The endocrine Fibroblast Growth Factor family:

Possible insulin-sensitizing therapeutics

Summary:

This thesis focuses on the endocrine members of the Fibroblast Growth Factor (FGF) family. The 23 FGFs, polypeptides that perform different actions in an organism, can be divided into seven subfamilies, functioning intracrine, paracrine or endocrine. The endocrine family consists of FGF19, the human ortholog of FGF15, FGF21 and FGF23. To function in the body, endocrine FGFs signal through FGF receptors (FGFR) with the use of co-receptor Klotho. Tissue specific expression of FGFR and Klotho determines in which tissue endocrine FGF functions. FGF23 regulates phosphate and calcium plasma levels. Via active vitamin D (AVD) FGF23 lowers insulin sensitivity. FGF15/19 and FGF21 are regulated by food intake. FGF15/19 expression is regulated by bile acid (BA) uptake in the ileum after feeding. FGF15/19 inhibits bile acid synthesis, increases energy expenditure and improves metabolic parameters. FGF21 increases in a lower energy state. FGF21 also increases energy expenditure, improves metabolic parameters and improves insulin-sensitivity. FGF23 has a huge impact on phosphate en calcium serum levels which makes it unsuitable as insulin sensitizer. Besides improving metabolic parameters, FGF15/19 regulates BA synthesis and induces liver tumors. FGF21 improves metabolic parameters, increases insulin sensitivity, and most likely does this without big negative side effects. Furthermore, FGF21 is suggested to be safe for healthy organisms and in total shows to be the most promising target as future therapeutic for diabetes mellitus type 2 and obesity.

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Abbreviations:

ACC: Acetyl CoA Carboxylase, **ADHR**: autosomal dominant hypophosphataemic rickets, **AKT**: v-akt murine thymoma viral oncogene, **ATGL**: adipose triglyceride lipase, **AVD**: Active Vitamin D (1,25$(OH)_2$ vitamin D_3), **BA**: Bile acid, **BaF**: murine bone barrow derived pro-B-cell line, **BAT**: Brown Adipose Tissue, **BBMW**: Brush border membrane vesicles, **BMI**: Body Mass Index, **CCK**: CholeCystoKinin, **CDCA**: chenodeoxycholic acid **CNS**: Central Nervous System, **CPT1a**: carnitine palmitoyl transferase 1a, **CREB**: cAMP response element-binding protein, **Cyp24**: 24-OHase, **Cyp27b1**: 1α-OHase, **DAG**: DiAcylGlycerol, **DGAT1**: diglyceride acyltransferase-1, **DIO**: Diet Induced Obesity, **DMT2**: diabetes mellitus type 2, **ECM**: Extra Cellular Matrix, **Egr1**: ETS related gene 1, **FAS**: fatty acid synthase, **FGF**: Fibroblast Growth Factor, **FGFR**: Fibroblast Growth Factor Receptor, **FHF**: Fibroblast growth factor Homologous Factors, **FoxA2**: forkhead transcription factor A2, **FRS2a**: Fibroblast Growth Factor Receptor Substrate 2a, **FTC**: familial tumoral calcinosis, **FXR**: Farnesoid X Receptor, **G6Pase**: Glucose 6-phosphatase, **GAB1**: GRB2-Associated Binding protein 1, **GH**: Growth Hormone, **Glut1**: Glucose transporter 1, **GPAT**: glycerol-3-phosphate acyltransferase, **GRB2**: Growth Factor Receptor-Bound protein 2, **H4IIE**: Rat hepatoma cells, **HB**: Heparin Binding Site, **HBS**: Heparin Binding Site, **HCC**: HepatoCellular Carcinoma, **HEK**: Human embryonic kidney, **HMGCS2**: hydroxymethylglutaryl-CoA synthase 2, **hMSC**: human Mesenchymal Stem Cells, **HNF4α**: hepatocyte nuclear factor 4α, **HSL**: high sensitive lipase, **HSPG**: Heparin Sulfate ProteoGlycans, **Ig**: Immunoglobulin-like domain, **IGF-1**: Insuline-Like Growth Factor 1, **IP3**: Inositol 1,4,5-triPhosphate, **IR-1**: Inverted Repeat-1, **KD**: Ketogenic Diet, **KO**: Knock out, **MAPK**: Mitogen-Activated Protein Kinase, **NaPi**: sodium-phosphate cotransporter, **PEPCK**: PhosphoEnolPyruvate CarboxyKinase, **PGC-1α**: peroxisome proliferator-activated receptor γ coactivator protein-1α, **PI3K**: PhosphoInositide-3 Kinase, **PIP2**: PhosphatIdylinositol 4,5-biPhosphate, **PKC**: Protein Kinase C, **PLCγ**: phospholipase Cγ, **PPAR**: peroxisome proliferator-activated receptor, **PTH**: Parathyroid hormone, **RAS**: Rat Sarcoma, **ROS17/2.8**: rat osteosarcoma cells, **RXRα**: Retinoid X Receptor α, **SCD1**: stearoyl-CoA desaturase 1, **SOS**: Son Of Sevenless, **SP**: Signaling Peptide, **SP***: uncleaved bipartite secreted signal sequence, **STAT5**: Signal Transducer and Activator of Transcription 5, **TK**: Tyrosine Kinase, **TPTX**: thyroparathyroidectomy, **UCP1**: uncoupled protein 1, **VDR**: Vitamin D receptor, **WAT**: White Adipose Tissue, **WT**: Wild Type.

Introduction

Fibroblast Growth Factors (FGF) are polypeptides which perform different actions in organisms. FGF has first been isolated in the 1970s, from the bovine brain and pituitary gland. Acidic FGF and basic FGF, now referred to as FGF1 and FGF2 respectively, are the first reported FGF. The FGF were functioning as growth factors for fibroblasts.[1-3] Nowadays it is known that the mammalian FGF family compromises 23 members. Humans express 22 of these FGF members.

The FGF family can be divided into seven subfamilies.[2] By phylogenetic analysis, shown in figure 1A, and the analysis of gene loci, shown in figure 1B, the evolutionary relationships of the FGF gene family has been studied. Phylogenetic analysis looks at the "age" of the genes in the evolution. Based on differences between organisms which developed at different time points and from different species, you can extract information to link genes together into a subfamily since they are more related towards each other.[2] A gene loci analysis bases its findings on the location of the genes on a chromosome. The conservedness of gene locations on the chromosome can tell something about the overall relation between genes. And this helps to formulate a subfamily.[2] The seven subfamilies differ slightly between the phylogenetic and gene loci analysis. The phylogenetic analysis shows the following subfamilies: FGF1/2, FGF4/5/6, FGF3/7/10/22, FGF9/16/20, FGF8/17/18, FGF11/12/13/14 and FGF15/19/21/23 (figure 1A). Two subfamilies would be different in de gene loci analysis: FGF1/2/5 and FGF3/4/6 (figure 1B).[1, 2]

All the members within one FGF subfamily act intracrine, paracrine or endocrine. The intracrine FGF subfamily consists of FGF11/12/13/14.[2] The intracrine subfamily is not secreted, and has an intracrine, also called autocrine, function. Figure 1B shows that the intracrine FGF family members lack a signal sequence to be secreted, which is present in the paracrine and endocrine subfamilies.[2] The intracrine FGFs have been shown to regulate voltage-gated sodium channels.[4] Not much is reported about the intracrine FGF family. Knock out (KO) mice models for fgf12 and fgf14 have been reported.[4] Both KO mice are viable, but have several neurological deficiencies, indicating a neurological role for at least part of the intracrine FGFs.[2, 4]

5 of the 7 FGF subfamilies are known for having a paracrine function. Figure 1B shows the main differences in domains of the subfamilies. All these subfamilies have a signal peptide (SP) and a heparin binding-site (HB). The functions of the paracrine FGFs are diverse. In 2009 Beenken *et al.* made an overview of the function of the paracrine FGFs.[5] The functions of the paracrine FGF mostly point out towards a role in the development. Paracrine FGF has been reported to function in the development of the inner ear, limbs, brain, eyes, gonads, heart, bone, neural tissue and more.[5]

The last FGF subfamily functions in an endocrine manner. This difference in function can be explained by the differences in domain structures which are shown in figure 1B. Paracrine and endocrine FGF are secreted out of the cell, while intracrine FGFs remain inside of the cell. Paracrine and endocrine FGFs posses a signal peptide (SP) on the N-terminus, something which intracrine FGFs lack.[2] This indicates that the SP domain in the FGF families regulates secretion. One paracrine subfamily differs from the others for having an additional SP region in the conserved core. It has been shown that the SP region in the conserved core is responsible for the secretion of these FGFs (Figure 1B).[2] The difference between the paracrine and endocrine FGFs is that endocrine FGFs can function in the whole body, while paracrine FGFs function locally. This difference is explained by the HB domain. Paracrine FGFs posses the HB domain and have a high affinity for heparin sulfate proteoglycans

(HSPG).[2] HSPG are present in the extra cellular matrix (ECM). The HB domain of paracrine FGFs mediates binding to HSPG. This traps the paracrine FGFs in the ECM outside of the cells from which they are secreted. Paracrine FGFs are therefore unable to diffuse and thus work in an endocrine fashion. Endocrine FGF do not possess the HB domain. The affinity for HSPG is low, which enables endocrine FGF to diffuse and reach the circulation.[2]

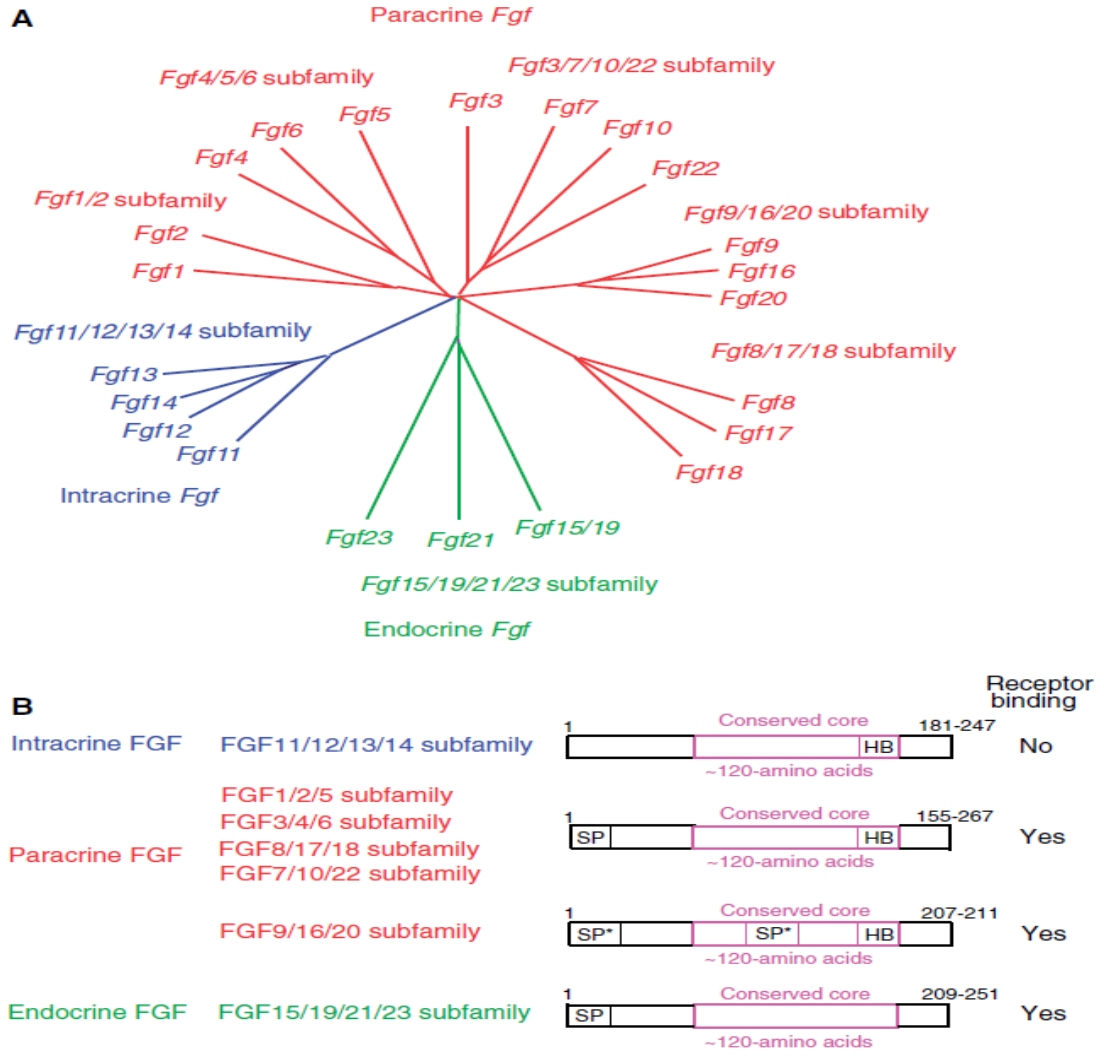

Figure 1 **Phylogenetic and gene loci analysis (A)** Phylogenetic analysis of the FGF family shows 7 different subfamilies. 5 of the 7 subfamilies are known for having a paracrine function (red). One subfamily is functioning intracrine (blue) and one family functions endocrine (green). **(B)** Gene loci analysis also shows 7 subfamilies of FGF, which are slightly different. The domain and domain functions of the FGF are explained in the text. HB= Heparin binding site. SP = signal peptide. SP*= uncleaved bipartite secreted sequence domain. Figure used from Itoh *el al.*[2]

This thesis focuses on the endocrine subfamily. First the receptors which are used by the endocrine FGFs will be discussed. Then the separate endocrine FGFs will be discussed in which production, action and mechanism of actions will be discussed. The purpose of this thesis is to get more insight in the way endocrine FGFs work in an organism. Ultimate goal is to use the endocrine FGFs as future therapeutics. This thesis is indented to give insights in these possibilities.

Tissue specific FGFR and Klotho mediate endocrine FGFs signaling

Endocrine and paracrine FGFs activate Fibroblast Growth Factor Receptors (FGFRs). The intracrine FGFs are unable to activate FGFRs, and are therefore sometimes also considered to be excluded from the FGF family.[5, 6] To activate the FGFRs, both the paracrine and endocrine FGFs use a co-receptor. This co-receptor differs between the paracrine and the endocrine FGFs.[7] Paracrine FGFs possess a HB domain (figure 1B). This HB domain enables FGF to bind to HSPG in the ECM.[2] These HSPG are needed to bind to FGF, so when FGF binds to FGFR it can exert its function.[8-10] Insoluble HSPG in the ECM prevent the paracrine FGF from diffusing. This leads to only local levels of FGFs, in the absence of soluble glycosaminoglycans, thus a paracine effect.[7] Endocrine FGFs have low affinity for HSPGs and use Klotho proteins to activate FGFRs.

FGFR is a ligand-dependent FGF receptor tyrosine kinase molecule.[11] The first FGFR was discovered in chicken, using an affinity column. Once the first FGFR was identified, PCR techniques soon revealed three more FGFRs, which led to the 4 FGFRs which are known to date (FGFR1-4).[10] Figure 2 gives an overview of FGFR's structure.[9] FGFRs have three immunoglobin-like domains (IgI-IgIII), which are all outside of the cell membrane. IgI is proposed to have an auto-inhibition function, of which the role in FGFR functioning is not clear yet.[10] IgII contains a HB domain. The HB domain of FGFs can bind to the HBS of IgII for more efficient FGF-FGFR binding.[10] Since the endocrine FGFs have lost their HBS, they need to bind Klotho proteins to form a FGF-FGFR-Klotho complex which can activate the FGFRs.[8-10] IgIII can be encoded in 2 forms by alternative splicing.[10] IgIII is the part of the FGFR which contains the FGF binding-domain. FGFR1-3 have alternative exons, which means that there are 3 extra forms of FGFRs. The FGFR1-3s are called FGFR1b, 1c, 2b, 2c, 3b and 3c.[10] For FGFR4 alternative splicing has not been described. The alternative splicing in FGFR1-3 is reported to be tissue specific. Mesenchymal tissue expresses the c- isoform, where epithelial tissue expresses the b-isoform.[10]

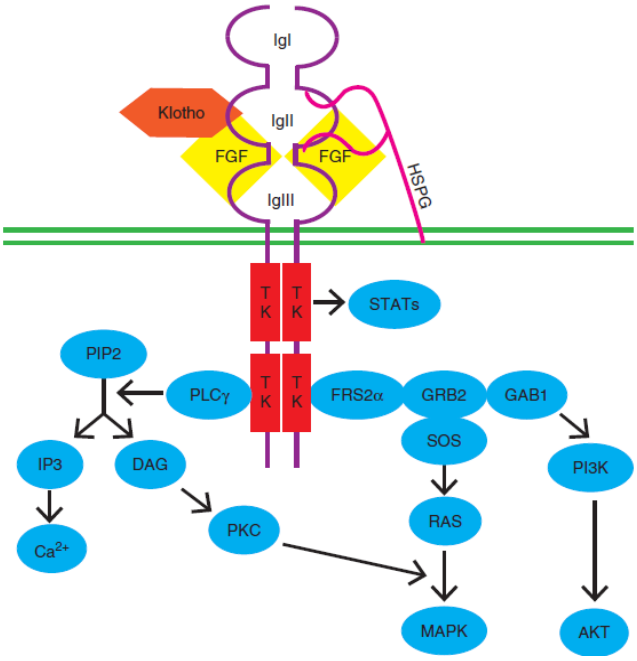

Figure 2 **Overview of FGFR structure and signaling** The FGFR consists of 3 immunoglobin-like domains (IgI-IgIII) and tyrosine kinase domains (TK). IgI might have an auto inhibition fuction.[10] IgII contains a heparin binding site which is involved in efficient FGF-FGFR binding.[10] IgIII contains the FGF binding site.[10] FGFR is activated by FGF and a co-receptor (Klotho for endocrine FGF and heparin sulfate proteoglycans (HSPG) for paracrine FGF). Once the FGFR is activated, the TK are cross-phosphorylated. Docking proteins can bind phosphorylated TK, and the signaling cascade starts. 4 main pathways are found in FGFR signaling, where activation of each of them relies on FGFR type and cell type. The main pathways are PLCγ, STATs, AKT, and MAPK. Abbreviations can be found on page 2. Image taken from Tenhagen *et al.* [9]

Endrocrine FGFs are unable to bind HSPG, which also impairs activation of FGFRs. FGFs need a co-receptor in order to activate their FGFRs. Instead of HSPG, endocrine FGFs bind Klotho, a protein which is expressed as isoforms αKlotho and βKlotho.[8] Figure 2 shows the activation of FGFR both for the endocrine and the paracrine FGFs.[9] Without stabilization by Klotho or HSPG, FGFs cannot activate

the FGFRs. Once the FGFR is activated by FGF and the co-receptor, FGFR dimerizes with another FGFR subunit and the tyrosine kinase domains (TK) cross-phosphorylate. Docking proteins can bind the phosphorylated TK and activate signal pathways.[9] In general, FGFRs are reported to function through 4 main pathways: phospholipase Cγ (PLCγ), signal transducer and activators of transcription (STATs), v-akt murine thymoma viral oncogene (AKT) and mitogen-activated protein kinase (MAPK).[9] The 4 pathways are not always activated. Although FGFRs signal via similar pathways, activation of downstream effectors appears to differ. The activation may depend on crosstalk between pathways in a cell, which makes the signaling cell specific.[9]

It has been reported that FGFR and Klotho are not expressed everywhere in the body.[12] Tissue specific expression of FGFR and Klotho can suggest where the functions of endocrine FGFs are exerted. With the real-time quantitative PCR technique, mRNA levels for FGFRs and Klothos were measured in 39 different mice tissues of the C57/BL6 and 129x1/SvJ mice.[12] Table 1 shows the most important outcome of this study for this thesis, where expression (+) or no expression (-) is indicated.

FGFR/ Klotho	CNS	Liver	Kidney	BAT	WAT	Muscle	Bone
FGFR1c	+	-	+	+	+	+	+
FGFR3c	+	-	+	-	-	-	-
FGFR4	-	+	+	-	-	-	-
αKlotho	+	-	+	-	-	-	-
βKlotho	-	+	-	+	+	-	-

Table 1 **FGFR and Klotho expression is tissue specific** Data from Fon Tacer *et al.* [12] The table show the important outcome of this a real-time quantitative PCR study, performed by Fon Tacer *et al.*, for our thesis. The table shows expression (+) and no expression (+) of a (co-)receptor. CNS = Central Nervous System. BAT= Brown Adipose Tissue. WAT= White Adipose Tissue.

Endocrine FGFs cannot activate all the different FGFRs or use both Klotho proteins. Figure 3 is a scheme which can be drawn from literature which will be discussed in more detail further on in this thesis. Figure 3 shows us that FGF15/19 and FGF21 are βKlotho dependent and that FGF23 is αKlotho dependent.[13-16] In literature, FGF15/19 is reported to exert its functions in adipose tissue and the liver. FGF15/19 acts through FGFR1c and FGFR4 with the help of co-receptor βKlotho.[14] In line with table 1, the effects seen in the liver are suggested to be regulated via FGFR4/βKlotho, where FGFR1c/βKlotho regulates FGF15/19s effect in adipose tissue.[12, 14] FGF21 is reported to act through FGFR1c/βKlotho, FGFR3c/βKlotho and a pathway independent of βKlotho is reported.[15-17] Although FGF21 is capable to form a complex with FGFR4/βKlotho, no activation of the FGFR is observed.[14] Like FGF15/19, FGF21 exerts functions in adipose tissue through FGFR1c/βKlotho and also through a rather unknown βKlotho independent pathway.[16, 17] In which tissue FGF21s property to activate the FGFR3c/βKlotho complex is exerted is unknown. Table 1 also does not suggest a target tissue for FGFR3c/βKlotho complexes.[12] FGF21 and FGF15/19 both exert metabolic functions in the liver. How this is regulated by FGF21 is still rather unknown, but will be discussed. FGF23 signals mainly through the FGFR1/αKlotho complex, which extremely pronounced in the kidney.[12, 16] FGFR3/αKlotho and FGFR4/αKlotho are suggested to play a role in FGF23 signaling, but it is reported that this is not

exerted in the kidney.[18] Table 1 suggests that the FGFR3c/αKlotho complex is present in the central nervous system (CNS), but this is unknown.[12]

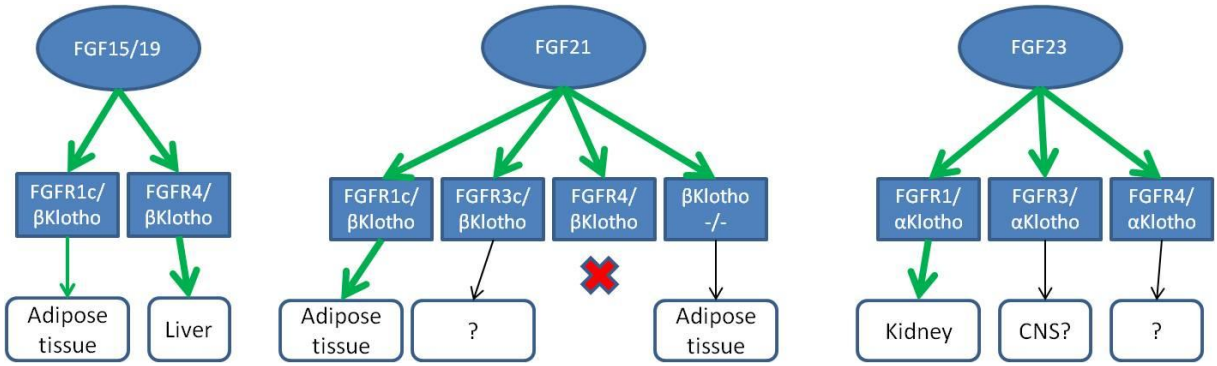

Figure 3 **Endocrine FGF signaling is FGFR/Klotho specific** The endocrine FGF do exert their functions through all FGFRs and Klothos. FGF15/19 exerts its function through FGF15/19 FGFR1c/βKlotho in adipocytes and FGFR4/βKlotho in the liver. FGF21 exerts functions through FGFR1c/βKlotho and an unknown βKlotho independent pathway in adipose tissue. FGF21 can bind FGFR4/βKlotho complexes, but does not exert a function there. Where the ability of FGF21 to activate FGFR3c/βKlotho occurs in an organism is unknown. FGF23 dominantly exerts its function through the FGFR1/αKlotho complex in the kidney. FGFR3/αKlotho and FGFR4/αKlotho are also reported to be activated by FGF23. Expression of FGFR3 and αKlotho could indicate a role of FGF23 in the central nervous system (CNS).

The endocrine FGFs will now be discussed separately.

BAs induced FGF15/19 regulates metabolic system

FGF15/19 is an endocrine FGF which has metabolic functions. FGF15 and FGF19 are taken together in the literature. FGF19 is present in humans and FGF15 is an ortholog of FGF19 present in mice. Although FGF15 and FGF19 only share 51% amino acid identity, conservations of regions within the genes (synteny) do show that they are orthologs.[19] Furthermore, the activation site of FGF19 and FGF15, an inverted repeat with a single nucleotide spacer, is also conserved in both genes.[19]

In primary human hepatocytes Farnesoid X Receptor (FXR), activated by BA (or an agonist) regulates FGF15/19 transcription. The aim was to identify changes in gene expressions following FXR activation, using a synthetic FXR agonist; GW4064. FGF19 appeared to be the most induced gene after GW4064 administration.[20] Together with a natural FXR agonist, chenodeoxycholic acid (CDCA, a BA), they tested for the response on various agonist concentrations. In absence of these agonists, FGF19 mRNA is undetectable.[20] The effect of the FXR agonist is dose dependent, which is much stronger in synthetic agonist GW4064, a potent selective agonist. The use of a protein synthesis inhibitor, cycloheximide, prior to FXR agonist treatment did not influence the FGF19 mRNA levels after GW4064 administration, which indicates a direct effect.[20] To determine whether the FGF19 gene has a FXR promoter, they isolating fragments of the FGF19 promoter and inserted it into a luciferase vector. A human liver-derived cell line was transfected with the chimeric FGF19-luciferase reporter gene construct. GW4064 administration could not induce luciferase activity.[20] After trying the full FGF19 gene (fragmentated) they ultimately discovered that the luciferase expression which was observed could be explained by a perfect inverted repeat-1 (IR-1) motif in the second intron of the FGF19 gene. Since mutations induced in this IR-1 motif abolished the luciferase activity, the FGF19 gene contains a functional FXR responsive element within the second intron.[20] They also showed that a FXR - Retinoid X Receptor α (RXRα) heterodimer was responsible for this binding, by

using radiolabeled probes and competition binding analyses. Radiolabels probes bind to the FXR-RXRα complex when IR1 mutated FGF19 is around, where normal IR1 FGF19 shows no binding of probes to the FXR-RXRα complex.[20] Also in fgf15, it is shown that the IR1 motif in the fgf15 gene is bound by a FXR-RXR heterodimer. This time, they observed FGF15 expression *in vivo.* After administrating GW4064 or cholic acid, no FGF15 was detected in the liver.[19] The ileum, jejunum and duodenum did express FGF15 mRNA.[19] Using in situ hybridization analysis, FGF15 mRNA expression was shown in the enterocytes of the villus epithelium.[19] This means that BAs can activate FXR, which will promote FGF15/19 transcription in the enterocytes.

FGF19 has shown to be functional in mice. The use of transgenic FGF19 mice can give information about the function of FGF15/19. In these transgenic FGF19 mice, the human FGF19 gene is incorporated in the genome of the mice. Here human FGF19 cDNA was incorporated behind the myosin light chain promoter. Transgenic FGF19 express the FGF19 mainly in the muscle, where the myosin light chain promoter is active.[21] The myosin light chain promoter is more active than the FGF15/19 promoter, causing over expression of FGF19 in the transgenic mice.[21] Transgenic FGF19 mice on a normal (chow) diet have a lower weight, caused by a lower fat content. Increased energy expenditure is observed in the FGF19 transgenic mice and increased brown adipose tissue (BAT) mass.[21] The fat lowering activity of FGF19 was further investigated using a high fat diet. Here also, the fat pads of mice are smaller and this also results in lower leptin levels, a hormone produced by adipose tissue.[21] Glucose tolerance tests have shown that glucose levels of transgenic FGF19 mice are significantly lower upon high fat diet as compared to wild type (WT) mice.[21] Compared to controls, transgenic FGF19 mice insulin levels are significantly lower, just like glucose levels after an insulin suppression test.[21] This also makes FGF19 able to prevent obesity and diabetes mellitus type 2 (DMT2) in mice which lack leptin, thus no inhibition signal for feeding, or do not express BAT, which normally leads to lower energy expenditure and increased weight.[22] Not only transgenic mice show this effect, also intra venous injection leads to the same effect in mice.[22] The increased metabolic rate in transgenic FGF19 mice shows an increase in lipolysis. Both in mice on a high fat diet or in leptin resistant mice, recombinant FGF19 caused a rapid decrease in Acetyl CoA Carboxylase 2 (ACC2). ACC2 is an enzyme which is the rate-limit in entrance of fatty acid into the mitochondria. The down-regulation of ACC2 means that fatty acids can enter the mitochondrion faster which is thought to increase the lipolysis.[22]

Instead of increasing FGF15/19 levels, FGF15/19 levels can also be abolished in a FGF15 KO mouse. Most of these mouse die shortly after birth, due to a morphological dysfunction in the cardiac tissues.[19] This shows that FGF(15) is also involved in the development of an organism. FGF15/19 has not been shown to have a cardiac function in adults. The FGF15 KO mice that were viable have increased fecal bile acid excretion. Connected with this, the mice have increased mRNA and protein levels of CYP7A1, an enzyme involved in bile acid synthesis.[19] Gallbladders of FGF15 KO mice also show a different phenotype compared to WT mice. The gallbladders hardly contained BAs.[23] Administration of FGF15 or FGF19 could restore the volume of the gallbladder. Even in WT mice, this led to a 2-fold increase in volume.[23] Responsible for gallbladder emptying is the hormone cholecystokinin (CCK). Administration of both CCK and an excess of FGF15 leads to a blockage in gallbladder contraction.[23] BA, which stimulates FGF15 production, at the same time inhibits CCK production in the duodenum. This is why after feeding, when BAs are released from the gallbladder, the FGF15/CCK ratio increases and the gallbladder refills.[23] FGF15 KO mice also show to be glucose intolerant and have reduced levels of hepatic glycogen.[24] It is shown that FGF19 administration

increased glycogen levels, by increasing glycogen synthase activity. But also other hepatic proteins, like albumin, production is increased by FGF19 administration.[24] FGF19 does not alter lipid synthesis, unlike insulin. This is explained by the fact that FGF19 does not use the Akt-pathway (at least in the liver).[24] The Akt-pathway, which insulin does use, regulates SREBP-1c, important regulator in lipgenesis.[24] It is proposed that the use of an alternative pathway may also be the reason why FGF19 lowers glucose- and insulin levels in diabetic rodents, something which insulin cannot achieve.[24] Analysis of several proteins on phosphorylation/activation by FGF19 in mice liver proposes the Ras-ERK-p90RSK pathway to be responsible for the up-regulation of glycogen and protein synthesis. This pathway does not regulate Akt or SREBP-1c, and thus no lipogenesis.[24] Besides increasing the glycogen synthesis, FGF19 also inhibits the formation of new glucose by inhibiting gluconeogenesis. Mice who are fasted overnight, to induce gluconeogenesis, got administered FGF19. 6 hours later, the mice were sacrificed and the expressions of important regulators of gluconeogenesis (peroxisome proliferator-activated receptor γ coactivator protein-1α (PGC-1α), Phosphoenolpyruvate carboxykinase (PEPCK) and Glucose 6-phosphatase (G6Pase)) are measured. Indeed, these regulators of gluconeogenesis are downregulated in the liver.[25] This makes sense, since FGF15/19 is normally produced upon BA activation. And BA is available when there is food intake, which usually means glucose. cAMP response element-binding protein (CREB) is an inducer of PGC-1α. FGF19 reduced the activity CREB in fasting mice, which could be measured by phosphorylation levels of CREB.[25] This effect of FGF19 was not observed in a FGFR4 KO mouse. This indicates that FGF19 is inhibiting gluconeogenesis players through inhibition of CREB via FGFR4 signaling in the liver.[25]

FGF15/19 also exerts a function in adipocytes. In 3T3-L1 adipocytes (mouse cell line), FGF19 increases glucose uptake upon administration. When βKlotho, the co-receptor required for FGF19, was knocked down in the 3T3-L1 cells, glucose uptake was diminished.[14] In FGF21 it has been shown that glucose transporter 1 (Glut1) was responsible for this glucose uptake, but that is not reported yet for FGF15/19. [14, 26]

Although there are therapeutic implications seen for FGF19 regarding DMT2 and obesity, FGF19 is involved in the formation of Hepatocellular Carcinoma (HCC, liver tumors).[27] In a *in vivo* mice study, 53% of the transgenic FGF19 mice between the ages of 10-12 months showed to have HCC.[27] Quantification of hepatocyte proliferation was performed using BrdU, which is incorporated in the DNA upon proliferation. Antibodies against BrdU, eventually with peroxidase, visualized the proliferation. Injection of recombinant FGF19 in non-transgenic mice led to a 3- to 5-fold increase in proliferation already after 6 days.[27] Analyzed HCCs showed nuclear β-catenin staining in 4 of the 9 tumors (44%).[27] β-catenin is downstream of the Wnt-pathway, a pathway which is associated with several tumors, with colon cancer as the most pronounced one. The fact that FGF19 antibodies and FGFR4 KOs abolish the effect seen in colon cancer cells tells us that FGF19, through FGFR4, is involved in inducing β-catenin production. Since only 44% of the tumor cells in the *in vivo* experiment show β-catenin expression, it seems that there are also other mechanisms through which FGF19 can cause tumor formation. Interestingly, tumor formation in transgenic FGF19 mice is only seen in the liver, not even in the producing skeletal muscles, where these transgenic mice expressed FGF19.[27, 28]

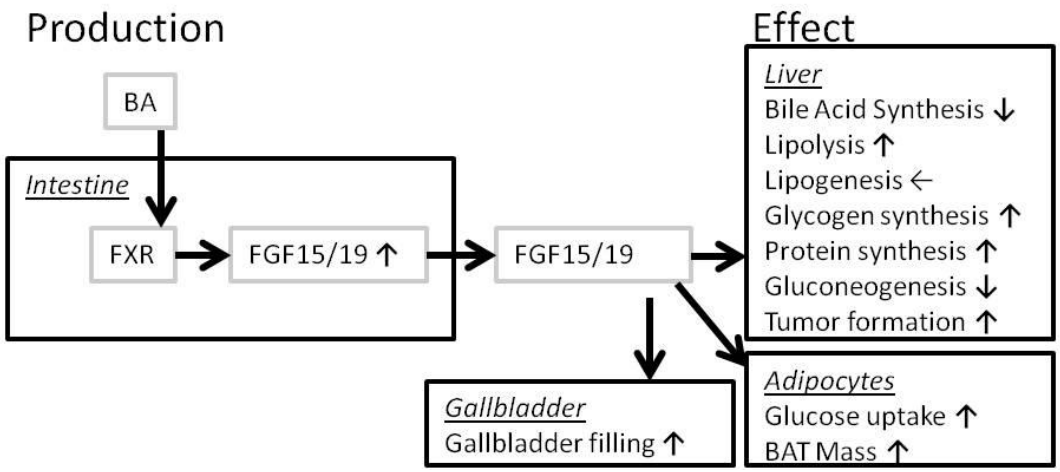

Figure 4 **Production and actions of FGF15/19** Bile Acids (BAs), which are released in the intestine after feeding, binds FXR in the intestine. FXR facilitates the transcription of FGF15/19, mainly occurring in enterocytes. FGF15/19 is secreted in the circulation where it has multiple effects in the liver. FGF15/19 decreases BAs synthesis. FGF15/19 does not change the production of lipids in the liver (lipogenesis), but it does increase the breakdown of lipids (lipolysis). FGF15/19 up-regulates protein synthesis, glycogen synthesis and inhibits the gluconeogenesis. FGF15/19 also causes the formation of tumor growth in the liver, which becomes clearly visible in transgenic FGF15/19 mice. In adipocytes FGF15/19 is involved in the increased glucose uptake, and transgenic FGF19 mice show increased brown adipose tissue (BAT) mass. Finally, FGF15/19 increases the volume of the gallbladder.

Figure 4 shows the scheme which can be drawn out of the literature that is just discussed.

FGF15/19 exerts functions through FGFR4/βKlotho and FGFR1c/βKlotho

FGF15/19 is a secreted hormone which exerts its functions through the FGFR family and βKlotho. A βKlotho KO mouse, of which the start codon was removed using a gene-targeting vector, is viable and, besides from small significant lower weight, appears mostly normal under normal conditions.[13] βKlotho is not expressed everywhere in WT (table 1). Tissues which express βKlotho are related to lipid metabolism, but βKlotho KO mice do not have statistical differences in lipid metabolism levels.[13] Cholesterol levels were slightly reduced and the liver, organ which is processor of cholesterol, normally highly expresses βKlotho.[12, 13] In the liver the BAs excretion was highly increased in βKlotho KO mice.[13] The enzyme which is involved in processing cholesterols into BAs, CYP7A1, was also highly up-regulated in livers of βKlotho KO mice.[13] FGF15 decreases CYP7A1 expression and therefore decrease BA synthesis. FGF15-expressing adenoviruses in WT mice led to a 5-fold decrease of CYP7A1.[19] Binding studies using recombinant FGF19 indicates FGF19 binds to FGFR4.[29] Beside binding studies also a fgfr4 KO mouse model indicates the role of FGFR4. In the fgfr4 KO mouse model, gallbladders are smaller with less BA content. Both mRNA and protein levels of CYP7a1 in fgfr4 KO mice were elevated in the same manner, which was independent of diet.[30] To confirm whether FGF15/19 would decrease levels of CYP7A1 through FGFR4, fgfr4 KO mice were injected with FGF15-expressing adenoviruses. This would not lead to a repression of CYP7A1. The levels of FGF15 mRNA between WT and fgfr4 KO mice were comparable, which points out that FGF15 repression of CYP7A1 requires FGFR4.[19] Figure 5 shows the discussed mechanism behind the inhibition of CYP7A1.[31] That FGFR4 and βKlotho are dominantly expressed in the liver indicates that FGF15/19 acts mostly on the liver.[14] fgfr4 KO mice indicate whether FGFR4 is involved in FGF15s effect on the gallbladder. The gallbladder volumes of fgfr4 KO mice are reduced, but not to the extent of fgf15 KO mice. Furthermore, administration of FGF15 to fgfr4 KO mice increases gallbladder volume, meaning FGFR4 is not responsible for FGF15/19s action in the gallbladder.[23]

Other experiments show molecular and cellular evidence that indicates FGF19 require βKlotho to stably bind to FGFR. FGF19, just like FGF21, only activate a signaling cascade in HEK293 (human embryonic kidney) cells when βKlotho is expressed, and this is dose-dependent.[14] Also in 3T3-L1 adipocytes FGF19 increases activation, measured by phosphorylation of FRS2α, and this is attenuated by knocking down βKlotho with siRNA.[14] Interestingly, the 3T3-L1 adipocytes predominantly express FGFR1. They also demonstrate that βKlotho binds to FGFR1c and FGFR4 strongly, indicating that FGF19 probably also can activate cells through FGFR1.[14]

FGF19 will bind βKlotho as a co-receptor, and form a complex with either FGFR4 or FGFR1c to exert its function. FGF19 had a powerful activation of FGFR4 when in complex with βKlotho, while FGF19 was gave a weaker response with the FGFR1c-βKlotho complex.[14] Figure 3 shows a brief overview of FGF15/19 and its receptors.

FGF15/19 as a possible therapeutic for DMT2 and obesity in humans

FGF19 in humans is not directly known to be involved in a genetic disorder. There are reports of malfunctioning in the human body where FGF19 is involved. As reported, FGF19 regulates the BAs synthesis. Chronic diarrhea can be caused by excessive BA synthesis.[31] In humans there are two mechanisms reported on why the BA synthesis is increased, shown in figure 5.[31] Increased BA synthesis results from a reduced action of FGF19. FGF19 production is induced by BAs. When BA cannot enter the FGF19 producing cells, FGF19 is not produced. Mostly this is caused by malabsorption BA, but also removal of a part of the ileum, for instance in Crohns Disease, may remove FGF19 producing cells, and therefore lowering FGF19 levels.[31] A second possibility is that FGF19 is not secreted from the cell. Without FGF19 out in the circulation, BA synthesis also is not impaired. The mechanism behind this impaired FGF19 release however is unknown.[31] Besides BA synthesis, FGF19 has many functions in the metabolism processes in an organism. FGF19 is reported to be protective against obesity and DMT2. One study pointed out FGF19 to be a marker for the metabolic syndrome.[32] In their study already 1 risk component for the metabolic syndrome correlates with a significant decrease of FGF19 levels. The FGF19 levels were corrected for FGF19 associated parameters, like Body Mass Index (BMI), triacylglycerol levels and glucose levels.[32] BMI and FGF19 levels were not related to each other, while BMI normally is a marker for obesity.[32]

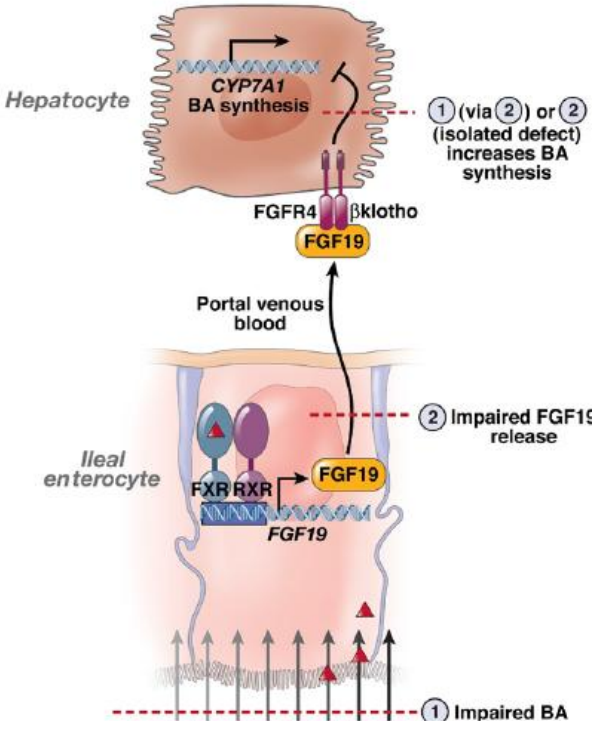

Figure 5 **Overproduction of BAs in humans** BAs are absorbed by enterocytes, and act as an agonist for FXR. The FXR-RXR complex mediates transcription of FGF19. Secreted FGF19 binds the FGFR4/βKlotho complex which eventually inhibits CYP7A1 expression, thus BA synthesis. In humans, excessive BA synthesis can usually be caused in 2 ways. First (1) is the impaired BA absorption from the colon. As BA is an inducer of FXR, and thus FGF19 production, impaired BA absorption lowers FGF19 levels. Second (2), via unknown reasons FGF19 is not released from the enterocytes. Via both ways, FGF19 cannot activate FGFR4/βKlotho. CYP7A1 is not down-regulated, keeping BA synthesis highly active. Figure from Hofmann *et al.* [31]

The effects of FGF19 which are described in literature suggest that FGF19 could be beneficial for DMT2 and obesity patients. But, the great pitfall of FGF15/19 is that it has also been reported to induce HCC.[27] Without this side-effect, FGF5/19 is promising as future therapeutic. And research is going on to abolish the tumor stimulating role of FGF15/19. One study used a variant of FGF19, which they called FGF19-7, to learn more about the mechanisms behind the positive and negative effects of FGF19. FGF21, which will be discussed next, also holds promises to become a potential therapeutic for DMT2 and obesity. FGF19-7 is a protein which is based on FGF19, but has two substitutions at the 23-42 and 50-57 amino acid residues which are corresponding sequences from FGF21.[33] *In vitro* FGF19-7 lost its ability to signal through FGFR4, and is shown to be biased to signal through FGFR1c.[33] Using FGF19-7 in a study using diet induced obese (DIO) and *ob/ob* mice, both mimicking a obese and DMT2 state, concludes that the positive effect of FGF19 is still present in FGF19-7 treated mice.[33] *Ob/ob* mice are leptin deficient, removing the internal inhibition on food intake. The effect of FGF19-7 and FGF19 on body weight, oral glucose tolerance test, and triglyceride and insulin levels are at least equal after 1 year treatment.[33] But in the liver, FGF19-7 is now hardly able to signal on hepatocytes. Also, a test with BrdU labeling showed that FGF19-7 lost its ability to induce HCC, and had the same or even lower levels in HCC formation compared to the control group.[33] No HCC were visible in the FGF19-7 group, while the FGF19 group did show HCC.[33]

FGF19 is reported to be a hormone which exerts several functions in the metabolic system. While FGF19 mostly exerts its functions on the liver, as FGF19-7 it is still able to exert most of the functions when it is hardly able signal in the liver, since FGF19-7 lost its FGFR4 binding capacity. FGF19-7 is biased towards FGFR1c, which is dominantly expressed in adipocytes. It seems that the metabolic effects on the liver are regulated indirectly, and that FGFR4 signaling was responsible for the formation of HCC. With this knowledge, further research is needed to help us understand the exact mechanisms by which FGF19 exerts its functions. FGF19-7 holds promise to make FGF19 a possible therapeutic against DMT2 and obesity.

Fasting induced FGF21 increases insulin sensitivity

FGF21 is an endocrine FGF exerting metabolic functions. FGF21 mRNA is dominantly in the liver.[34] FGF21 is present during a lower-energy state. During a ketogenic diet (KD), high in fat and low in carbohydrate, of 30 days in mice, FGF21 expression is up-regulated in the liver. This is also seen after fasting for 24 hours. The effect vanishes after re-feeding.[35] The up-regulation of FGF21 in the KD mice was observed using a microarray analysis. The microarray analyzed hepatic gene expression changes. This microarray detected peroxisome proliferator-activated receptor α (PPARα) activated transcripts were up-regulated.[35] Experiments were performed to conclude whether FGF21 up-regulation was directly induced by PPARα. Using C57/BL6 mice primary hepatocyes, a PPARα agonist (fenofibrate) was administered and indeed induced FGF21 expression. Also, pparα KO mice which were put on a KD or fasting after having a normal diet, had no FGF21 expression in fasting mice and a big reduction in KD mice compared to WT mice.[35] Not only PPARα induces FGF21. A KD leads to several fatty acid components, therefore primary hepatocytes were also administered with these major fatty acid components. After 24h exposure, Oleic acid, a predominant fatty acid in KD, significantly induced FGF21 mRNA in these primary hepatocytes.[35] The production of FGF21 is regulated by PPARα in the liver. Besides PPARα also PPARγ is associated with FGF21, not in the liver but in adipocytes. In a study on PPARγ in white 3T3-L1 (pre)adipocytes, FGF21 transcription was targeted by PPARγ. Administration of PPARγ agonist Troglitazone rapidly increases FGF21 mRNA levels. After adding a protein synthesis inhibitor (cycloheximide) transcription of FGF21 was not blocked, indicating direct

activation of PPARγ.[36] Glucagon, important hormone in glucose homeostasis, also increases FGF21 levels. Glucagon has similarities with FGF21, since they are both fasting hormones, naturally present in a lower energy status. Administration of a glucagon receptor agonist in mice increased circulating FGF21 levels.[37] Glucagon in obese healthy humans also significantly increases the FGF21 circulating levels.[37] In glucagon receptor KO mice, the glucagon agonist does not increase FGF21 circulating levels.[37] Weight lowering and lipid metabolism effects of glucagon diminish in fgf21 KO mice, indicating glucagon induces FGF21 expression to exert part of, what was thought, glucagon functions.[37]

The knockdown of FGF21 by adenoviruses causes a big change on circulating metabolites in KD-fed mice. These mice show a fatty liver, lipemia, which is increased lipid serum levels, and reduced serum ketones, pointing out to a role of FGF21 in lipolysis, triglyceride clearance and ketogenesis.[35] Besides knock down of FGF21, also transgenic FGF21 mice are reported. Transgenic FGF21 mice contain the human FGF21 gene after the apoe promoter, so over-expression of human FGF21 occurs in the liver. These mice are viable and have similar glucose levels compared to WT mice.[26] After 9 months, there are differences with their control littermates. FGF21 transgenic mice weight less, had lower glucose fasting levels, less fat in the liver, more BAT, smaller size subcutaneous adipocytes and had higher insulin sensitivity.[26] Doubling the calorie intake of FGF21 transgenic mice would not increase weight, indicating resistance to diet-induced obesity.[26] Also, FGF21 transgenic mice have increased keton bodies circulating during the fed state, again indicating the role of FGF21 in ketogenesis. But also in pparα KO mice, where FGF21 expression is low, administration of FGF21 increases keton bodies in both fed and fasted states.[38] In WT mice, FGF21 administration significantly increases keton bodies only during a fed state. Protein levels of important regulators of ketogenesis, carnitine palmitoyl transferase 1a (CPT1a) and hydroxymethylglutaryl-CoA synthase 2 (HMGCS2), are higher in the liver after FGF21 administration. The mRNA levels of these regulators are not up-regulated, suggesting a posttranscriptional mechanism.[38] Administration of FGF21 in DIO or *ob/ob* mice lowered the glucose levels, fat levels and weight, without a lower calorie intake. This indicates FGF21 to increase energy expenditure.[39] Analysis of transcriptional changes in DIO mice by FGF21 suggests mechanisms by which FGF21 achieves this anti-diabetic and anti-obesity effect. The lowering of fat levels is accounted towards both the decrease in lipogenesis and increase of lipolysis in the liver. Key enzymes/proteins for both processes were differently transcribed. For example stearoyl-CoA desaturase 1 (SCD1), glycerol-3-phosphate acyltransferase (GPAT) and fatty acid synthase (FAS), all involved in the lipogenesis, were down regulated. Down regulation of ACC1 and ACC2, the rate-limiting proteins in transporting fatty acids to the mitochondria, increases lipolysis.[39] Regarding the positive effects on the glucose levels, hepatocyte nuclear factor 4α (HNF4α) and forkhead transcription factor A2 (FoxA2) were up-regulated. Both factors are described to be important to maintain a normal glucose metabolism. FoxA2 over-expression is associated with insulin sensitivity, normal plasma glucose levels and improves a fatty liver (hepatosteatosis).[39] Gluconeogenesis is also affected by FGF21. FGF21 administration to WT mice elevates the mRNA levels of PGC-1α, G6P and PEPCK in the liver. In fasted fgf21 KO mice, both gluconeogenesis and ketogenesis was impaired compared to WT mice.[40]

In white adipose tissue (WAT) FGF21 changes gene expression associated with both lipogenesis and lipolysis. This is concluded from studies with DIO mice, *ob/ob* mice and also transgenic FGF21 mice. In WAT, opposite to the liver, players involving lipogenesis were up-regulated by FGF21 (SCD1, diglyceride acyltransferase-1 (DGAT1), ACC). Also lipolysis players, high sensitive lipase (HSL) and

adipose triglyceride lipase (ATGL), are up-regulated just like uncoupled protein 1 (UCP1).[39] The increase in lipogenesis, lipolysis and UCP1 expression implicates a futile cycling mechanism in WAT. Futile cycling is a process in which two metabolic pathways run in the opposite direction. In this case, the formation of lipids and the breakdown of lipids occur at the same time in adipocytes. This results in a "waste" of energy, mostly converted into warmth. UCP1 helps to convert energy into warmth, and therefore indicates that FGF21 indeed induced futile cycling in WAT tissue. Also in brown adipose tissue (BAT), UCP1 is up-regulated.[38, 39] Contrary to the mice situation, lipolysis is inhibited by FGF21 in human adipocytes. This is the opposite of what has been reported in *in vivo* mice models. Inagaki *et al.* their transgenic FGF21 mice have increased lipolysis in adipocytes, and also confirm it in *in vitro* studies using 3T3-L1 mice adipocytes.[38] Arner *et al.* could not reproduce the increase of lipolysis in adipocytes differentiated from human Mesenchymal Stem Cells (hMSC). The expression of HSL and ATGL was unchanged.[41] Surprisingly using the same 3T3-L1 cell line as Inagaki *et al.* used, it was impossible for them to replicate their results. Differences seen in the *in vitro* experiment could not be explained. The differences seen between *in vitro* and *in vivo* could point towards an indirect of FGF21 on the lipolysis in adipocytes, which suggests a more adaptive mechanism that initiates lipolysis in WAT. [38, 41] There is more evidence that FGF21 exerts a function in lipid metabolism in adipocytes. FGF21 increased glucose uptake in both mouse 3T3-L1 adipocytes and primary human adipocytes. This effect is not present in human pre-adipocytes.[26] Up-regulation of the GLUT1 transporter, a transporter which enables glucose to enter cells, was identified. This up-regulation is also seen *in vivo*, where *ob/ob* mice show increase in GLUT1 mRNA levels in adipocytes after FGF21 administration. This up-regulation of GLUT1 is not seen in other organs.[26]

Increased uptake of glucose also helps to reduce hyperglycemic levels. It is thought that improvement in insulin sensitivity is how FGF21 lowers glucose plasma levels towards normal. FGF21 administration in mice up-regulates mRNA expression of insulin receptors in the liver.[39] Furthermore, chronic administration of FGF21 to *ob/ob* mice lowers insulin levels, increases basal glucose clearance and causes a higher glucose influx during an insulin clamp.[42] These results suggest increased insulin sensitivity. Increased insulin sensitivity is a desired effect of drug in order to reverse DMT2. During DMT2, there is insulin resistance, which causes an overproduction of insulin in the pancreatic βcells. Ultimately, this leads to amyloid formation in the pancreatic island, and apoptosis of the βcells. Due to FGF21 increased insulin sensitivity and lower glucose levels the apoptosis of βcells in DMT2 patients is indirectly inhibited. FGF21 also directly targets βcells. When pancreatic islands of diabetic mice were incubated with FGF21, insulin content increased and glucose-induced insulin secretion increased compared to controls.[43] This increased secretion is unseen in healthy controls.[43] Besides this positive effect on βcell functioning, FGF21 improves survival during glucolipotoxicity and cytokine-induced apoptosis. In pancreatic islands cultured in very high glucose levels or with cytokines, FGF21 lowers the caspase levels, marker for apoptosis, and increased βcell insulin secretion and content.[43]

FGF21 has also been linked to growth hormone (GH) signaling inhibition. It has been reported before that GH signaling is blocked by starvation. Since FGF21 is induced by fasting, it is not surprising that a link is observed. Transgenic FGF21 mice are significantly smaller than WT mice.[44] Measuring GH and insulin-like growth factor 1 (IGF-1), important hormone stimulating growth, showed an increase in GH and decrease in IGF-1 levels in FGF21 transgenic mice. Signal transducer and activator of transcription 5 (STAT5), a regulator of IGF-1, and other growth related factors, was indeed less active

in livers of FGF21 transgenic mice, indicating that FGF21 also inhibits growth by inhibition of STAT5 in the liver.[44]

Figure 6 shows the scheme which can be drawn out of the literature that is just discussed.

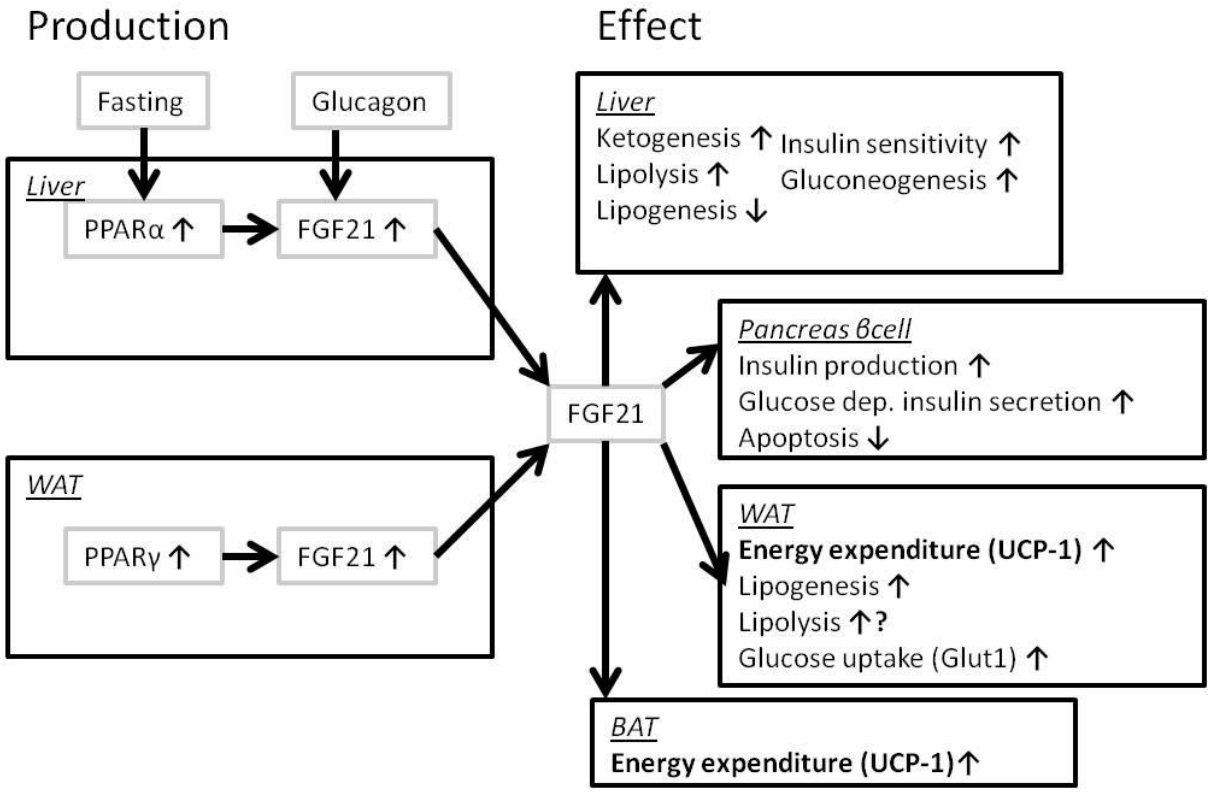

Figure 6 **FGF21 regulation in different tissues** Fasting or a ketogenic diet increases peroxisome proliferator-activated receptor α (PPARα) in the liver, which increases FGF21 transcription in the liver. Increased glucagon levels increase the levels of FGF21. Also white adipose tissue (WAT) expresses FGF21 when PPARγ levels increase. FGF21 has been shown to regulate several processes. In the liver, ketogenesis, lipolysis, insulin sensitivity and gluconeogenesis increases. Lipogenesis decreases by FGF21. In the insulin producing βcells in the pancreas, FGF21 increases the production of insulin and the glucose dependent insulin secretion. The caspase activity in βcells is deceased by FGF21, which results in a lower rate of apoptosis. In WAT FGF21 increases the energy expenditure by futile cycling: both lipogenesis and lipolysis are increased in WAT, just like glucose transporter 1 (Glut1). *In vitro* data exists which shows lipolysis to be inhibited by FGF21. FGF21 also increases brown adipose tissue energy expenditure, with UCP-1 up-regulated.. And finally, FGF21 causes Growth Hormone (GH) resistance.

FGF21 mainly signals through FGFR1c/βKlotho on adipocytes

Just like FGF15/19, FGF21 requires βKlotho to stably bind to FGFRs. FGF21 only activates a signaling cascade in HEK293, measured by phosphorylation of FRS2α, cells when βKlotho is expressed, and this is dose-dependent.[14] In 3T3-L1 adipocytes FGF21 activates a signaling cascade, measured by phosphorylation of FRS2α, and this is attenuated by knocking down βKlotho with siRNA. Interestingly, the 3T3-L1 adipocytes predominantly express FGFR1. This indicates FGF21 exerts its role in adipocytes through FGFR1 and βKlotho.[14] This is also proved in BaF3 cells, a murine bone barrow derived pro-B-cell line. WT BaF3 cells do not express FGFRs. BaF3 cells may be transfected to over-express FGFRs, βKlotho or both. FGF21 had no effect on BaF3 transfectants expressing only FGFR1c without βKlotho.[15] Also, BaF3 transfectants only over-expressing βKlotho do not respond to FGF21. But stimulation of BaF3 transfectants expressing both FGFR1c and βKlotho did show an effect, namely significant increase in proliferation.[15] This effect occurs without heparin, since the heparin binding site of the endocrine FGF is not conserved. In the presence of heparin, the effect of FGF21 on

proliferation does increase.[15] Besides FGFR1c, also the other 6 FGFRs were expressed, after transfection, in the BaF3 cells. Only FGFR1c and FGFR3c, in the presence of βKlotho, gave an increased proliferative response by FGF21 in BaF3 cells.[15] This indicates FGF21 is working via FGFR1c and FGFR3c, only when βKlotho is also present.

FGF21 depends on a βKlotho as co-receptor in order to activate FGFR. A study suggests an extra alternative route. WT mice were injected with hFGF21, to first prove that hFGF21 works. This was confirmed by measuring ETS related gene 1 (*Egr1)* mRNA levels, which is increased upon FGF21 administration.[16] After hFGF21 injection in βKlotho KO mice, *Egr*1 was significantly up-regulated in WAT and brown adipose tissue BAT, just as in WT mice.[16] Also, the comparison between the βKlotho KO mice and fgf21 KO mice indicate an alternative pathway. In WAT of fgf21 KO mice, HSL and ATGL expression levels were decreased 2-fold in feeded mice compared to WT.[45] But in βKlotho KO mice, both in WAT and BAT, the levels of HSL and ATGL were not significantly different between the WT mice.[17] These studies suggest that βKlotho, in adipose tissue, is not required for all the effects of FGF21.[16, 17] The mechanism behind the effects noticed independent of βKlotho are yet unknown. It is therefore unfortunate that ATGL and HSL levels are not measured in βKlotho KO mice administered with FGF21. In my opinion that is missing to make the independent pathway more convincing.

When FGF21 binds βKlotho, it is also possible to form a complex with FGFR4. This was concluded from a pull down experiment where the βKlotho-FGFR4 complex was able to pull down FGF21 efficiently.[46] In rat hepatoma cells (H4IIE), which co-express both βKlotho and FGFR4, there was only a very poor response in the cells. H4IIE cells did respond to FGF19. This indicates that even though FGF21 does form a complex with FGFR4 and βKlotho, it seems unable to exert its actions through FGFR4.[14] This proofs *in vitro* binding does not mean that it actually functions *in vivo*.

Also *in vivo* it is tested whether the conclusions drawn from most *in vitro* experiments seem valid. In an *in vivo* study in mice they first determined the levels of the several FGFRs on different organs. The liver has a high expression of FGFR4 and adipocytes have a high expression of FGFR1.[14] Immunoprecipitate assays from cell lysates conclude that βKlotho form complexes more efficiently with FGFR1 and FGFR4.[14] After adding recombinant FGF19 or FGF21, FGF21 activated the FGF signaling in WAT, but not in the liver. FGF19 was activated the FGF signaling strongly in the liver, but weakly in WAT. This was concluded by measuring ERK phosphorylation, one of the main signaling pathways of FGFR (see figure 2).[14] This shows FGF21 functions mainly through FGFR1/βKlotho.

The data indicates that FGF21 binds βKlotho to form a complex with FGFR1c, FGFR3c or FGFR4. The FGF21-FGFR4-βKlotho complex is not capable to signal.[14] Since most of FGF21 effects are metabolic, and the presence of FGFR3c is very low in liver and adipocytes, it is likely that most effects of FGF21 are achieved through the FGF21-FGFR1-βKlotho complex. Although, there also may be a βKlotho independent pathway, which is not described well in literature yet.[16, 17] FGF21 exerts metabolic activities in the liver while it is unable to directly activate hepatocytes FGFR4, which suggests an in-direct effect of FGF21 on the liver.[5] In H4IIE hepatic cells FGFR3 is also expressed, but in a 5-fold lower mRNA expression then FGFR4.[14] FGF21 can act through FGFR3 when βKlotho is present as well in BaF3 cells.[15] Still, in the H4IIE cells FGF21 does not induce signal transduction.[14] This means further research will have to elucidate how the effects in the liver are achieved by FGF21. Figure 3 shows a brief overview of FGF21 and its receptors.

Safety profile makes FGF21 promising insulin sensitizer for humans

FGF21 is not reported to be involved in any genetic disorder in humans. FGF21 does show some differences between humans and animals. For instance the up-regulation of FGF21 after fasting occurs in humans as well, but only after 7 days where in mice 24 hours of fasting elevated FGF21 levels.[5, 35] This indicates that, at least in humans, ketogenesis may occur independent of FGF21, since keton bodies already present way earlier.[5] It does not mean that FGF21 has no effect on ketogenesis in humans. FGF21 levels may play a smaller role in healthy persons. The FGF21 levels are independent of the regular metabolic parameters in healthy individuals, and also differ to a 250-fold between healthy persons.[5] These big differences are not concluded to be dangerous. In mice, it has already been reported at the discovery of FGF21 that administration of FGF21 does not cause hypoglycemia, tumor formation or weight gain (by edema). This indicates the perhaps low risks to use FGF21 as a therapeutic.[5, 26] Cross-sectional studies in humans conclude FGF21 has positive associations with adiposity, insulin resistance and adverse lipid profiles. The levels of FGF21 in fasting DMT2 patients are shown to be higher. Just like insulin, FGF21 is also increased compared to healthy humans. This could also be a compensatory mechanism, which would mean administration of even more FGF21 could be beneficial for diabetic patients. It could also be a sign of resistance, like also occurs with insulin. Chronic administration of FGF21 in *ob/ob* mice does lead to positive effects compared to no response to acute FGF21 administration. This also indicates a resistance mechanism for FGF21.[5, 42]

Most promising is the insulin sensitization effect of FGF21 for DMT2 persons. Since in DMT2 insulin is less active, sensitization could normalize the metabolic parameters of the DMT2. Furthermore, during the pathophysiology of DMT2, more and more insulin producing βcells become apoptotic which is at some point irreversible. FGF21 has also, directly and indirectly, been proven to promote βcell functioning and reduce apoptosis.[43] Besides the insulin sensitizing effects, FGF21 increases energy expenditure in animals which causes weight loss. Dose dependent effects of FGF21 are reported in literature. It is reported that at lower doses, hyperglycemia can already be lowered, where weight loss is achieved at higher concentrations.[5, 39]

FGF23 regulates calcium and phosphate homeostasis via AVD

FGF23 is the only endocrine FGF which has been reported in human hereditary diseases. FGF23 was first discovered by the hereditary diseases and therefore is discussed here. Both gain- and loss-of-function gene mutations are reported for FGF23. Gain-of-function gene mutations are the cause of the disease autosomal dominant hypophosphataemic rickets (ADHR), where point mutations in amino acid 176 and 179 of FGF23 are reported to be the cause. [47] ADHR is characterized by short stature, bone pain, fracture and limb deformity. Also ADHR is associated with hypophosphatemia, decreased levels of active vitamin D ($1,25(OH)_2$ vitamin D_3, in this thesis referred to as AVD) and reduced absorption of phosphate in the kidney. The missense mutations in ADHR make FGF23 less sensitive for protease cleavage, which prolongs the lifetime of FGF23 and increases its serum levels.[47] Loss-of-function mutations in FGF23 have been reported in familial tumoral calcinosis (FTC). FGF23 loss-of-function results in hyperphosphatemia.[48] This is concluded in fgf23 KO mice, which are viable, but have growth retardation, abnormal bone phenotype and generally die within 13 weeks after birth.[49] The serum levels of phosphate, calcium and AVD levels in fgf23 KO mice are increased. Parathyroid hormone (PTH), the main calcium regulating hormone, levels are normal after 10 days.[49] Together this indicates that FGF23 plays a role in the phosphate and calcium homeostasis.

AVD levels are regulated by the renal enzymes 1α-OHase (Cyp27b1), which increases active AVD, and 24-OHase (Cyp24), which decreases AVD levels. Northern blot analysis revealed that CYP27b1 mRNA expression in fgf23 KO mice increased at the same time when AVD levels increased, which was 10 days after birth.[49] Cyp24 mRNA expression also increased, only after 3 weeks. It is proposed that the increase of CYP27b1 expression causes the AVD levels to rise, and that the AVD in turn increased Cyp24 to try to compensate.[49]

Serum phosphate levels are usually maintained by renal phosphate reabsorption. The amount of type IIa sodium-phosphate cotransporter (NaPi-2a) in renal proximal tubular cells determines the amount of reabsorption. Immunohistochemistry confirmed the increase of NaPi-2a localized in the apical membranes of the proximal tubules in FGF23 KO mice.[49] NaPi is not only active in the kidney, also in the (small) intestine. Brush border membrane vesicles (BBMV) are the cells which can express the NaPi. BBMV were isolated from rats which got administered FGF23 with a normal or prolonged life time. Compared to rats that were treated with normal FGF23, the prolonged FGF23 caused a 45% decrease in Na^+ dependent phosphate uptake in BBMWs from the kidney, and 30% in BBMWs from the small intestine.[50] PTH is also reported to down regulate NaPi cotransporters, which decreases phosphate levels. By performing a thyroparathyroidectomy (TPTX) on rats, PTH production is completely abolished. TPTX rats have increased levels of phosphate and decreased calcium levels. Administered FGF23 with prolonged lifetime can restore phosphate levels without restoring the calcium levels. Administration of PTH in TPTX rats restored both phosphate and calcium levels.[50] Since calcium levels were not restored by FGF23 proves that FGF23 can work independently of PTH to regulate phosphate levels.[50]

Besides the role of FGF23 in regulation of mostly phosphate homeostasis, fgf23 KO mice also have significant lower glucose and triglyceride serum levels and increased total cholesterol.[49] In a double KO study, where both fgf23 and Vitamin D receptor (vdr) are knocked out, AVD regulates the effects observed in both phosphate and carbohydrate metabolism.[51] The fgf23/vdr KO mice had normal calcium and phosphate levels (after a rescue diet with extra minerals) and had a normal weight.[51] The phenotype of vdr KO and fgf23/vdr double KO mice were strikingly similar. Also the hypoglycemia of fgf23 KO mice, proposed to be induced by higher insulin sensitivity, was unnoticed in the fgf23/vdr KO mice, just like the hyperphosphatemia and hypercalcemia.[51] This indicates AVD regulates many of FGF23s actions in mineral and glucose metabolism.

The production of FGF23 dominantly takes place in the bone-like cells. FGF23 mRNA is detected in osteocytes and flattened bone-lining cells. In osteogenic cells during phases of active remodeling, FGF23 mRNA is detected more intensively.[52] The production of FGF23 is induced by AVD. *In vitro* studies using both mice and rat osteoblast-like-cells revealed that 24 hour administration of AVD leads to a 100-fold increase in FGF23 mRNA levels.[53] An *in vitro* luciferase assay was performed where the 3500 base pair sequence prior to the ATG start codon was cloned into a firefly luciferase vector, pGL3-Bacic vector. This vector was transfected into rat osteosarcoma cells (ROS17/2.8). After administration of VDR, the ROS17/2.8 cells transfected with the vector containing the FGF23 promoter, luciferase activity increased 6-fold compared to the empty luciferase vector.[53] After more specific cloning, a 15 bp deletion could stop the luciferase activity, proving FGF23 to have a vitamin D receptor element.[53] The expression of FGF23 in bone cells can also be induced by leptin. Treatment of the leptin deficient *ob/ob* mice with leptin shows significant lower calcium and phosphate levels, while FGF23 concentration was significantly elevated. When leptin was administrated to leptin

receptor deficient *db/db* mice, then FGF23 levels were not significantly different, indicating leptin as an inducer of FGF23 levels.[54]

Figure 7 shows the scheme which can be drawn out of the literature that is just discussed.

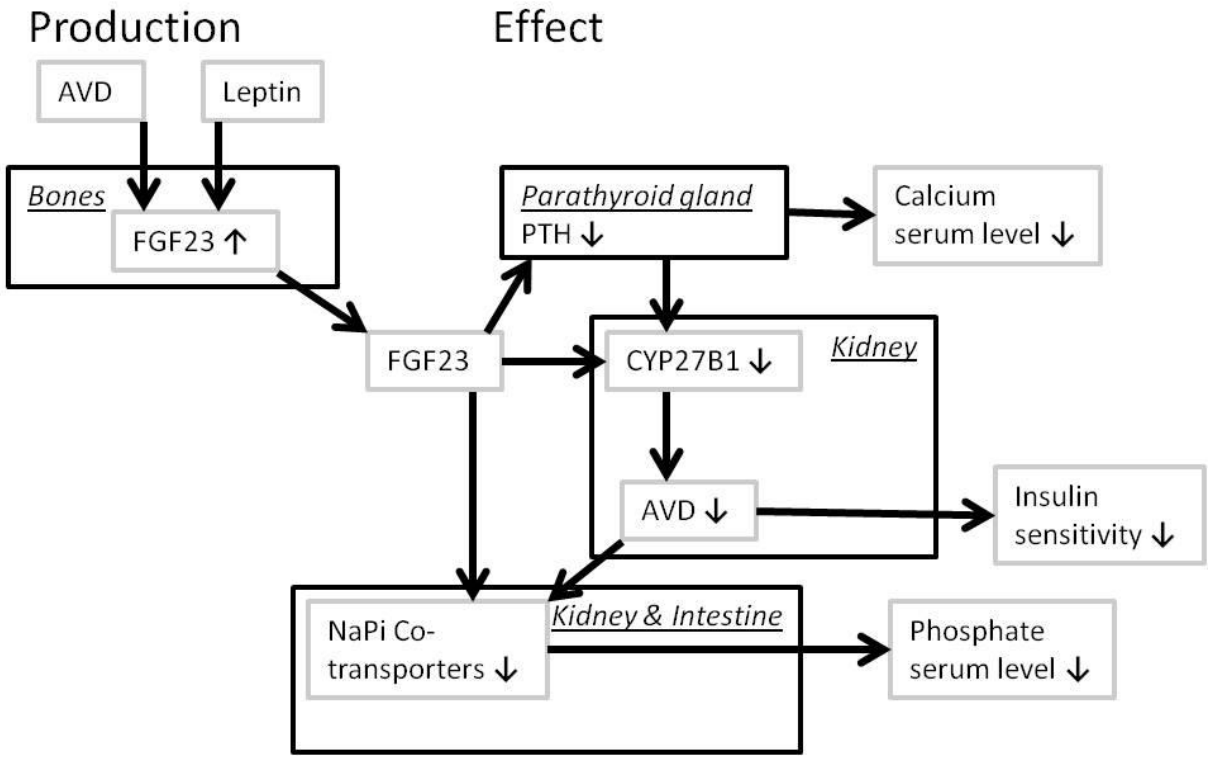

Figure 7 **The mechanism how FGF23 regulates and is regulated** FGF23 can be induced by leptin and active vitamin D (AVD) in the bones. FGF23 lowers the Parathyroid hormone (PTH) levels which lowers calcium serum levels. FGF23 and PTH both inhibit 1α-OHase (Cyp27b1), a renal enzyme which increases AVD levels. AVD increases insulin sensitivity, which means FGF23 and PTH lowers the insulin sensitivity by lower AVD levels. FGF23 lowers sodium-phosphate cotransporters (NaPi) which are present in kidney and intestine. AVD can increase NaPi cotransporter levels. NaPi cotransporters (re)absorb phosphate to increase phosphate levels, which is inhibited by FGF23 directly and indirectly through AVD.

FGF23 signals mainly through FGFR1c/αKlotho in the kidney

FGF23 is αKlotho dependent, unlike FGF15/19 and FGF21. In the study in which BaF3 (murine bone barrow derived pro-B-cell line) transfected cells, to let them expresses FGFRs and Klothos, were used to prove FGF21 binds FGFR1c and FGFR3c through βKlotho, they also tested whether FGF23 was capable to activate the BaF3 cells to proliferate. βKlotho/FGFR1c expressing BaF3 cells would not proliferate upon FGF23 administration, while they did on FGF21 administration.[15] The αKlotho/FGFR1c expressing BaF3 cells did proliferate upon FGF23 administration, while they did not on FGF21 administration.[15] This indicates that FGF23 is αKlotho dependent, unlike FGF19 and FGF21 which are βKlotho dependent.

More studies concluded FGF23 is αKlotho dependent. When hFGF23 is administered in WT mice, there is a reduction in AVD levels and hypophospathemia. AVD levels in either fgf23 or αKlotho KO mice are increased compared to WT mice.[16] Furthermore, the FGF23 serum levels in αKlotho KO mouse was over 8,000 fold increased, indicating a compensatory mechanism of FGF23.[16] This suggests FGF23 is unable to exert its function without αKlotho. αKlotho mice have side effects with low AVD levels. The AVD levels can be restored to normal with a special diet. When hFGF23 is

administered to αKlotho KO mice with normal AVD serum levels, caused by a diet, they could not see changes, while in WT mice the AVD levels would decrease significantly.[16]

Vitamin-D metabolizing enzymes Cyp27b1 and Cyp24 are involved in the action of FGF23. When kidneys of WT mice are injected with hFGF23, Cyp27b1 expression reduced over 10-fold and Cyp24 expression increased over 5-fold after 4 hours. The AVD levels decreased around 2-fold after 4 hours.[16] hFGF23 in αKlotho KO kidneys however do not lead to a significant effect.[16] hFGF23 administration results in down-regulation of αKlotho in the kidney of WT mice, suggesting a negative feedback system.[16] Using co-precipitations to prove which FGFRs FGF23 activates, they concluded *in vivo* that αKlotho was both co-precipitated with FGFR1 and FGFR4 in the kidney.[16] After injection of hFGF23, FGFR1 was activated, which they measured by phosphorylation of the FGFR. FGFR4, which also interacts with αKlotho, was not activated after hFGF23 injections.[16] The effect of FGFR1 activation was abolished when a αKlotho KO model was used. This experiment exposes that the effects seen in the kidney by FGF23 are both αKlotho and FGFR1 dependent.[16]

Another *in vivo* study excludes other FGFRs which could account for the effect of FGF23 observed in the kidney. The Hyp mouse, which is a mouse that has elevated levels of circulating FGF23, was used in the mice study. FGF23 has an effect on the kidneys, and because FGFR3 binds FGF23 *in vitro* and FGFR3 is expressed most of all FGFRs in the proximal tube, the effect of FGF23 on FGFR3 was aim of the study.[18] Hyp mice were crossed with fgfr3 KO mice, to create fgfr3 KO hyp mice. Apart from some abnormalities in these mice, the phosphate serum levels, AVD serum levels and Cyp27b1 levels were not significantly changed, which indicates that FGF23 exerts its renal functions not through FGFR3.[18] The same experiment has also been performed with fgfr4 KO, which also failed to lead to changes in the mentioned parameters. But both mouse models did induce higher FGF23 serum levels, which does indicate that both FGFR3 and FGFR4 do exert some functions of FGF23, only most likely not in the kidney.[18]

FGF23 is αKlotho dependent, while FGF19 and FGF21 are (mostly) βKlotho dependent. The most pronounced effects of FGF23 are the changes in phosphate serum levels by regulating AVD levels. FGF23 exerts its main effects through FGFR1c in the kidney.[16] But KO of fgfr3 and fgfr4 in mice suggests a function of these receptors outside of the kidney.[18] Expression levels of αKlotho and FGFR3 (table 1) may show a possible role for the central nervous system.[12] Further research is needed to elucidate these mechanisms. Figure 3 shows a brief overview of FGF23 and its receptors.

FGF23 is not suited as a insulin sensitizer in humans

FGF23 has been reported in a gain- and loss-of-function mutation in human heritable diseases. The role of FGF23 in these diseases, and also in animal models, seems rather clear. Although there are uncertainties still. FGF23 is also linked to renal failure. Humans with chronic kidney disease have 100-1000 fold higher FGF23 levels in the circulation.[55] This correlated with creatinine levels in blood, a protein which is normally used as a marker for kidney failure.[55] Interestingly, in healthy individuals, lowering the phosphate intake resulted in a lower secretion of phosphate in the urine, since more phosphate was reabsorbed to compensate. During phosphate loading, the healthy individuals had higher phosphate levels in their urine. But in both cases, the levels of FGF23 did not change significantly. Just like phosphate, PTH and AVD levels did not change.[55] This suggests that short term changes in (re)absorption are regulated independent of FGF23. Analysis of patients who are starting dialysis revealed that FGF23 levels are also correlated to survival prediction. There is a correlation

between the height of the FGF23 levels and 1-year all-cause mortality. After multivariable adjustment of crude data, humans in the third or fourth quartiles of FGF23 serum levels have a 500 to 600% increased risk for death.[56] Although it is not sure whether FGF23 here is a biomarker which only gives information about the condition of the patient, or that FGF23 is actually harmful in the process. It is reported that FGF23 levels go up before patients become hyperphosphatemic, which may indicate that another processes elevate FGF23 than the phosphate levels feedback loop.[56]

Discussion

In this thesis an overview of the role and regulation of the endocrine FGF has been given. The FGF family consists of 23 members, which can be divided into 7 subfamilies.[1, 2] The endocrine subfamily differs from the rest of the FGF family because they lack a HB domain. Without this HB domain, endocrine FGF can diffuse freely and enter the circulation.[2] Endocrine FGFs use FGFRs and Klotho proteins to exert their function.[8] FGF15/19 and FGF21 are only capable to bind βKlotho, while FGF23 is capable of binding only αKlotho.[13-16] Figure 3 shows which endocrine FGF can activate which receptor complexes. FGFR and Klotho expression is tissue specific.[12] This also makes the endocrine FGFs site of action more specific. Table 1 shows the expression of Klotho and FGFR proteins in the most important organs.

This thesis shows that FGF15/19 and FGF21 have similar functions. Both FGF15/19 and FGF21 are reported to hold therapeutically benefits for DMT2 and obesity. The production of these endocrine FGFs are both regulated by food intake. Difference is that FGF15/19 is produced after food intake, while FGF21 levels increase after fasting or KD.[23, 35] The production of FGF15/19 is regulated by BAs, which activates FXR and leads to the production of FGF15/19.[20] In the liver, the production of BAs is stopped by FGF15/19.[19] In the gallbladder FGF15/19 mediates the refilling with BAs.[23] FGF15/19 further improves metabolic parameters. Glucose levels are improved by stimulation of glycogen synthesis and decreasing gluconeogenesis both in the liver. Also, adipocytes have an increase inflow of glucose. The liver also increased lipolysis and protein synthesis.[25, 57] The increased BAT mass indicates an increased energy expenditure, which may also explain the obesity resistant effect seen in FGF19 transgenic mice.[21] All actions of FGF15/19 are shown in figure 4. FGF15/19 exerts its functions through FGFR4 and FGFR1.[14] FGFR4 is abundantly expressed in the liver, FGFR1 in adipocytes.[14] A negative effect noticed in FGF19 transgenic mice is the formation of HCC.[27, 28] FGF19-7 is a modified FGF19 which has small amino acid residues replaced by those of FGF21. FGF19-7 is unable to activate FGFR4 anymore. Without the activation of FGFR4, FGF19-7 still improves the metabolic parameters to the same extend as FGF19.[33] BA regulation and HCC however are not affected anymore.[33] This suggests that modified FGF19 may be a promising therapeutic in order to improve metabolic parameters, and reverse the starting DMT2 and obesity phenotype. It is striking however that although the livers FGFR4 is not activated anymore, FGF19-7 is still capable of regulating metabolic processes in the liver.[33] This suggests an indirect effect of FGF19-7 on the liver. Processes of FGF15/19 and FGF21 in adipocytes are regulated via FGFR1 directly.[14] Perhaps FGF19-7 induced metabolic processes in the adipocytes which changes plasma glucose of lipid levels. These levels may cause a change in the metabolism in the liver which leads to the effects in the liver.

Not only FGF15/19 exerts positive effects on metabolic parameters. FGF21 also regulates a lot of metabolic processes which are thought to be beneficial. Compared to FGF15/19, FGF21 is normally produced by fasting.[35] Also glucagon, a hormone which is secreted during low glucose levels,

stimulates the production of FGF21.[37] Compared to FGF15/19, FGF21 only seems to improve metabolic parameters. In the liver there is increases ketogenesis, although not necessary for functioning in humans.[5, 35] In the liver also lipolysis, gluconeogenesis and insulin receptor expression increase. The formation of lipids, lipogenesis, is inhibited and there is GH resistance.[26, 35, 38, 39, 44] FGF21 also directly targets the insulin producing βcells. The production of insulin increases, just like the glucose dependent secretion of insulin and apoptosis of the βcells is inhibited by FGF21.[43] In adipose tissue there is an increase in energy expenditure. In WAT FGF21 may induce futile cycling by activating both lipogenesis and lipolysis.[39] However, the increase of lipolysis is unsure, due opposing and irreproducible results.[38, 41] Up-regulation of UCP-1 in both WAT and BAT indicates increased energy expenditure.[38, 39] For the effects of FGF21 see figure 6. Overall FGF21 make mice DMT2 and obesity resistant, just like FGF15/19. Compared to FGF15/19, FGF21 does not regulate other processes or posses a harmful side effect.

So both modified FGF19 and FGF21 have promising effects *in vivo* in mice studies. I think that FGF21 holds most promise to become a future DMT2 and obesity therapeutic. This is because FGF21 is reported to be more safe and diverse. *In vivo* transgenic FGF21 mice are not hypoglycemic. The transgenic FGF21 mice are significantly smaller compared to WT mice, but this is most likely caused by the GH resistance which is induced by FGF21.[26, 44] Also, administration of FGF21 has not shown to lead to weight loss in mice. And doubling the calorie intake of FGF21 transgenic mice did not increase their weight.[26] There are thoughts that FGF21, just like insulin, becomes resistant in obese patients.[5, 42] With insulin resistance, administration of more insulin helps on the short term. But eventually, when life style is not changed, the insulin creating βcells will die. It is not known whether FGF21 production will also eventually diminish, but that would mean that hepatocytes and adipocytes would go into apoptosis, which would be unlikely. But not only *in vivo* mice experiments suggests FGF21 may be safe. There may be a 250-fold difference in FGF21 levels between healthy humans.[5] Beside the safety profile of FGF21, a huge benefit of FGF21 over FGF19 is the role of FGF21 on the insulin producing βcell.[43] In DMT2 patients, the βcells become apoptotic. However both FGF19 and FGF21 are improving metabolic parameters and increasing insulin sensitivity, FGF21 directly inhibits the apoptosis of βcells. Furthermore, βcell functioning increases after FGF21 administration in DMT2 mouse models.[43] In my opinion, this makes FGF21 more favorable above FGF19 as a therapeutic.

FGF23 also is an endocrine FGF. Besides the main role of FGF23, regulation of phosphate and calcium plasma levels, FGF23 also decreases insulin sensitivity.[49, 51] In theory, this would mean that decreasing FGF23 would lead to increased insulin sensitivity. This is not a promising idea. The regulation of insulin sensitivity via FGF23 is regulated by AVD. AVD is also involved in regulation of phosphate serum levels.[49] When insulin sensitivity is regulated via FGF23, also phosphate levels will be changed.[49, 51] FGF23 is known in hereditary diseases, in which both gain-of-function and loss-of-function of FGF23 have been reported.[47, 48] The main problems in these diseases are based on the changed phosphate and calcium serum levels. This makes FGF23 unsuited as future therapeutic against DMT2 and obesity. In regulation of phosphate and calcium, FGF23 may play a therapeutic role, however more research is needed since results in humans' shows large variations in FGF23 serum levels and that phosphate absorption can change without changes in FGF23 levels.[55, 56]

In conclusion, FGF15/19 and FGF21 show to play a role in several metabolic processes in the body. AdAdministration of these endocrine FGFs improves metabolic parameters, such as glucose and triglyceride homeostasis and insulin levels and sensitivity. FGF21 holds most promise as therapeutic

based upon the safer profile, its positive effects on βcells and the smaller amount of undesired effects. FGF23 only plays a minor role in changing metabolic parameters, but may be investigated to become a therapeutic for odd phosphate and calcium serum levels.

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