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Introductory Chapter: Vitamin K2

Jan Oxholm Gordeladze

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http://dx.doi.org/10.5772/66384

Before going into detail the biological effects of vitamin K2, it must be emphasized that vitamin K2, also known as menaquinones with varying side chain lengths, may in fact associate with other genes and modulate their effects substantially.

In fact, vitamin K2 may associate with a protein nuclear factor or intra-nuclear hormone receptor, just like what is known for vitamin A and vitamin D. This protein has many names, such as NR1I2, PXR or SXR. Vitamin K2 with its isoprenoid side chain of varying length will, for reasons of simplification, now be referred to as MK-7, even though there are moieties which are both shorter and longer. MK-7 is chosen, due to the fact that this molecule might be the more abundant, as well as the more active moiety.

First, it might be of interest to see which the genes are, and thus, what are the cellular or biological functions being 'determined' or 'modulated' by MK-7 and its 'relatives'. A scrutiny of the interactions of NR1I2 taken from 'Gene Cards': http://string-db.org/cgi/network. pl?taskId=I2KNgtQWbVsT looks like this (**Figure 1**).

It has been well established that NR1I2-PXR-SXR is the receptor, which binds vitamin K2 analogues of different chain lengths, e.g. MK-4 and MK-7, and NR1I2 may thus communicate the effects of vitamin K2 via associating with RXRA, forming a heterodimer. Furthermore, one may postulate that vitamin K2 may influence several other genes indirectly, by 'impinging' on elements or members of a 'gene lattice' like the one shown in **Figure 1**. Some of the genes being putatively strongly affected by vitamin K2 are (text from the same web-page as referred to **Figure 1**):

RPS6KB1 ribosomal protein S6 kinase, 70 kDa, polypeptide 1; Serine/threonine-protein kinase that functions downstream of signalling by mTOR responding to growth factors, as well as nutrients in order to sustain cell proliferation, growth and progression of the cell cycle. It modulates protein synthesis via phosphorylation of EIF4B, RPS6 and EEF2K, and ensures cell survival via repression of pro-apoptotic functions of BAD. When nutrient depletion occurs, inactive forms associate with EIF3 to build a translation initiation complex.

PPARGC1A peroxisome proliferator-activated receptor γ, co-activator 1α; Serves as a transcriptional co-activator of steroid hormone receptors, as well as nuclear receptors. It greatly enhances transcription of the PPARG and thyroid hormone receptors, and can regulate mitochondrial genes, contributing to adaptive thermogenesis. It plays a pivotal role in metabolic adaptation in response to the availability of various diets via coordinating the expression of a large spectrum of genes mandatory for glucose and fatty acid metabolism.
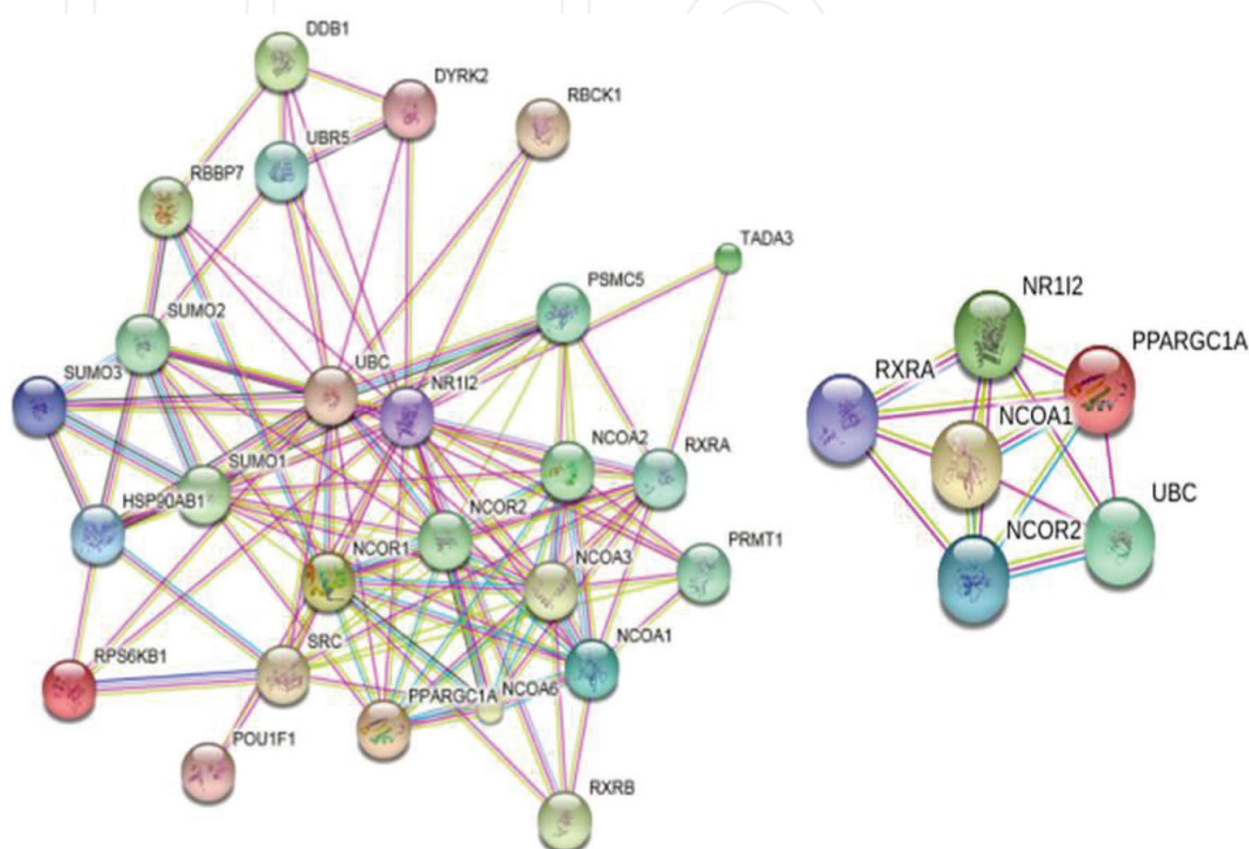

Figure 1. Genes interacting with the vitamin K2 (MK-7 or MK-4), which bind to the nuclear (transcription factor) NR1I2 being connected to a network of associated genes. Right panel: high stringency emulation and left panel: intermediate stringency.

NR1I2 nuclear receptor subfamily 1, group I, member 2; this is the nuclear receptor actually binding vitamin K2, and also goes by the names of SXR and PXR.

DYRK2 dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2; this serine/threonine-PK is involved in modulatory control of mitosis, proliferation of cellular entities, apoptosis, as well as cytoskeleton organization encompassing neurite outgrowth. It functions partly via its role in ubiquitin-dependent degradation of proteasomes. It works downstream of ATM to phosphorylate p53/TP53 at position 'Ser-46', thus contributing to induce apoptosis as a response to DNA damage. It phosphorylates NFATC1, thus inhibiting its accumulation in the nucleus, as well as its activity as a transcription factor.

RBCK1 RanBP- and C3HC4-type zinc finger containing 1; E3 ubiquitin-protein ligase, accepting ubiquitin delivered by E2 ubiquitin-conjugating enzymes, one of which is UBE2L3/

UBCM4, thereafter transferred to its substrates. It serves as E3 ligase for oxidized IREB2, while heme and oxygen are both necessary for the ubiquitination of IREB2. It promotes ubiquitin-coupling to TAB2 and IRF3, as well as their proteasome-mediated degradation. It is a component of the LUBAC complex, which conjugates ('M-1'-linked) polyubiquitin to substrate molecules, and plays a key role in NF- kappa-B activation and modulation of inflammation.

SRC v-src sarcoma viral oncogene homolog; non-receptor protein tyrosine kinase being activated subsequent to engaging of several various classes of cellular receptors, encompassing immune response receptors and integrins as well as adhesion receptors, TyrK-receptor protein kinases, G protein-sensitive receptors, and cytokine receptors. It participates in signalling, controlling a plethora of biological phenomena encompassing transcription of genes, immune responses, adhesion of cells, progression of the cell cycle, apoptosis, migration as well as transformation.

NCOA6 nuclear receptor co-activator 6; co-activator of nuclear receptor, directly binding nucleoceptors, stimulating transcriptional characteristics in a hormone-like manner. It co-activates expression in an agonist- and AF2-mediated fashion. It is implicated as co-activator of various nuclear receptors, like those binding steroids (GR & ERs), retinoids (RARs & RXRs), thyroid hormone (TRs), cholecalciferol (D3, VDR), as well as prostanoids (PPARs). It probably serves as a ubiquitous co-activator, instead of just another nucleoceptor co-activator. It may furthermore serve as a co-activator within the NF-kappa-B signalling pathway.

RXRA & RXRB retinoid X receptor, alpha & beta; Retinoic acid receptor. These receptors associate as heterodimeric entities to target response elements, elicited by their ligands, which are all-trans or 9-cis retinoic acid, thus regulating the expression of genes responsible for various biological processes. The heterodimers of RAR/RXR associate with response elements specific for retinoic acids (RAREs) comprising tandem 5'-AGGTCA-3' binding sites, DR1-DR5 (by similarity). They preferentially associate with 9-cis retinoic acid.

RBBP7 retinoblastoma binding protein 7; nucleocore histone-associating subunit targeting the chromatin-bound remodelling factors, histone acetyltransferases, as well as and histone deacetylases to histone substrates in a way compatible to 'its' nucleosomal DNA. It is a constituent of a plethora of complexes, regulating chromatin turnover, which include the type B histone acetyltransferase (HAT) complex, required for chromatin assembly subsequent to DNA replication; core histone deacetylase (HDAC) complexes, promoting deacetylation of histones and consequently transcriptional repression.

SUMO1&2 SMT3 'suppressor of mif two 3, homolog 1'; ubiquitin-like protein which may be covalently bound to proteins as a monomeric entity or as a lysine-linked polymer. It is covalently associated via an iso-peptide bond to its substrates–necessitates prior activation by an E1 complex (SAE1-SAE2) and firm linkage to the E2 enzyme UBE2I. It can be promoted/facilitated by E3 ligases, such as PIAS1-4, RANBP2 or CBX4. The present post-translational alteration of lysine-containing protein residues serves a decisive role in a series of cellular processes, such as intra-nuclear transport, replication and repair of DNA, mitosis, as well as signal transduction.

NCOA2 nuclear receptor co-activator 2; transcriptional co-activator for both steroid receptors in general, as well as any type of nuclear receptors. It is the co-activator of the AF-2 steroid binding domain, but not of the AF-1 modulating N-terminal domain. It is required along with NCOA1 to master the cellular energy balance between white and brown adipose tissues.

UBR5 ubiquitin protein ligase E3 component n-recognin 5; E3 ubiquitin-protein ligase, which serves as one member of the N-end rule pathway. It recognizes and associates with specific N-terminal protein residues, destabilizing in accordance with the 'N-end rule', leading to a final ubiquitination with subsequent degradation. It is involved in the maturation and/or transcriptional modulation of mRNAs by activating CDK9 (via multiple ubiquitinations). It may serve a role in the progression and control of the cell cycle. It may also exert tumor suppressor activity. It modulates the DNA topoisomerase II binding protein (TopBP1) activity within in the DNA.

A short and non-exhaustive summary (for details, see above) of the potential biological impact exerted by MK-7 is quite impressive, and encompasses phenomena like the regulation of: protein synthesis, the cell cycle, cell survival, cell adhesion, nuclear transport, DNA replication and repair, cell phenotype regulation and stabilization via impact on histone deacetylases (HDACs), as well as inflammatory processes [1]. As seen from the few genes described above, it may be asserted that vitamin K2 is also able to influence bodily phenomena like energy (lipid and glucose) metabolism, due to a balance between the amount of bodily distribution of white and brown/beige adipocyte phenotypes. It is well known that white adipocytes store fat, while brown and beige adipocytes are either directly burning fatty acids only or performing both tasks simultaneously, in order to balance the characteristics of the other two. A few articles describe the versatile biological effects of vitamin K2 analogues in detail, and are referred to in the following paragraphs.

The nuclear receptors (NRs), the pregnane X receptor (PXR) and the related constitutive androstane receptor (CAR), play important roles as part of the xenobiotic detoxification reactions by modulating the expression of drug-metabolizing enzymes and transport molecules, to aid in the degradation and excretion of foreign chemical substances, as well as endogenously produced metabolites. The present survey is seeking to expand on the perceived biomedical relevance of both the PXR and CAR beyond their established role as master xenosensors, rather focusing on disease-oriented subjects, and with emphasis on their ability to be modulated by small molecules. These nuclear receptors are apparently involved in both the development and the treatment of non-alcoholic fatty liver disease (NAFLD). These receptors are, in fact, transcription factors (TFs), that are able to sense altering environmental and/or hormone-like signals, thus effectuating transcriptional changes in order to balance vital functions like cell/organ growth, but also development and reproduction. To be able to sustain this function, the following ligand-induced activation by xenobiotics (but also by liganding vitamin K2 (e.g. MK-7), 'members of subfamily 1 nuclear receptors' (NR1s) will heterodimerize with retinoid X receptor (RXR) and thus regulate gene transcription modulated processes being engaged in energy metabolism, but also inflammation. Many of these receptors, including PPARs (peroxisome proliferator-activated receptors), PXR, CAR, LXR and FXR (pregnane

and xenobiotic, constitutive androstane, liver and X) receptors serve as key regulatory elements of the gut-liver-adipose biological axis, but also coordinate metabolic responses within organs, when oscillating between the fed and fasting states [2].

Non-alcoholic fatty liver disease (NAFLD) happens to be the most common liver ailment, which may eventually progress to cirrhosis and thereafter develop into hepatocellular carcinoma. NAFLD is characterized by insufficient nuclear receptor activity, leading to disturbed signalling through the gut-liver-adipose axis, which encompasses obesity, increased 'leakage' or permeability of the bowel system, with ensuing systemic inflammation, 'derangements' of the hepatic lipid metabolism, as well as insulin resistance. Unfortunately, environmental chemicals may complicate the issue by directly interfering with these nuclear receptors, conferring 'metabolic confusion' and thus the inability to distinguish feeding from starvation hours. Clinical investigations including conducted in the past (cfr. the PIVENS and FLINT trials) have shown that treatments aimed at these nuclear receptors may cause paradoxical reactions characterized by a dissociation of phenomena like inflammation, fibrosis, insulin resistance, dyslipidemia, steatosis and obesity. However, novel strategies (e.g. tissue-specific ligand molecules and/or dual receptor agonists) may be mandatory to be able to separate beneficial effects of nuclear receptor activation from untoward metabolic adverse effects [3].

In another investigation, one was looking into the effect of vitamin K2 on aortic calcification induced by warfarin via the Gas6/Axl survival pathway. A calcification rat model was established where warfarin was given to rats, which were divided into the following groups: controls and calcification groups, where some of the animals received vitamin K2. And the effect measurements/analyses were as follows: aortic calcium depositions (with Alizarin red); alkaline phosphatase activity in serum; apoptosis was evaluated by the TUNEL assay; and protein expression levels of Gas6, Axl, phosphorylated Akt (p-Akt) and Bcl-2 were analysed using western blotting. To summarize the results: the calcium content, calcium depositions, ALP activity and apoptosis were significantly higher in the calcification groups than control group. Furthermore, Gas6, Axl, p-Akt and Bcl-2 expression was lower in the calcification group than in the control group. Interestingly, 100 μg/g vitamin K2 administration reduced calcium depositions, ALP activity, as well as apoptosis, while Gas6, Axl, p-Akt and Bcl-2 expression were enhanced. Furthermore, vitamin K2 reversed almost half of the calcification. Finally, there was a positive correlation between formation calcification and apoptosis with $P < 0.0001$. This data therefore provides a sound theoretical basis for future treatments of aortic calcification [4].

The impact of vitamin K2 on apoptosis in different types of cancer cells have been shown in previous studies. But, the apoptotic effect of K2 on bladder cancer cells has not yet been evaluated. Hence, apoptotic activity and the underlying mechanism of K2 in bladder cancer cells were investigated. It was shown that K2 induces apoptosis in these cells via the 'mitochondria pathway' (i.e. membrane potential, cytochrome C release and the caspase-3 cascade). Also, phosphorylation of c-Jun N-terminal kinase (JNK) and p38 MAPK was detected in the vitamin K2-treated cells. Generation of ROS (reactive O_2 species) was observed in the cancer cells, however, treatment with K2 and the anti-oxidant N-acetyl cysteine virtually

blocked the K2-triggered apoptosis, loss of mitochondrial membrane potential, as well as the JNK and p38 MAPK phosphorylation. These findings show that vitamin K2 clearly induces apoptosis in bladder cancer cells via the ROS-mediated JNK/p38 MAPK and mitochondrial pathways [5].

The pregnane X receptor (PXR) was until recently considered to serve as a nuclear receptor deemed to be specialized for detecting exposure from xenobiotics. In concurrence with this characteristic, PXR was identified to modulate drug-metabolizing enzymes. During the previous decades, PXR shown to harbour a broader spectrum of features. It is now evident that ligand-activated PXR modulates hepatic glucose turnover and lipid metabolism, while also affecting metabolic homeostasis throughout the whole body. At present, the consequences of PXR-elicited modulation on overall metabolic health, are not fully investigated, however, it has been shown that Rifampicin as well as St. John's wort, both serving as prototypical human PXR agonists, impair glucose tolerance in healthy individuals. Therefore, chronic exposure to naturally occurring PXR-agonists could be construed as a risk factor for diabetes and the metabolic syndrome [6].

As one associate of heterodimeric couples (ligand-receptor complexes), the retinoid X receptor (RXR) plays a leading role within the superfamily of nuclear receptors (NRs). Some heterodimers, e.g. PPAR&RXR, LXR&RXR, as well as and FXR&RXR are perceived as 'permissive', i.e. they turn into active transcriptional moieties, when an RXR-selective ligand ('rexinoid') or an NR partner ligand is present. In contrast, the so-called 'non-permissive' heterodimers (such as RAR&RXR, VDR&RXR or TR&RXR) are non-responding (inert) to the 'rexinoids' in question, when alone. Nonetheless, the agonists turn into 'transcriptional activators', when appearing together with a synergizing partner. However, despite their constellations assumed, when serving within the heterodimer formation/activation of multiple pathways, RXR appears as a target for drug discoveries. Interestingly, a rexinoid is applied in the clinic for the treatment of cutaneous T-cell lymphoma. More importantly, a plethora of RXR modulators also beholds a therapeutical ability for the treatment of metabolic diseases. The modulatory 'skill' of the rexinoids lies in the ligand-receptor complex conformation, as well as the wide spectrum and extent of their association with co-regulators, thus sustaining the specificity of the physiological response elicited. Interestingly, collected genetic and pharmacological data, emanating from investigations of insulin sensitivity, diabetes and obesity, definitely conclude that RXR agonists and antagonists show great promise as anti-obesity agents [7].

Unfortunately, the 'therapy' with rexinoids enhances plasma triglycerides, suppresses the hypothalamic-pituitary-thyroid hormone axis, as well as induces hepatomegaly, which complicates further search for compounds to treat insulin resistance and type 2 diabetes mellitus (T2DM). The recently developed PPARγ/RXR and LXR/RXR heterodimer-selective rexinoids, acting differently from PPARγ or LXR agonists alone, could possibly circumvent these limitations. Suffice to say, therapy with vitamin K2 alone (either as MK-7 or MK-4) in combination with a suitable rexinoid, might overcome the treatment 'paradox' described above [7].

The steroid and xenobiotic receptor (SXR) and its murine orthologue, the pregnane X recep-tor (PXR), are, as mentioned many times over, expressed in the liver and intestine where, they function as xenobiotic sensors involved in detoxification and drug excretion. However, it has recently been shown that both SXR and PXR are present in osseous tissue, where they facilitate bone metabolism [8]. It was shown that a deletion of PXR provokes in an ageing-dependent wearing of the articular cartilage in knee joints. A histomorphometrical analysis demonstrated a marked diminution of the width of, as well as an enlarged gap between, articular cartilage of the femur and tibia in PXR-knockout mice. It was therefore speculated that the up-regulated SXR in the chondrocytes play a protective role in articular cartilage. Interestingly, the Fam20a (family-with-sequence-similarity-20a) gene proved to be an SXR-sensitive gene, known to be induced by SXR ligands, such as rifampicin and vitamin K2 (MK-7) [8].

Furthermore, it was proven that the expression of Fam20a was primarily seen in articular chondrocytes. Consistent with the epidemiological data given, the present results strengthen the notion that SXR/PXR may protect against ageing-dependent detrimental wearing of artic-ular cartilage, and, therefore, one may assert that ligands for SXR/PXR (such as vitamin K2) may prevent the induction of osteoarthritis due to 'old age' [9].

The fat-soluble vitamin K1 is involved in blood coagulation mediated by maintaining the activity of coagulation factors in the liver. However, the vitamin K2 variety exerts extrahe-patic 'abilities' known to prevent bone fractures. In addition, epidemiological studies suggest that a lack of vitamin K (mainly K2) is associated with several geriatric diseases, including osteoporosis (bone brittleness), osteoarthritis (inflamed joints), dementia (mental retardation) and arteriosclerosis (blood vessel stiffness/narrowing). Furthermore, it was demonstrated that vitamin K may serve as an important factor in the prevention and/or treatment various types of cancers [10].

Recently, a novel role was discovered for vitamin K, serving as a ligand of the nuclear, steroid and xenobiotic receptor (SXR), as well as its murine, pregnane X receptor (PXR) orthologue. As a supplement to its published function as a co-factor for the γ-glutamyl carboxylase (GGCX), which effectuates a plethora of post-transcriptional changes, vitamin K also displays an addi-tional mode of action, which is conveyed via transcriptionally modulations of the SXR/PXR target genes. Investigations of bone chips from PXR-deficient animals (mice) demonstrated that the osseous protection impact of vitamin K is partially conveyed via SXR/PXR-dependent signals. The discovery of new ways, by which vitamin K acts, has unravelled novel hope that this little molecule might come in handy in the prevention and/or treatment of a series of dis-eases affecting the elderly [10].

The steroid and xenobiotic/pregnane X receptor (SXR/PXR) is a nuclear receptor, which is located mainly in hepar and intestine, where they serve as xenobiotic sensors. However, many groups have identified SXR/PXR as a ubiquitous mediator of bone homeostasis. It was shown that systemic deletion of PXR brings about a marked loss of bone tissue (osteopenia) characterized by mechanical fragility in experimental animals (mice) down to 4 months of

age. Values for BMD (bone mineral density) of PXR knockout (PXRKO) animals decreased substantially, when compared with BMD-values of wild-type animals. Micro-computed tomography measurements of trabecular bone (femur) from PXRCO animals unravelled a 3D-bone volume fraction markedly lower than what was found for WT mice [9].

Furthermore, histomorphometrical measurements of the trabecular bone material from the proximal tibia demonstrates a marked diminution bone mass from the PXRKO mice. Furthermore, bone formation was reduced, whereas bone resorption was augmented in the PXRKO mice. Histomorphometrical measurements of femoral cortical bones unravelled larger cortical areas in control animals than in PXRKO animals. The WT mice displayed an augmented cortical width, compared with PXRKO controls, and the so-called 'three-point bending test' showed that the 'new' morphological phenotypes in fact led to significant mechanical fragility. And, not to forget, serum levels of phosphate, as well as calcium and alkaline phosphatase remained unaltered in the PXRKO mice, as compared with their WT littermates. Consequently, it was concluded that SXR/PXR-activation augments the bone formation/resorption ratio, thus carving out a role for SXR/PXR as the key 'provider' of bone health [9].

The steroid and xenobiotic receptor (SXR = PXR = NR1I2) is activated by a plethora of endogenous hormones, pharmaceutics (drugs), as well as xenobiotics. SXR exhibits an enlarged, flexible and hydrophobic domain (LBD) for the ligand binding, and this is markedly divergent across different species of mammals. SXR also displays a pronounced difference in its pharmaco-dynamics and -kinetics amongst different mammalian species. The versatile response profile of SXR has paved the way for launching a 'steroid and xenobiotic binding hypothesis'. SXR is well-established as a xenobiotic sensor, which in a coordinated fashion rules the clearance of xenobiotics from the liver and intestine, through the up-regulation of genes being instrumental in both drug and xenobiotic turnover and excretion. Recently, it was revealed that SXR (most unexpectedly so) in fact makes a major contribution to (1) subduing inflammation, (2) bone homeostasis, (3) vitamin D turnover, (4) lipid metabolism, (5) energy homeostasis, as well as (6) the body's defence against cancer. Hence, the discovery of SXR as more than a xenobiotic sensor enables the use of a powerful tool for scrutinizing new mechanisms via which dietary factors, chemicals and the environment ultimately impact health, as well as disease development, prevention and treatment [10–13].

It has since long been shown that the xenobiotic receptors (XRs) have functionally evolved into cellular sensors for both endogenous and exogenous stimuli. Over the past decade, it has been demonstrated that regulating of the transcription of genes encoding drug-metabolizing enzymes and transporters, as well as the genes involving energy homeostasis, cell proliferation and/or immune responses are sensitive to the XRs. Unlike prototypical steroid hormone receptors, XRs are activated through both direct ligand-binding and ligand-independent (indirect) mechanisms by a plethora of structurally unrelated chemicals. In particular, scrutiny has been on the signalling control of the constitutive androstane receptor (CAR), the pregnane X receptor (PXR), as well as the aryl hydrocarbon receptor (AhR), which, in this context, function together in a synergistic manner. The latter (AhR) normally activates natural killer (NK) cells residing within the liver [10–13].

Ever since the identification of the retinoid X receptor (RXR) being a member of the nuclear receptor (NR) superfamily, the interest it has created has unravelled new understanding of its physiological regulation by nuclear receptors. Biologically, RXR serves an important role via its potent ability to associate with a variety of nuclear receptors. RXR possesses the ability to modulate nutrient metabolism via forming so-called 'permissive' heterodimers with PPAR (peroxisome proliferator-activated receptor), the liver-X-receptor (LXR), the farnesoid X receptor (FXR), the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR), of which all of them start being functional, when ligands are associated to one or both of these heterodimers [10–13].

On the other hand, RXR will form 'non-permissive' heterodimers with the VDR (vitamin D receptor), TR (thyroid receptor), as well as RAR (retinoic acid receptor), which function properly only when vitamin D, T3 and retinoic acid, respectively, are present. Furthermore, RXR can form homodimers in the presence of a selective agonist, rexinoid, to regulate gene expression and to either inhibit proliferation or induce apoptosis in human cancers. Thus, over the last 25 years there have been several reports on the design and synthesis of small molecule RXR selective agonists, rexinoids [10–13].

A summary of some of the end point effects of agonists of the make: PXR/LXR (**Figure 2**, left panel), which appear to be working through the PI3K-AKT signalling pathway (**Figure 2**, right panel).

http://www.biochemj.org/content/453/1/71   http://www.genome.jp/kegg-bin/show_pathway?hsa04151
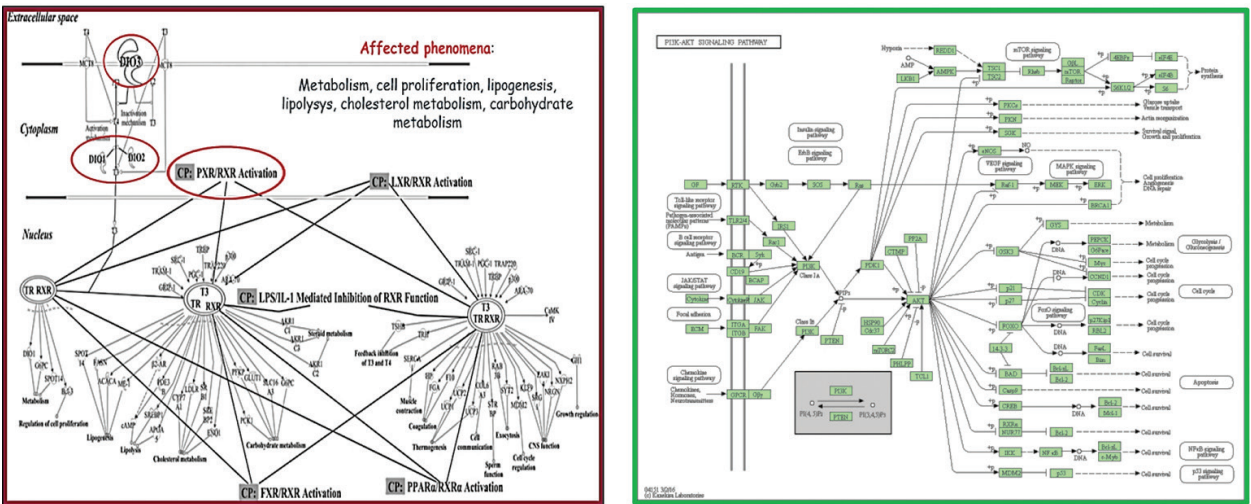

Figure 2. Summaries of genes impinging on metabolic pathways in general, cell proliferation, lipid (including cholesterol) metabolism with emphasis on the impact of DIO3 (deiodinase 3), and 'KEGGs Pathways' showing how the PI3K-AKT regulatory system affects a plethora of metabolic functions. *Source:* http://www.biochemj.org/content/453/1/71; http://www.genome.jp/kegg-bin/show_pathway?hsa04151.

On the left-hand figure, the pathways of T4 (via the deiodinase DIO3) and T3 are indicated. The keen reader will definitely study the chart for her- or himself, however, suffice to emphasize that there are the biological pathways or cellular functions on which receptors PXR/RXR and LXR/RXR exert powerful biological impacts: metabolism in general, cell

proliferation, lipogenesis, lipolysis, cholesterol metabolism, carbohydrate metabolism, steroid metabolism, feedback inhibition of T3 and T4, muscle contraction, coagulation, thermogenesis, cell communication, sperm function, exocytosis, cell cycle regulation, and CNS function [14]. On the right-hand figure, the reader can observe that the PI3K-AKT signalling pathway, used by both osteoblasts or fibroblasts/HUVEC/VIC cell phenotypes to ensure or block the development of mineralizing properties [1], or described as modulators of GSK3, Bcl-2, RXRα and NFκB, and the FOXO-family of transcription factors, which are involved in biological phenomena summarized as glycolysis/gluconeogenesis, cell cycle, apoptosis, and NFκB/p53- signalling, of which all of them can and should be construed as adaptors of cell 'lifetime' = survival and 'phenotype' = functional stabilization [14].

As can be seen from **Figure 2**, it should be quite clear (or rather obvious?) that vitamin K2 (e.g. MK-4 or MK-7) exerts a spectrum of effects on various organs, and not only serves as a vitamin/hormone with an impact on coagulation mineralization of bone and demineralization of soft tissues like blood vessel walls (ref). And there are numerous reports to be found asserting a much broader function of vitamin K2, here are but a few:

(1) 'The steroid and Xenobiotic receptor (SXR), beyond xenobiotic metabolism' [15], which describes the impact of vitamin K2 on cholesterol and lipid homeostasis, bile acid homeostasis, inflammatory bowel disease (featuring SXR and NFκB), interplay between SXR and other receptors like CAR, FXR, VDR, the implication of SXR in cancer development and treatment, the implication (synergy) of SXR, and FoxO1, and FoxA2 in energy homeostasis.

(2) 'Role of Pregnane X Receptor in Obesity and Glukose Homeostasis in Male mice' [16], where PXR (the receptor binding vitamin K2) knock-out mice display weight increase, with a concomitant increase in liver weight (hepatomegalia), hyperinsulinemia and hypoadiponectinemia, as well as a loss of R2 receptors for adiponectin. These trends are all associated with the development of type II diabetes (T2DM).

(3) 'Targeting xenobiotic receptors PXR and CAR for metabolic diseases' [17], where the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) was found to be involved in normalizing metabolic disorders like the metabolic syndrome, T2DM, dyslipidemia, as well as atherosclerosis.

(4) 'Novel Functions of PXR in cardiometabolic disease' [18], where the authors in the abstract asserts that recent studies have revealed novel and unexpected roles of PXR in modulating obesity, insulin sensitivity, lipid homeostasis, atherogenesis, and vascular functions. These studies suggest that PXR signalling may contribute significantly to the pathophysiological effects of many known xenobiotics on cardiometabolic disease in humans. Genes mentioned in this article are many, and classified under functions like: 'cholesterol and lipid metabolism', 'lipoprotein metabolism', glucose metabolism', cholesterol and lipid metabolism', 'inflammation and atherosclerosis', detoxification and oxidative stress protection'.

(5) 'Regulation of PXR and CAR by protein-protein interaction and signalling crosstalk' [19], where the authors assert that 'it is clear that PXR and CAR perform a much broader range of cellular functions through protein-protein interactions and signalling crosstalk, which typically mutually affect function of all the partners involved. Future research on PXR and CAR should, therefore, look beyond their xenobiotic functions'.

(6) 'Mechanism of xenobiotic receptor activation: Direct vs indirect' [20], where the authors focus on the nuclear receptor (NR; remember that SXR = PXR also goes by the name NR1/2 = Nuclear Receptor 1/2) and its mode of activation, via pairing with a different nuclear receptor partner (activators/inhibitors, such as RXR, CCRP, NCOR, SMRT and several others). PXR may also be activated indirectly, which contributes to a meticulous and intricate regulatory system. Suffice to say that vitamin K2 (e.g. MK-7 or MK-4) is, but one factor contributing to the activation of NR1I2 = SXR = PXR, and is probably dependent on a certain intra-cellular/intra-nuclear level (as a 'cut-point' for proper activation) to exert a significant/maximal effect.

These six reports (of several more) signify that vitamin K2 may and will be of significance as to optimize and/or maximize certain toward effects, beneficial to organ systems in the body, as well as optimization of inter-organ cross-talk for the benefit of organ health and homeostasis. Finally, it should be mentioned that microRNAs play an important part in the regulatory network determining the impact of nuclear proteins responsible for proper gene expression—metabolic function—cell phenotype 'development' and stabilization.

1. Role of microRNAs in the determination of cellular phenotype

MicroRNAs (miRNAs or miRs) are conserved, small non-coding RNAs (18–25 nucleotides long) instrumental in the regulation of gene expression, and serve as a part of a network of factors, including transcription factors determining the phenotype of a certain cell in the body (ref). The transcription factors, such as the SXR = PXR = NR1I2 serve as receptors, much the same way as the receptors for vitamin A (RXR) and D (VDR), and may modulate gene transcription to determine the cell phenotype with its defined phenotypic characteristics (e.g. mineralizing osteoblast or non-mineralizing vessel-lining epithelial cells or heart valve fibroblast). It is well known that vitamin K2 (MK-7) stabilizes the two phenotypes (also in the presence of an inflammatory environment), thus preserving 'correct' inter-organ cross-talk, as would be expected in a healthy organism.

First, it should be emphasized that vitamin K2 binds to a nuclear receptor, which is part of a regulatory network consisting of microRNAs and other transcription factors. Second, this network represents a minimal lattice of regulatory factors, which may be manipulated in order to breach the stability of a certain cell phenotype (e.g. a cancer cell), or doing the opposite: reinforcing the phenotype in question (e.g. mineralizing osteoblast and non-mineralizing fibroblast during renal failure, uremia, for instance). Without going into detail, it is asserted that the

regulatory networks presented here bargain for: (1) how vitamin K2 is involved in the stabilization of the osteoblastic phenotype, and (2) which are the major players (microRNAs and genes) determining whether a cell will adapt mineralizing properties or not (osteoblast or fibroblast).

Suffice to say (with reference to **Figure 3**), the master transcription factor JUN, impinges on a set of microRNAs (let-7 species) in a hierarchical structure of traditional genes and other microRNAs, which are well known in the literature, as being part of the WNT-Notch, the TGFβ and the BMP pathways (determining the osteoblast phenotype) (see KEGGs pathways), where specific markers like WNT6, DKK1, and CTNNB1 (β-catenin, activator of Runx2, the most referred marker of osteoblastic cells), are represented, and where some of the major microRNA-species, like miR-125, miR-21, miR-221, miR-27 and miR-23, known to be important in the differentiation and stabilization of the osteoblast phenotype is ensured.
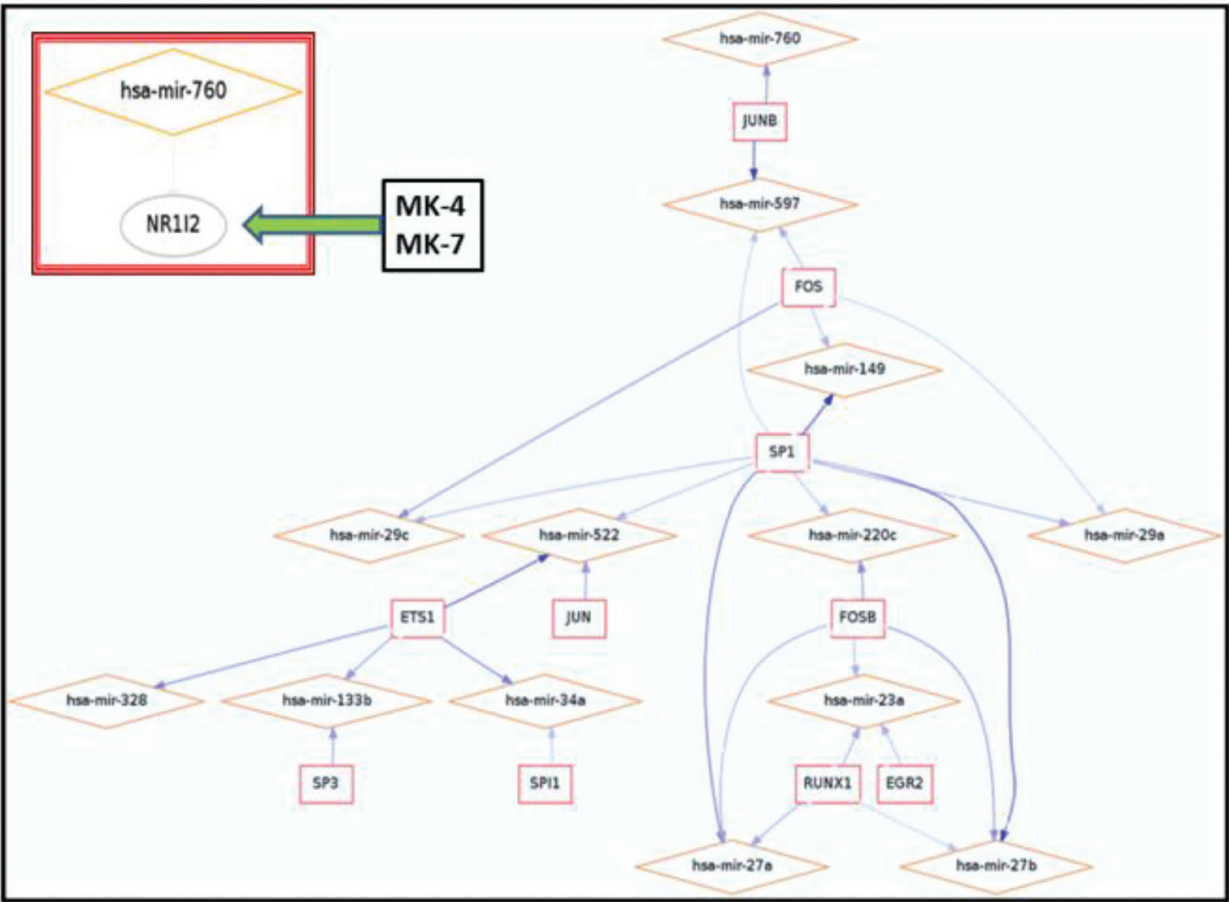

Figure 3. The involvement of vitamin K2 (MK-4 and MK-7), binding to NR1I2 = SXR = PXR, exerting its effect via hsa-miri-760 on regulatory loops in osteoblasts (according to the **Mir@nt@n** algorithm). This chart shows how vitamin K2 may affect the regulatory system determining the phenotype of osteoblast as a mineralizing cell in the body, involving microRNAs, transcription factors (e.g. FOS, JUN, SP1 & SP3) and 'functional' or 'marker' genes, like RUNX1.

In very much the same way, one may analyse the gene-transcription factor—microRNA axis in conditions like non-alcoholic fatty liver disease (NAFLD) or non-alcoholic steatohepatitis (NASH), and hopefully arrive at blood-born microRNAs, representing the diseases in

question and/or their severity. MicroRNA species and putative target genes related to liver disease (NAFLD & NASH), as well as cardio (vascular) affection were pooled from three different articles (**Figure 5**).
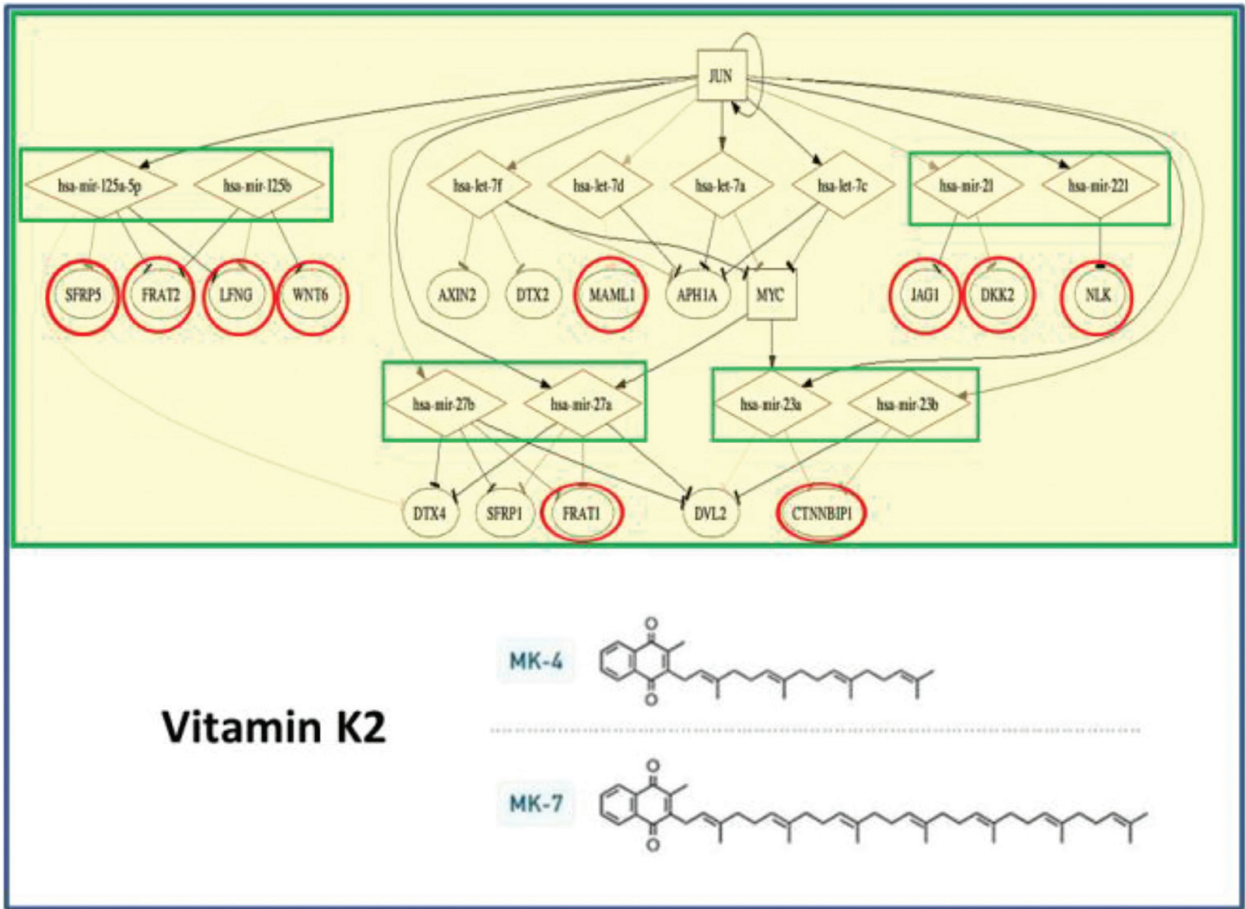

Figure 4. Hierarchical regulatory system consisting of microRNAs, transcription factors and genes involved in the mineralization phenotype of osteoblastic cells, but also present in fibroblasts having attained untoward/unwanted mineralizing properties during exposure to inflammatory cytokines and/or co-cultured with Th-1 and Th-17 cells.

At highest stringency applied, there are but a few genes and microRNAs emerging as 'connected': hsa-mir-122 with FBXO32 (F-Box protein 32; involved in FOXO-mediated signalling) and STAT4 (transcription factor); hsa-mir-144 with ABCA1 (ATP binding cassette subfamily A member 1, involved in cholesterol and sphingolipids transport from golgi and ER to the apical membrane and regulation of lipid metabolism by PPARα), hsa-mir-33b with ABCA1 and SLC25A25 (solute carrier family 25 member 25), and finally hsa-mir-145 with TFAM (transcription factor A, mitochondrial). Without going into details, all three articles [21–23], emphasize microRNA species 122, 144, and 33b, as instrumental in regulating hepatic lipid metabolism, with emphasis on hsa-mir-122. This microRNA-species is instrumental in the optimization of fatty acid oxidation vs synthesis, cholesterol production, as well as VLDL secretion to the circulation, and thus determining the health status of any individual in terms of risk of incurring atherosclerosis. The fact that this regulatory system, shown in **Figure 4**, lacks reciprocal regulatory loops makes it more vulnerable and

unstable, when threatened by 'disease states', like NAFLD/NASH, than systems found in the osteoblast, which apparently appears more resilient to change, when exposed to conditions where inflammation prevails.
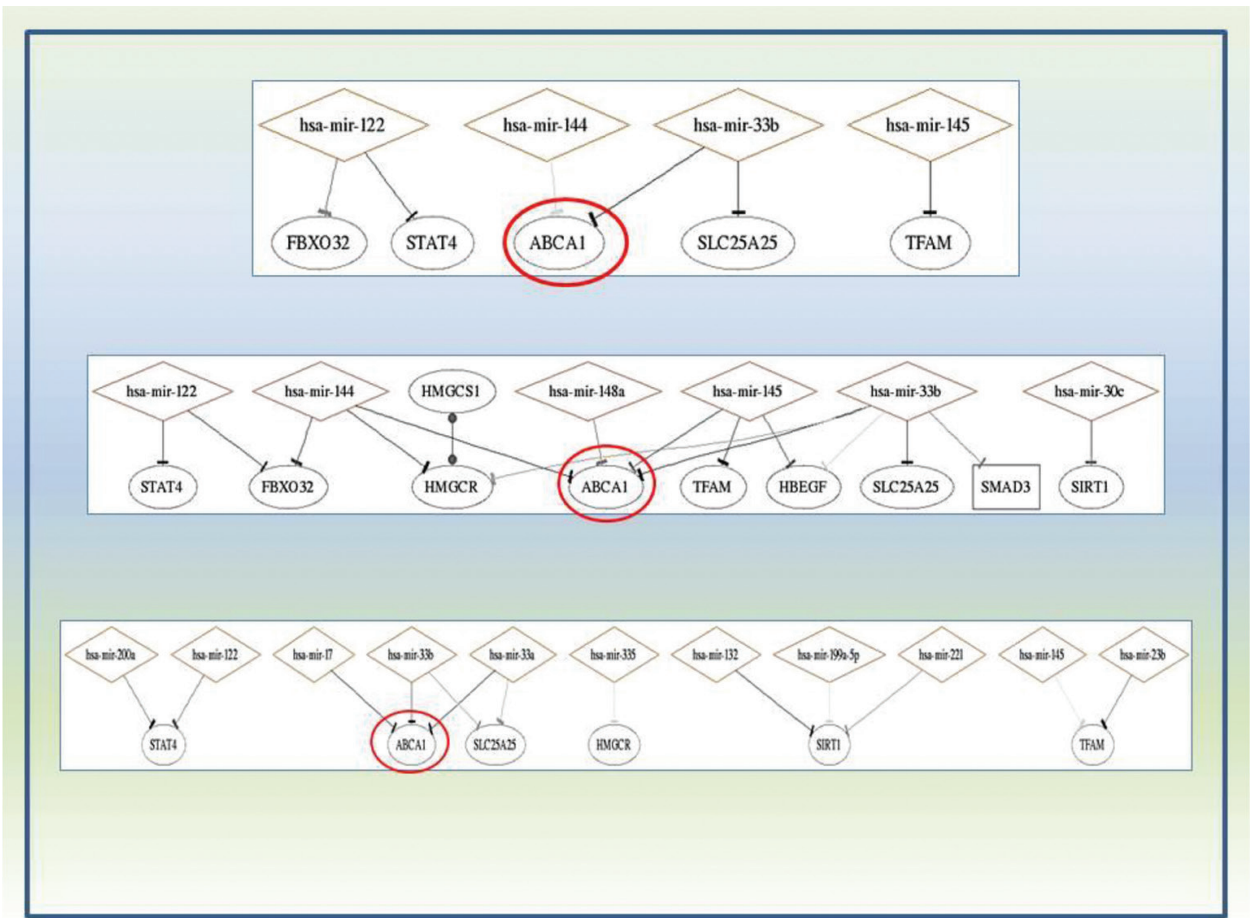

Figure 5. MicroRNAs, transcription factors and 'functional' genes related to liver function in patients with NAFLD/NASH with or without metabolic cardiovascular disease (see Refs. [21–23]). The three charts represent decreasing stringency/from top to bottom), and it should be emphasized that the regulatory system does not contain any reciprocal regulatory feedback systems, as was shown for the 'stabilization' of the osteoblast (or mineralizing phenotype).

Finally, it should be emphasized that bioinformatics analyses of the 'NRI2-relative' NR1I3, the constitutive androstane receptor (CAR), which interacts with NR1I2 = SXR = PXR, is also biologically interfering with many of the same factors (e.g. PPARα, CEPBα, STATs and T3) [24], thus linking them together in a very tight regulatory network, affecting lipid metabolism. Understanding the impact of vitamin K2 on these regulatory systems seems to be mandatory to grasp and acknowledge the idea that this fat-soluble molecule exerts such a tremendous effect on biological processes compatible with organ health, disease free old age, and thus 'longevity'.

Addendum 1

List of microRNAs and genes used as 'input' into the Mir@nt@n-algorithm, asking the program to 'retrieve' regulatory networks

hsa-mir-223, hsa-mir-320, hsa-mir-122, hsa-mir-27b, hsa-mir-30c, hsa-mir-27a, hsa-mir-378, hsa-mir-335, hsa-mir-148a, hsa-mir-370, hsa-mir- 33a, hsa-mir-33b, hsa-mir-144, hsa-mir-10b, hsa-mir-17, hsa-mir-19b, hsa-mir-26, hsa-mir-33a, hsa-mir-33b, hsa-mir-93, hsa-mir-101, hsa-mir-106b, hsa-mir-128, hsa-mir-144, hsa-mir- 145, hsa-mir-148a, hsa-mir-302a, hsa-mir-758, hsa-mir-223, hsa-mir 320, hsa-mir-122, hsa-mir- 613, hsa-mir-155, hsa-mir-143, hsa-mir-132, hsa-mir-145, hsa-mir-161, hsa-mir-211, hsa-mir-241, hsa-mir-146a, hsa-mir-30c, hsa-mir-99a, hsa-mir-122, hsa- mir-29c, hsa-mir- 320a, hsa-mir- 133a, hsa-mir-7, hsa-mir- 93, hsa-mir-98, hsa-mir-161, hsa-mir-191, hsa-mir- 195, hsa-mir-203, hsa-mir- 425, has-mir-422, hsa-mir-200a, hsa mir- 519d, hsa-mir-23b, hsa-mir-199a-5p, hsa-mir-199a-3p, hsa-mir-107, hsa-mir-103, hsa-mir-203, hsa-mir-21, hsa-mir-23, hsa-mir-24, hsa-mir-221, hsa-mir-223, hsa-mir-224, and hsa-mir-320b,

with or without:

hsa-mir-122, hsa-mir- 33a, hsa-mir-33b, hsa-mir- 132, hsa-mir-145, hsa-mir- 211, hsa-mir-144, hsa-mir-148a, hsa-mir-161, hsa-mir- 241, hsa-mir-146a, hsa-mir-30c, hsa-mir-223, hsa-mir-24, and hsa-mir-29,

combined with:

FOXO3, p300, fbxo32, ABCA1, SREBP1, SREBP2, PGC-1α, SLC25A25, NRF1, TFAM, HMGCS1, SMO1 SMO2 , LDLR, HMGCS2, hfe, hjv, LPL, HMGCR, LDLR, MTP, ASO, LPL, smad3, c/EBP, VIM, ADRP, DGAT, CPTA1, FABP4, LOX, ago2, STAT4, HBEGF, and Sirt1,
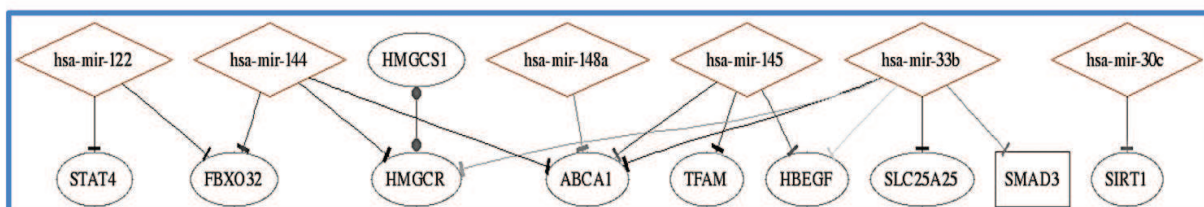
Author details

Jan Oxholm Gordeladze

Address all correspondence to: j.o.gordeladze@medisin.uio.no

Institute of Medical Biochemistry, Medical Faculty, University of Oslo, Oslo, Norway

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