU/ml and fasting plasma glucose in mmol/L.
The HOMA-IR index which relies on fasting plasma glucose and fasting plasma insulin concentrations, has been shown to provide a reasonable estimate mainly of hepatic insulin sensitivity.

### 2.6.4 Insulin sensitivity from euglycemic - hyperinsulinemic clamp

An insulin sensitivity index ($ISI_{clamp}$) for systemic glucose uptake was calculated as the mean infusion rate of glucose necessary to maintain euglycemia during the last 60 min of the euglycemic-hyperinsulinemic clamp divided by the steady-state plasma insulin concentration. Accordingly, for the determination of insulin sensitivity by euglycemic hyperinsulinemic clamp, the following index was used:

$ISI_{clamp}$= mean (steady-state) of glucose infusion rate / mean (steady-state) of plasma insulin concentrations

where $ISI_{clamp}$ in µmol · $kg^{-1}$ · $min^{-1}$ · $[pmol/l]^{-1}$, mean infusion rate of glucose in µmol · $kg^{-1}$ · $min^{-1}$ and mean rate of insulin infusion in mU · $kg^{-1}$ · $min^{-1}$

**2.7 Statistical analyses**

Whether the FGFR4 Gly388Arg SNP was in Hardy-Weinberg equilibrium and the relationship of the SNP with gender was tested by $\chi^2$-test. For statistical analysis continuous variables that were non-normally distributed (Shapiro-Wilk *W*-test) were logarithmically transformed. Multivariate linear regression models were used to determine relationships of the SNP with the parameters of interest, that is, primarily liver fat and insulin sensitivity and secondarily other metabolic characteristics, like intramyocellular- total- and visceral fat, plasma glucose and insulin and free fatty acids. In all models these parameters were included as dependent variables. Their association with the SNP at baseline (cross-sectional analysis), was tested by using multivariate linear regression models with gender, age, total body fat and the genotype included as independent variables. Changes in the traits during the intervention (longitudinal analyses) were included as dependent variables, whereas the traits at baseline, age, gender and the genotype represented the independent variables. Changes in metabolic characteristics were additionally adjusted for body fat at baseline and at follow-up. For the association of the SNP besides the additive (Gly388Gly vs Arg388Gly vs Arg388Arg), also a dominant model (Gly388Gly vs Arg388Gly and Arg388Arg) is presented to confirm the findings of the additive model and to show if an allele-dose effect is present.

Data are given as mean ± SE (standard error). A p-value < 0.05 was considered statistically significant. The statistical software package JMP 4.0 (SAS Institute Inc., Cary, NC, USA) was used.

# 3. Results

## 3.1 Cross-sectional associations of the Gly388Arg SNP in *FGFR4* at baseline

### 3.1.1 Subject characteristics at baseline

Demographics and metabolic characteristics of the participants at baseline are shown in table 1. Eighty three subjects (48.8%) were found to be homozygous for the 388Gly allele, 70 subjects (41.2%) to be heterozygous and 17 subjects (10%) to be homozygous for the 388Arg allele. Thus, the minor 388Arg allele had a frequency of 0.31 in our population and the SNP was in Hardy-Weinberg equilibrium ($\chi^2$ - test, p=0.92).

### 3.1.2 Univariate relationships at baseline

At baseline insulin sensitivity correlated negatively with total body fat, visceral fat, liver fat and intramyocellular fat. Liver fat was positively correlated with total body fat, visceral vat and intramyocellular fat and negatively with insulin sensitivity, as shown in table 2.

### 3.1.3 Effects of Gly388Arg polymorphism on demographics and body composition characteristics at baseline

Associations of the Gly388Arg SNP in FGFR4 with subjects' characteristics at baseline are presented in table 3. There was no significant difference in body weight, body and visceral fat, IMCL of tibialis anterior and liver fat, between the three genotypes. The SNP was also not associated with fasting and post-load blood glucose and insulin levels, as well as with insulin sensitivity (all p>0.13, after adjustment for age, gender and total body fat).

In a subgroup of 45 individuals (table 5) we have measured the whole body insulin sensitivity, using the euglycemic hyperinsulinemic clamp. However, the effect of FGFR4 SNP on baseline in insulin sensitivity during the clamp did not reach statistical significance.

**Table 1.** Demographics and metabolic characteristics of the subjects at baseline and after 9 months of follow-up

| | Baseline | Follow-up | p-value |
|---|---|---|---|
| **Demographics and body composition** | | | |
| Gender (males / females) | 68 / 102 | | |
| Age (years) | 46 ± 1 | 47 ± 1 | --- |
| Body Weight (kg) | 85.4 ± 1.2 | 82.9 ± 1.2 | <0.0001 |
| BMI (kg/cm²) | 28.9 ± 0.3 | 28.1 ± 0.3 | <0.0001 |
| Body fat (kg) | 25.2 ± 0.7 | 23.0 ± 0.7 | <0.0001 |
| Visceral fat (kg) | 3.0 ± 0.1 | 2.6 ± 0.1 | <0.0001 |
| $IMCL_{tibialis\ anterior}$ (arb. units)* | 4.0 ± 0.1 | 3.7 ± 0.1 | <0.0001 |
| Liver fat (%) | 5.1 ± 0.4 | 3.6 ± 0.3 | <0.0001 |
| **Metabolic characteristics** | | | |
| Fasting glucose (mM) | 5.23 ± 0.04 | 5.16 ± 0.04 | 0.01 |
| 2h glucose (mM) | 6.87 ± 0.12 | 6.62 ± 0.12 | 0.02 |
| Fasting insulin (pM) | 59 ± 3 | 51 ± 2 | 0.0002 |
| 2h insulin (pM) | 480 ± 30 | 420 ± 33 | 0.007 |
| Fasting FFA (µM) | 655 ± 17 | 611 ± 15 | 0.02 |
| 2h FFA (µM) | 84 ± 6 | 76 ± 10 | 0.002 |
| HOMA-IR index | 1.89 ± 0.09 | 1.60 ± 0.08 | <0.0001 |
| $Insulin\ sensitivity_{OGTT}$ (arb. units) | 13.3 ± 0.5 | 15.1 ± 0.6 | <0.0001 |

Values represent means ± SE (standard error); p for paired differences after log transformation of non-normally distributed parameters. *****available in 149 subjects. FFA, free fatty acids; HOMA-IR, homeostasis model assessment of insulin resistance.

**Table 2.** Univariate relationships at baseline

| | Insulin sensitivity | | Liver fat | |
|---|---|---|---|---|
| | **r** | **p** | **r** | **p** |
| **Total body fat** | -0.35 | <0.0001 | 0.30 | <0.0001 |
| **Visceral fat** | -0.41 | <0.0001 | 0.62 | <0.0001 |
| **IMCL** | -0.33 | <0.0001 | 0.16 | <0.0001 |
| **Insulin sensitivity$_{OGTT}$** | ---- | ---- | -0.49 | <0.0001 |
| **Liver fat** | -0.49 | <0.0001 | ---- | ---- |

### 3.2 Longitudinal analyses. Associations of the Gly388Arg SNP in *FGFR4* with changes during the intervention

#### 3.2.1 Changes in subject characteristics during the lifestyle intervention

The demographics and metabolic characteristics of the subjects at follow-up are presented in table 1. During the follow-up of nine months, there was a mean decrease in body weight by 3% and in total body fat by 9%. Intramyocellular fat decreased by 7%. Larger decreases were found for visceral fat (-13%) and particularly liver fat (-29%). Fasting as well as 2h glycemia and insulinemia also decreased significantly and insulin sensitivity increased (14%).

#### 3.2.2 Effects of the Gly388Arg SNP with changes in parameters during the lifestyle intervention

The relationships of the Gly388Arg SNP with changes in parameters during the lifestyle intervention are presented in table 4. The Gly388Arg SNP was not associated with the magnitude of changes in body weight, total body fat (table 4 and figure 1, panel A), visceral- (table 4 and figure 1, panel C) or intramyocellular fat (table 4). In contrast, the minor 388Arg allele was associated with less decrease in fasting and 2h insulinemia as well as 2h glycemia compared to homozygous carriers of the 388Gly allele. Of note, 2h glycemia and insulinemia actually increased in homozygous carriers of the 388Arg allele. Furthermore, subjects carrying the 388Arg allele had less

increase in insulin sensitivity (figure 1, panel D) and less decrease in liver fat content (figure 1, panel B) compared to individuals who were homozygous for the Gly allele.

In the subgroup of 45 individuals (table 5) which underwent measurements of whole body insulin sensitivity using the euglycemic hyperinsulinemic clamp the effect of FGFR4 SNP on change in insulin sensitivity during the clamp did not reach statistical significance. In contrast, change in insulin sensitivity estimated by the OGTT was still depended on the FGFR4 SNP, in this small group.

**Table 3.** Associations of the Gly388Arg SNP in FGFR4 with subject characteristics at baseline

| | Gly/Gly | Gly/Arg | Arg/Arg | p-value additive | p-value dominant |
|---|---|---|---|---|---|
| **Demographics** | | | | | |
| Gender (males/females) | 29/54 | 31/39 | 8/9 | 0.41[#] | 0.19[#] |
| Age (years) | 46±1 | 46±1 | 45±4 | 0.79 | 0.66 |
| **Body composition** | | | | | |
| Body weight (kg) | 85.8±1.7 | 83.3±1.8 | 89.6±4.3 | 0.22 | 0.24 |
| Body mass index (kg/m²) | 29.0±0.5 | 28.5±0.5 | 30.1±1.0 | 0.42 | 0.83 |
| Body fat (kg) | 25.9±1.1 | 23.7±1.1 | 28.2±2.2 | 0.14 | 0.68 |
| Visceral fat (kg) | 3.02±0.20 | 2.84±0.19 | 3.26±0.51 | 0.20 | 0.31 |
| IMCL tibialis anterior (arb. units)* | 3.91±0.20 | 4.09±0.20 | 3.90±0.53 | 0.29 | 0.36 |
| Liver fat (%) | 5.11±0.67 | 5.33±0.65 | 4.49±0.99 | 0.49 | 0.69 |
| **Metabolic characteristics** | | | | | |
| Fasting glucose (mM) | 5.24±0.05 | 5.25±0.06 | 5.11±0.14 | 0.39 | 0.83 |
| 2 h glucose (mM) | 6.95±0.18 | 6.93±0.18 | 6.16±0.26 | 0.13 | 0.71 |
| Fasting insulin (pM) | 55±4 | 62±5 | 67±8 | 0.24 | 0.10 |
| 2 h insulin (pM) | 465±41 | 506±47 | 442±105 | 0.31 | 0.35 |
| Fasting FFA (µM) | 674±22 | 618±27 | 708±68 | 0.37 | 0.35 |
| 2 h FFA (µM) | 92±11 | 73±5 | 92±17 | 0.51 | 0.30 |
| HOMA-IR index | 1.78±0.13 | 1.97±0.15 | 2.10±0.28 | 0.28 | 0.13 |
| Insulin sensitivity $_{OGTT}$ (arb. Units) | 14.08±0.78 | 12.52±0.81 | 12.48±1.82 | 0.24 | 0.13 |

Data represent unadjusted mean ± SE. For statistical analyses, non-normally distributed parameters were log transformed. IMCL, intramyocellular lipids, [#] $\chi^2$-test *available in 149 subjects (Gly/Gly n=74, Gly/Arg n=60, Arg/Arg n=15). The genotype effect was tested using an additive and a dominant model. Body weight, body mass index and total body fat were adjusted for age and gender. The other parameters were additionally adjusted for total body fat. FFA, free fatty acids; HOMA-IR, homeostasis model assessment of insulin resistance.

**Table 4.** Associations of the Gly388Arg SNP in FGFR4 with changes in subject characteristics during the lifestyle intervention

| | Gly/Gly | Gly/Arg | Arg/Arg | p -value additive | p -value dominant |
|---|---|---|---|---|---|
| **Demographics** | | | | | |
| Gender (males/females) | 29/54 | 31/39 | 8/9 | | |
| Age (years) | 46±1 | 46±1 | 45±4 | 0.79 | 0.66 |
| **Body composition** | | | | | |
| Body weight (kg) | -2.65±0.45 | -2.14±0.43 | -3.36±1.10 | 0.83 | 0.94 |
| Body mass index (kg/m²) | -0.90±0.16 | -0.72±0.14 | -1.12±0.37 | 0.82 | 0.95 |
| Body fat (kg) | -2.20±0.40 | -2.04±0.39 | -3.29±1.07 | 0.65 | 0.35 |
| Visceral fat (kg) | -0.41±0.06 | -0.39±0.07 | -0.54±0.15 | 0.56 | 0.95 |
| IMCL tibialis anterior (arb. units)* | -0.15±0.23 | -0.33±0.19 | -0.58±0.37 | 0.48 | 0.30 |
| Liver fat (%) | -2.05±0.46 | -1.22±0.34 | -0.39±0.70 | 0.02 | 0.007 |
| **Metabolic characteristics** | | | | | |
| Fasting glucose (mM) | -0.06±0.04 | -0.10±0.04 | -0.05±0.10 | 0.54 | 0.92 |
| 2 h glucose (mM) | -0.51±0.18 | -0.05±0.18 | 0.23±0.31 | 0.02 | 0.006 |
| Fasting insulin (pM) | -9±2 | -7±4 | -8±7 | 0.04 | 0.01 |
| 2 h insulin (pM) | -122±28 | -20±43 | 82±67 | 0.03 | 0.06 |
| Fasting FFA (µM) | -61±25 | -33±30 | -33±68 | 0.14 | 0.56 |
| 2 h FFA (µM) | -26±11 | -11±4 | -25±16 | 0.90 | 0.61 |
| HOMA-IR index | -0.32±0.09 | -0.27±0.14 | -0.22±0.26 | 0.049 | 0.017 |
| Insulin sensitivity OGTT (arb. units) | 2.67±0.82 | 1.40±0.65 | -0.10±1.36 | 0.009 | 0.003 |

Data represent unadjusted mean ± SE; changes (follow-up – baseline). For statistical analyses, non-normally distributed parameters were log transformed. IMCL, intramyocellular lipids, *available in 149 subjects (Gly/Gly n=74, Gly/Arg n=60, Arg/Arg n=15). The genotype effect during the intervention was tested using an additive and dominant model. For longitudinal analyses, fold-changes in the parameters (follow-up over baseline) were adjusted for baseline parameters. Body weight, BMI and body fat were adjusted for age and gender. The other parameters were additionally adjusted for body fat at baseline and at follow-up. FFA, free fatty acids. HOMA-IR, homeostasis model assessment of insulin resistance.

**Table 5.** Association of the Gly388Arg SNP in FGFR4 with changes in subject characteristics during the lifestyle intervention in the subgroup of 45 subjects which underwent measurements using the euglycemic hyperinsulinemic clamp

| | Gly/Gly | | Gly/Arg | | Arg/Arg | | p baseline | | p for changes between genotypes | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Demographics** | Baseline | Follow-up | Baseline | Follow-up | Baseline | Follow-up | additive | dominant | additive | dominant |
| Gender (males/females) | 12/11 | | 8/8 | | 5/1 | | | | | |
| Age (years) | 44.6±2.0 | 45.3±2.0 | 49.4±3.4 | 50.3±3.5 | 41.3±7.4 | 42.3±7.4 | 0.44 | 0.77 | 0.04 | 0.03 |
| **Body composition** | | | | | | | | | | |
| Body weight (kg) | 84.1±3.6 | 82.1±3.3 | 84.1±3.4 | 82.3±3.5 | 83.5±6.6 | 80.1±6.4 | 0.53 | 0.64 | 0.56 | 0.61 |
| Body mass index (kg/m²) | 28.0±1.0 | 27.3±1.0 | 28.4±1.0 | 27.9±1.2 | 29.5±1.3 | 28.2±1.3 | 0.37 | 0.40 | 0.52 | 0.63 |
| Body fat (kg) | 23.3±1.9 | 21.7±1.8 | 22.8±2.3 | 21.5±2.4 | 28.1±3.1 | 25.4±2.1 | 0.35 | 0.69 | 0.90 | 0.95 |
| Visceral fat (kg) | 3.00±0.42 | 2.57±0.37 | 3.21±0.43 | 2.80±0.41 | 2.29±0.43 | 1.71±0.30 | 0.42 | 0.20 | 0.84 | 0.56 |
| IMCL $_{\text{tibialis anterior}}$ (arb. units) | 4.08±0.45 | 3.81±0.34 | 3.39±0.26 | 3.34±0.38 | 4.32±0.81 | 3.95±0.82 | 0.98 | 0.91 | 0.90 | 0.67 |
| Liver fat (%) | 4.52±0.96 | 3.12±0.79 | 5.86±1.65 | 4.44±1.22 | 4.01±1.27 | 4.08±1.05 | 0.94 | 0.72 | 0.29 | 0.30 |
| **Metabolic characteristics** | | | | | | | | | | |
| Fasting glucose (mM) | 5.17±0.08 | 5.06±0.11 | 5.11±0.10 | 5.02±0.09 | 5.05±0.28 | 5.36±0.23 | 0.72 | 0.43 | 0.02 | 0.25 |
| 2 h glucose (mM) | 7.04±0.37 | 6.05±0.42 | 7.31±0.42 | 7.01±0.39 | 5.98±0.44 | 6.93±0.64 | 0.28 | 0.87 | 0.03 | 0.02 |
| Fasting insulin (pM) | 53.13±6.13 | 41.96±5.22 | 50.81±5.98 | 42.19±3.32 | 57.83±7.30 | 64.35±9.66 | 0.79 | 0.82 | 0.19 | 0.13 |
| 2 h insulin (pM) | 473±77 | 330±75 | 492±77 | 388±65 | 403±130 | 642±228 | 0.71 | 0.62 | 0.02 | 0.02 |
| Fasting FFA (µM) | 624±42 | 632±44 | 620±66 | 649±67 | 636±148 | 645±53 | 0.28 | 0.28 | 0.96 | 0.77 |
| 2 h FFA (µM) | 105±31 | 79±11 | 86±11 | 72±7 | 93±19 | 76±14 | 0.68 | 0.63 | 0.88 | 0.95 |
| HOMA-IR index | 1.66±0.20 | 1.27±0.15 | 1.59±0.20 | 1.28±0.10 | 1.75±0.23 | 2.11±0.37 | 0.81 | 0.93 | 0.12 | 0.12 |
| Insulin sensitivity $_{\text{OGTT}}$ (arb. units) | 14.34±1.60 | 19.31±2.08 | 14.17±2.01 | 14.33±1.68 | 11.38±1.65 | 10.62±2.79 | 0.86 | 0.77 | 0.04 | 0.02 |
| $ISI_{clamp}$ ($\mu mol \cdot kg^{-1} \cdot min^{-1} \cdot pM^{-1}$) | 0.074±0.009 | 0.090±0.013 | 0.062±0.006 | 0.063±0.007 | 0.060±0.009 | 0.067±0.010 | 0.64 | 0.34 | 0.36 | 0.18 |

Data represent unadjusted mean ± SE; changes (follow-up – baseline). For statistical analyses, non-normally distributed parameters were log transformed. IMCL, intramyocellular lipids. The genotype effect during the intervention was tested using an additive and dominant model. For longitudinal analyses, fold-changes in the parameters (follow-up over baseline) were adjusted for baseline parameters. Body weight, BMI and body fat were adjusted for age and gender. The other parameters were additionally adjusted for body fat at baseline and at follow-up. FFA, free fatty acids. HOMA-IR, homeostasis model assessment of insulin resistance. $ISI_{clamp}$, insulin sensitivity estimated by euglycemic hyperinsulinemic clamp

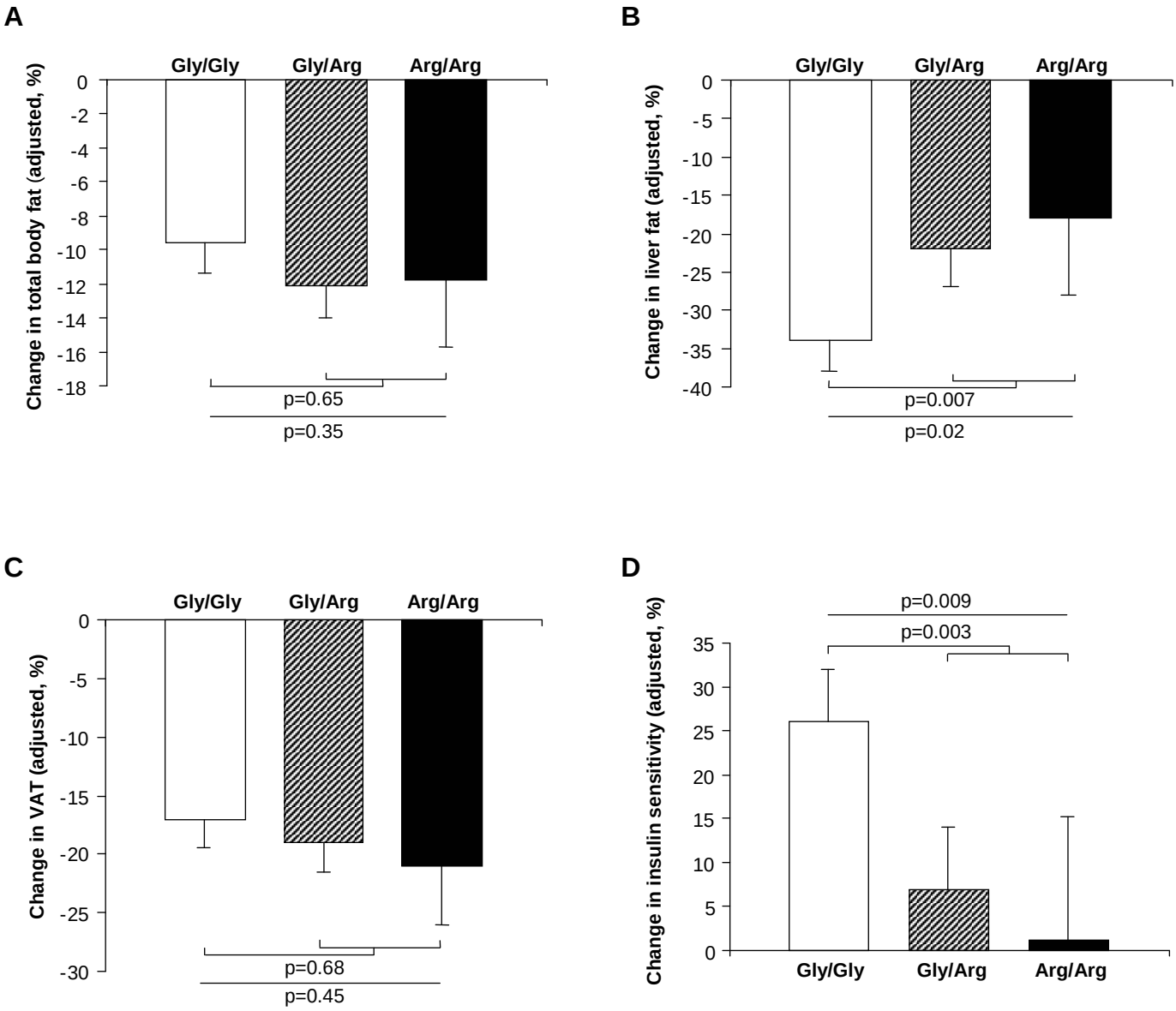


**Figure 1**. Percent of change (follow-up over baseline) in total body fat (A), liver fat (B), visceral adipose tissue (VAT) (C) and insulin sensitivity ($ISI_{OGTT}$) (D) during 9 month of lifestyle intervention in relation to the genotype of the Gly388Arg polymorphism of *FGFR4*. Change in total body fat was adjusted for total body fat at baseline, age and gender. Changes in the other parameters were adjusted for the respective parameters at baseline, age, gender, and body fat at baseline and at follow-up. Statistical significance is depicted using an additive as well as a dominant model for the 388Arg encoding allele.

# 4. Discussion

## 4.1 Genetic risk factors for both T2DM and cancer

Epidemiological studies suggest that parameters of the metabolic syndrome, such as obesity and insulin resistance, as well as type 2 diabetes mellitus (T2DM) are associated with the incidence of cancer (85,86). For instance there is some evidence that insulin resistance and resulting hyperinsulinemia promote the development and progression of colorectal cancer (86). T2DM was also found to be associated with a 10-20% excess in the relative risk of breast cancer. Conversely, up to 16% of patients with breast cancer have also diabetes (87). Total and especially visceral adiposity are thought to promote breast cancer via regulation of sex hormones and adiponectin (85,88). However, there is a large variability in the risk of breast cancer, even after controlling for total and visceral obesity (85). These findings suggest that a common genetic background for cancer and diabetes mellitus may exist. This is supported by recent studies pointing out to genetic variants that are closely related both with cancer and T2DM. A microsatellite, DG10S478 within intron 3 of the transcription factor 7-like 2 gene (TCF7L2) was reported to be associated with type 2 diabetes in a large Icelandic, Danish and European-American cohort, compared to non-carriers, heterozygous and homozygous carriers of the at-risk allele had a relative risk of 1.45 and 2.41, respectively to develop T2DM (89). These carriers are also at risk for familiar breast and colon cancer (90,91). The precise mechanism is not known yet, but it seems that TCF7L2 encodes transcription factor 4 which has a key role in the Wnt signaling pathway implicated in the pathogenesis of colorectal cancer (92-94). In addition, variants in the transcription factor 2 gene encoding for hepatocyte nuclear factor 1 beta, which is known to be mutated in individuals with maturity-onset diabetes of the young type 5 (95) were shown in genome-wide association studies to be associated with prostate cancer (96,97) and, in observational studies, also with other cancers (98).

### 4.2 The *FGFR4* Gly388Arg SNP as a potential candidate for the common background of cancer and T2DM

Recent data suggest that the single nucleotide polymorphism (SNP) at codon 388 in the gene coding for FGFR4 which represent a substitution of G for A and results in a change from glycine to arginine may represent another candidate for the common background of cancer and type 2 diabetes. Bange et al. discovered that the gene variant Gly388Arg in *FGFR4*, results in a higher *FGFR4* gene expression (70) and was found in several types of human cancers, including colon, liver, breast, pancreas, and neuroendocrine tumors. Furthermore, this SNP was found to be present at significantly higher frequency in cancer patients with aggressive disease and therefore represents a gene alteration that predisposes to a poor clinical outcome (70). In particular, Gly388Arg SNP was shown to be associated with a poor prognosis for positive node breast cancer, high-grade soft-tissue sarcoma, colon carcinoma, and head and neck squamous cell carcinoma (70,75,94). Additionally, emerging evidence supports a role of FGFR4 and its ligands in metabolism. Activation of FGFR4 by its major ligand, FGF15/19, transcriptionally downregulates CYP7A1 expression, thereby suppressing hepatic bile synthesis. Bile acid signaling is increasingly recognized as an important regulator of hepatic lipid metabolism and storage via regulation of fatty acid oxidation (57). Furthermore, a recent study in transgenic mice showed a critical role for FGFR4 in the maintenance of lipid and glucose metabolism, and particularly in the regulation of hepatic fat accumulation and of insulin sensitivity (99). Since both, liver fat and insulin sensitivity are major determinants of T2DM (100), if FGFR4 plays a similar to animal role also in humans and the Gly388Arg in *FGFR4* is associated with an altered expression and/or activity of FGFR4, then this SNP would be another good candidate for the common background of cancer and T2DM.

### 4.3 The Gly388Arg SNP affect glucose tolerance and insulin sensitivity, but not via regulation of total or visceral adiposity

In the present study, carriers of the 388Arg allele have significantly attenuated improvements in glucose tolerance and insulin sensitivity during a 9-month

lifestyle intervention compared to individuals with the Gly/Gly genotype. There was a clear allele-dose effect, with subjects heterozygous for the 388Arg allele displaying minimal improvement in glucose tolerance and insulin sensitivity and homozygous for the 388Arg allele displaying actually deterioration in glucose tolerance and insulin sensitivity. Total adipose tissue and especially visceral adipose tissue are important regulators of insulin sensitivity (101-104), but the SNP effect on glucose tolerance and insulin sensitivity seems unlikely to be mediated through effects on adiposity, because the magnitude of the reduction in both total- and visceral fat was similar among the three genotypes.

### 4.4 The Gly388Arg SNP regulates glucose tolerance and insulin sensitivity in humans, possibly via regulation of liver fat

The second possibility is that the SNP affects insulin sensitivity by regulating liver fat, which has been repeatedly reported to be associated with insulin resistance (33,100,105). Indeed, carriers of the 388Arg allele had less improvement in liver fat compared to individuals homozygous for the 388Gly allele. Also in this case an allele dose effect was apparent, with individuals homozygous for the 388Arg allele experiencing the least benefit in liver fat reduction. Since there was *a priori* hypothesis that the SNP is associated with liver fat and insulin sensitivity, these findings are true positive. The absence of a significant effect of the genotype in the cross-sectional analysis in our population may be the result of less variability, due to a bottom/ceiling effect. With the larger variability in the response to the intervention, the genotype effect may have become apparent.

### 4.5 The Gly388Arg SNP does not affect glucose tolerance and insulin sensitivity via regulation of IMCL

Apart from or in addition to liver fat, the effect of the SNP on insulin sensitivity could also have been mediated through regulation of fat accumulation in the skeletal muscle. As already discussed, intramyocellular lipids were shown to be related to whole body insulin resistance. FGFR4 is expressed in skeletal muscle (99,106) and is functional at least during myogenesis (107). Furthermore,

skeletal muscle in FGFR4$^{-/-}$ deficient mice exhibited elevated levels of lipids compared to wild-type mice (99). In the present study, however, the Gly388Arg SNP was not associated with intramyocellular fat at baseline nor was it with the change in intramyocellular during the lifestyle intervention. In addition, the clamp data support an effect of the SNP in the regulation specifically of the hepatic, rather than whole-body insulin sensitivity. In the subgroup of 45 individuals who had also measurements of whole-body insulin sensitivity using the euglycemic hyperinsulinemic clamp the effect of the FGFR4 SNP on the change in whole body insulin sensitivity did not reach statistical significance ($p=0.36$, table 5). In contrast, change in insulin sensitivity estimated by the OGTT still depended on the FGFR4 SNP in this small group ($p=0.04$, table 5). Whole-body insulin sensitivity measured by the clamp is a function of both, insulin stimulated glucose disposal (largely uptake by muscle) and insulin sensitivity to suppress endogenous glucose production from the liver. In contrast, estimates of insulin sensitivity obtained from fasting insulinemia, which is an important component in the estimation of insulin sensitivity by the OGTT (84), largely represents insulin sensitivity of the liver (108). Therefore, the closer relationship of the FGFR4 SNP with the aforementioned parameters may reflect the stronger effects of this SNP on the regulation of hepatic insulin sensitivity, than on insulin sensitivity of glucose disposal. Thus, the principal effect of the SNP on insulin sensitivity is probably not muscle-mediated, although a minor effect on glucose metabolism in muscle as proposed in animals (99), cannot be excluded.

Taking together, the consistent findings regarding liver fat and insulin sensitivity in our population, supports the hypothesis that the Gly388Arg SNP is involved in the pathophysiology of liver fat accumulation, and, thereby, insulin resistance in humans. Together with the recent findings that fatty liver is strongly associated with insulin resistance and the metabolic syndrome (109-111) and predicts type 2 diabetes (112), the data further support the causative role of hepatic fat accumulation in the pathogenesis of diabetes.

### 4.6 Molecular mechanisms that connect FGFR4 activity to hepatic lipogenesis and fatty acid oxidation

Most recently, a study with FGFR4 deficient mice confirmed the critical role of FGFR4 in mediating the effects of FGF19. In line with the findings in mice overexpressing or treated with FGF19 (106,113), compared to wild-type mice, FGFR4 deficient mice displayed increased mass of white adipose tissue, higher levels of triglycerides and cholesterol, as well as impaired glucose tolerance and insulin resistance. In these animals the expression of hepatic lipogenic transcription factors, such as PPAR-γ, and enzymes, particularly SCD-1 (stearoyl-Coenzyme A desaturase 1) was upregulated. However, and unexpectedly, FGFR4 deficient mice were somewhat protected from high fat diet - induced hepatic steatosis (99), because of an increased fatty acid oxidation and hepatic triglyceride secretion. In this case FGFR4 deficiency had the same effect as FGF19 overexpression/treatment. So far, the underlying mechanisms responsible for apparent controversy, as well as for the resulting dissociation of insulin resistance and fatty liver remain unclear. Because activated FGFR4 represses the rate-limiting enzyme of bile acid synthesis CYP7A1 (60,61,73,114), bile acids, are increased in serum of FGFR4 deficient mice (61,73). As already discussed, bile acids were proposed to reduce liver fat through several mechanisms (115). These data not only suggest that increased bile acid availability may partially compensate for FGFR4 deficiency in the liver, but also provide a general mechanism how genetic variability in FGFR4 may modulate liver fat content.

### 4.7 Conclusion

In this thesis was assessed that the *FGFR4* Gly388Arg SNP is associated with insulin resistance, probably by regulating liver fat content. A complete knowledge of FGFR4 signaling in the liver and how the *FGFR4* Gly388Arg SNP influences these pathways will be essential to design liver-specific agonists or antagonists of the receptor with the aim to alleviate fatty liver and improve insulin sensitivity. Finally, together with the previously discovered role of the *FGFR4* Gly388Arg SNP in the progression of breast- and colon cancer as well

as melanoma and sarcoma, our date indicate that the *FGFR4* Gly388Arg SNP may represent a new candidate for a common background of cancer, glucose- and lipid metabolism.

## 5. Abstract

The fibroblast growth factor receptor 4 (FGFR4), a monomeric receptor protein tyrosine kinase, regulates angiogenic, mitogenic and differentiation responses in cells, as well as insulin sensitivity and fat accumulation in the liver of mice. A functional polymorphism in *FGFR4* (Gly388Arg) was found to be associated with disease progression in cancer patients. In the present study it was investigated whether this polymorphism determines glucose tolerance, insulin resistance and hepatic steatosis in subjects at high risk for type 2 diabetes.

A total of 170 individuals participated in a lifestyle intervention program with diet modification and increase in physical activity. Total body fat and visceral fat were determined by magnetic resonance (MR) Tomography and liver fat and intramyocellular fat by $^{1}$H-MR spectroscopy. Insulin sensitivity was estimated from the oral glucose tolerance test.

At baseline the polymorphism was not associated with glucose tolerance, insulin sensitivity or liver fat (all p≥0,13). During 9 month of intervention, subjects carrying the minor 388 Arg encoding allele (n=87) displayed a mean increase in 2h glycemia (+3 %), less increase in insulin sensitivity (+21%) and less decrease in liver fat (-13%) compared to homozygous carriers of the 388 Gly allele (n=83; -5%, p=0,006; +34%, p=0,003; and -21%, p=0,007, respectively). In contrast, changes in total body fat, visceral fat and intramyocellular fat were not different between the genotypes (all p≥ 0, 30).

These data provide evidence that the functional Gly388Arg polymorphism in *FGFR4* is associated with the improvement of glucose tolerance and insulin sensitivity during a lifestyle intervention, possibly via regulation of liver fat.

## 6. Abbreviations

| | |
|---|---|
| A | Adenine |
| AMP | adenosine monophosphate |
| Arg | Arginine |
| AT | adipose tissue |
| Bp | base pair |
| BG | blood glucose |
| BMI | body mass index |
| CAP | c-Cbl-assosiated protein |
| Cbl | Casitas B lineage lymphoma |
| CYP7A1 | cytochrome P450 7a-hydroxylase |
| DNA | deoxyribonucleic acid |
| EMCL | extramyocellular lipid |
| FFA | free fatty acids |
| FGF | fibroblast growth factor |
| FGFR | fibroblast growth factor receptor |
| FPG | fasting Plasma Glucose |
| FPI | fasting Plasma |
| FXR | farnesoid X receptor |
| G | Guanine |
| $\hat{G}$ | mean plasma glucose concentration |
| Gly | Glycine |
| HCC | hepatocelular carcinoma |
| HCL | hepatocellular lipid |
| $^{1}$H-MRS | proton magnetic resonance spectroscopy |
| HOMA IR | homeostasis model assessment of insulin resistance |
| $\bar{I}$ | mean plasma insulin concentration |
| IL-6 | interleukin-6 |
| IMCL | intramyocellular lipid content |
| IRS | insulin receptor substrate |
| $ISI_{clamp}$ | insulin sensitivity index (euglycemic-hyperinsulinemic) clamp |

| | |
|---|---|
| $ISI_{OGTT}$ | insulin sensitivity estimated from OGTT |
| JNK | c-Jun $NH_2$-terminal kinases |
| LE | lower extremities |
| MAP | mitogen activated protein |
| mBAR | membrane-type bile acid receptor |
| MCP-1 | monocyte chemoattractant protein-1 |
| MR | magnetic resonance |
| MRS | magnetic resonance spectroscopy |
| NAFLD | non alcoholic fatty liver disease |
| NASH | nonalcoholic steatohepatitis |
| NEFAs | non-esterified-fatty acids |
| OGTT | oral glucose tolerance test |
| PGC-1a | peroxisome-proliferator-activated receptor γ co-activator 1a |
| PGK | phosphoglycerokinase |
| PI3K | phosphatidylinositol-3-kinase |
| PPAR-α | peroxisome proliferator activated receptor-α |
| PPAR-γ | peroxiisome proliferator activated receptor-γ |
| RBP-4 | retinol-binding-protein-4 |
| SCAT | abdominal subcutaneous tissue |
| SCD-1 | Stetoroyl-coenzyme A-desaturase-1 |
| SE | standard error |
| SNP | single nucleotide polymorphism |
| SREBP-1c | sterol-regulatory-binding protein 1c |
| STEAM | stimulated echo acquisition mode |
| TAT | total adipose tissue |
| TCF7L2 | transcription factor 7-like 2 gene |
| TE | echo Time |
| TGR5 | G-protein-coupled receptor |
| TNF-α | tumor necrosis factor-α , |
| TR | repetition Time |
| TT | total tissue volume |

| | |
|---|---|
| TULIP | Tübingen Lifestyle Intervention Program |
| T2DM | type 2 diabetes mellitus |
| UE | upper extremities |
| VAT | visceral adipose tissue |
| VLDL | very low density lipoprotein |
| WHO | world health organization |
| Wnt | Wnt was coined as a combination of Wg (wingless) and Int |

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