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Effect of flavonoids on fetal programming : implications for cancer susceptibility

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Effect of Flavonoids on Fetal Programming

Implications for Cancer Susceptibility

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Chapter 1

General introduction

Environmental and dietary exposures alter disease risk

Adult chronic illnesses, such as cancer, cardiovascular diseases and respiratory diseases account for the majority of deaths worldwide (1). Since the complete sequencing of the human genome, genes involved in disease susceptibility have been and will be distinguished, resulting in the improvement of predicting an individual's risk on disease (2). Cancer affects millions of people worldwide and represents a major burden of disease. However, only 5-10 % of all cancers are caused by genetic defects, while 90-95 % is caused by environmental factors of which the diet is the most important one (30-35 %) (3).

Throughout their life humans are constantly exposed to drugs and other foreign compounds. To prevent that these substances cause any damage, they are neutralized and excreted. Environmental genotoxicants and mutagens cause about 80 % of human malignancies (4). One important group of environmental contaminants are the polycyclic aromatic hydrocarbons (PAHs), which we are exposed to through inhalation of smoke from coal, wood, diesel fuel and tobacco; but also through ingestion of roasted, smoked or charbroiled foods. PAH can be metabolically activated to derivates that can damage our DNA, which is an important initiating event in carcinogenesis.

Another important source of DNA damaging compounds are the so-called reactive oxygen species (ROS). Enhanced exposure to ROS is linked to the onset and propagation of different diseases, for instance neurodegenerative diseases (5-9), cardiovascular diseases (10, 11), diabetes (12) and cancer (13-15), but are also believed to contribute to aging (16). They are regularly produced by mitochondria as a consequence of the aerobic metabolism. For instance 1-5 % of total oxygen consumed in aerobic metabolism gives rise to superoxide anion radicals ($O_2^{\cdot-}$) (17, 18). However, ROS are also formed by microsomal CYP450, flavoprotein oxidase and peroxisomal enzymes involved in fatty acid metabolism. Besides, they play a crucial role as signal transducers to regulate cell proliferation and they are also involved in the destruction of invading pathogens (18). The negative outcomes of ROS are thought to be the result of an imbalance between the amount of antioxidants and ROS, termed oxidative stress (17).

How can we protect ourselves against environmental exposure? Role of flavonoid supplementation

It is well known that a healthy diet contributes to our protection against chronic diseases. One major dietary group of components that receives a lot of attention in the scientific literature, because of their potential protective effects, are the flavonoids. Dietary flavonoids comprise a large group of polyphenolic compounds widely distributed throughout the diet as they play a role in plant's pigmentation and flavor (19). They have a common structure consisting of 3 phenolic rings defined as the A, B and C ring (Figure 1A). Depending on the oxidation level of the C ring flavonoids can be divided into 6 subclasses, namely flavones (apegenin, luteolin), flavonols (*quercetin*, kaempferol), flavanones (naringenin, hesperedin), flavanols (epicatechin, gallocatechin), anthocyanidins (malvidin,

cyanidin) and isoflavones (*genistein*, daidzein) (19-21). The daily dietary intake of mixed flavonoids in the human population ranges from 65 to 250 mg a day (22).

The most predominant flavonoid in the human diet is the flavonol quercetin (Figure 1B), which is mainly found in onions, apples, tea and red wine (19, 20). The average daily dietary intake of quercetin is estimated to be in the range of 10-100 mg (23). This polyphenolic compound is a potent free radical scavenger and iron chelator (24, 25).

Another important flavonoid is the isoflavone genistein (Figure 1C), which is mainly found in soybeans. Genistein is also known to be a phytoestrogen due to its structural similarities to 17β-estradiol and can therefore mimic or antagonize estrogen (26, 27). Due to the low soy-containing food consumption in Western countries the daily intake of isoflavones is in the range of several milligrams a day. However, isoflavone intake can increase dramatically when consuming soymilk, for instance by people allergic to cow milk, as soy milk contains between 30 and 175 mg/ L isoflavones. In Asian countries the daily intake of isoflavones is much higher due to the high consumption of soy and reaches levels of 25-40 mg a day (28). Moreover, the level of isoflavones, mainly genistein, found in soy-based infant formula lies even above the levels found in Asian adults (29).
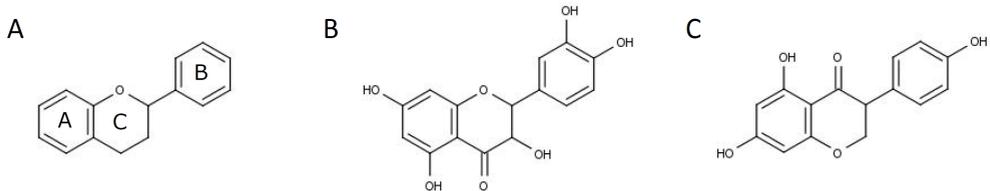
Figure 1. A. Basic flavonoid structure, B. Structure of quercetin, C. Structure of genistein.

In addition, as quercetin and genistein both are flavonoids, they possess besides the above mentioned properties very potent antioxidant capacities. Up till now, this is the best studied property of flavonoids to which several health benefits, like anti-inflammatory (30, 31), protection against cardiovascular diseases (32, 33), protection against neurodegenerative diseases (34, 35), protection against COPD (progression) (36, 37), and protection against ocular disease (38, 39) are attributed. This is also the main reason why flavonoids are gaining interest as treatment and prevention of adult diseases (19, 40-42). Flavonoids are therefore freely available as high dose supplements, with a daily recommended dose that can be as high as 1-2 gram per day (43, 44).

Moreover, studies concerning the use of dietary supplements during pregnancy are limited, it has been reported for the US that 78 % of the pregnant woman take supplements during pregnancy (45). Although it is know that flavonoids transfer across the placenta to accumulate in the fetus (46, 47), there is yet little known about their actions in pregnancy and the effects on the offspring.

Flavonoids during pregnancy: Infant leukemia

Besides the assumed health benefits, it is suggested that flavonoids can be detrimental when exposed to *in utero*, as they are thought to be involved in the onset of infant

leukemia (48, 49). Infant leukemia is characterized by an increased white blood cell count within the first twelve months after birth. It is the second most common malignancy within the first year of life (50, 51). Infant leukemia is thought to be the result of chromosomal translocations, creating fusion genes; deletions of chromosomal segments or of individual genes; duplication of segments or whole chromosomes (50, 52). The chromosomal translocations seen in infant leukemia are thought to be the result of the inhibition of topoisomersase II (topoII), for which fetal cells are more sensitive to as they are rapidly proliferating cells and have high topoII activity (53). This nuclear enzyme is normally involved in the relaxation of supercoiled DNA during replication and transcription. It binds to double-stranded DNA, cuts one of both helical chains, creating a transient double-strand break (DSB) through which the other helical chain passes after which the DNA strand is religated. This decreases the winding of the DNA, making it more available for replication or transcription. However, topoII-inhibitors can inhibit the religation, increasing the risk on translocations (Figure 2) (52, 54).
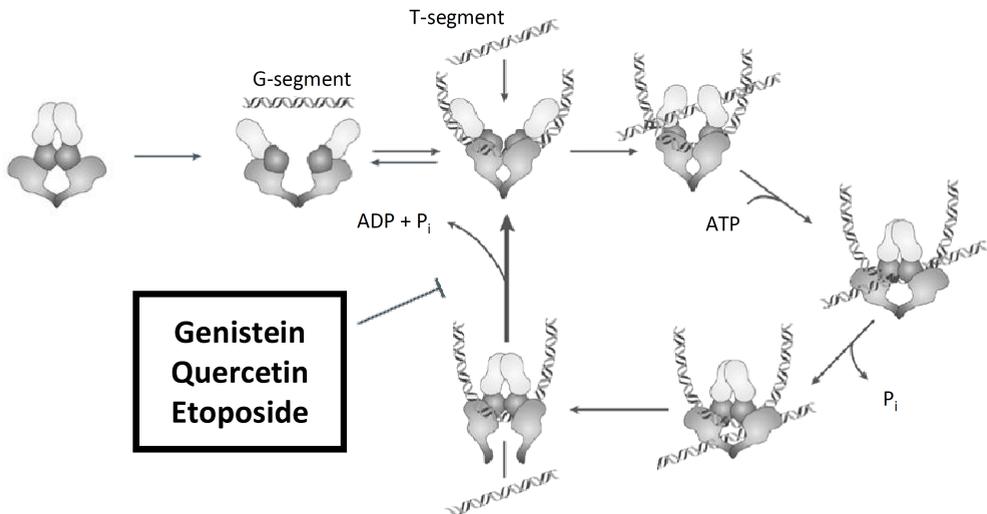
Figure 2. DNA helix relaxation by topoisomerase II.
TopoII relaxes the DNA helix by binding to topoII binding sites on both DNA chains. Next, it generates a transient DSB in one of the helical chains through which the other chain passes, followed by the resealing of the break. TopoII-inhibitors, like genistein, quercetin and etoposide inhibit the resealing of the DNA, increasing the risk on translocations. (The figure was adapted from Nitiss (55))

The most common genetic aberration associated with infant leukemia is a rearrangement in the mixed-lineage leukemia gene (*MLL*) found in up to 80 % of the children suffering from acute lymphoblastic leukemia (ALL; concerning abnormal expansion of T- or B-cells) and up to 50 % of children with myeloid leukemia (AML; concerning all other white blood cell types). This gene is involved in normal hematopoiesis and is located at chromosome 11q23. Largely all *MLL* translocations are located at a 8.3 kb breakpoint cluster region (BCR) which covers exon 5 to 11 of the gene. However, no specific translocation partners are identified. In addition, *MLL* rearrangements are also found in 25 % of patients suffering from secondary leukemia resulting from chemotherapy with DNA topoII-inhibitors (e.g. etoposide) (52). Strikingly, it has been

suggested that several dietary compounds, for instance flavonoids (48, 49, 56) but also caffeine (57) can inhibit the action of topoII. Still, the complex nature of human diets makes it impossible to elucidate the exact contribution of these dietary compounds to the onset of infant leukemia. It is generally accepted that childhood leukemia originates *in utero* as leukemia is present in newborns. Moreover, monozygotic twins suffering from leukemia have identical rearrangements of the *MLL* gene (49, 58).

Normally DSB are recognized by the ataxia-telangiectasia mutated (ATM) protein. This protein will activate checkpoints to slow down the cell cycling of cells carrying DNA damage and recruit proteins to repair the damage or if the DNA damage is too severe lead to apoptosis. Mutations in this gene result in the disease ataxia-telangiectasia (A-T). Patients suffering from this autosomal recessive disorder have an increased risk on developing cancer. One third of the A-T patients develop cancer of which the majority being lympoid malignancies (e.g. leukemia) (59). About 0.5-1 % of the human population carries at least one mutated *ATM* gene (59, 60).

Different studies suggest that mutations in *ATM* could play a role in the development of leukemia (61-63). Lavin *et al.* (64, 65) developed a murine model of A-T carrying a common mutation seen in humans with A-T, namely the 7636del9 deletion. These *Atm-ΔSRI* mice have an in-frame deletion of 9 nucleotides, resulting in a non functional Atm protein, lacking its kinase activity. Heterozygous *Atm-ΔSRI* mice have a mean age of tumor onset of 18.6 months, while only 30 % of homozygous *Atm-ΔSRI* mutant mice are still alive at 16 months of age. Heterozygous and homozygous *Atm-ΔSRI* mice suffer from different tumors of which one third is represented by leukemias and lymphomas.

Flavonoids during pregnancy: Fetal programming

Since the late 1980s the idea that maternal nutrition during pregnancy associates with an increased risk on degenerative diseases later in life gained interest (66, 67). A large number of studies on fetal over- or under-nutrition have been performed to investigate the adverse effects at adult age (66, 68-70). A clear example is given by the Dutch famine, which occurred during the winter of 1944-1945. Here, reduced nutrient supply during the first trimester of pregnancy resulted in a higher prevalence of obesity and cardiac heart diseases in the adult male progeny (71, 72). This study produced evidence for the 'thrifty genotype' also known as the `Barker Hypothesis`, defining the concept of fetal programming, namely an attempt of the fetus to adapt to adverse conditions encountered *in utero* resulting in adaptations that will be detrimental when these conditions will not prevail later in life (73). A general overview of the concept of fetal programming is given in Figure 3.

Over the years, it turned out that also other diseases at adulthood are linked to *in utero* malnutrition. For instance, maternal low caloric diets are also thought to increase the risk on diabetes type 2 in offspring at adult age (74-77). These studies on the concept of fetal programming showed that fetal nutritional deprivation (maternal caloric or macronutrient deficiency during pregnancy) is a strong programming stimulus (66, 68, 70). However, in many Western societies, maternal nutrition is sufficient or even excessive. For instance, maternal high saturated fat diet resulted in insulin resistance, obesity and hypertension in offspring at adult age (78), which indicates that developmental problems may not only be a consequence

of under-nutrition, but can also be achieved by an unbalanced diet during pregnancy. Thus, maternal over-nutrition can be deleterious to the health of the offspring and results in a phenotype of the offspring that is linked to increased health risks in adulthood.

The use of dietary supplements has tremendously increased over the past decades, and sometimes the manufacturer advices the intake of excessive amounts. Also for flavonoid supplements the recommended intake is high, because up till now no human data on long-term effects of high-dose flavonoid supplementation are available (21). As much as 78 % of woman use natural health products during pregnancy as these micronutrients are essential for a good pregnancy outcome (45), which includes the intake of folate. However, the ideal diet composition for pregnant woman has still to be found, especially in relation to dietary supplements. Although the intake may not be directly toxic to the developing child, the fetal programming hypothesis predicts that physiological alterations may occur that persist into adulthood. To fully understand the safety of supplements during pregnancy, such effects should be taken into account, and more research is needed in understanding the phenomenon of 'fetal programming'.
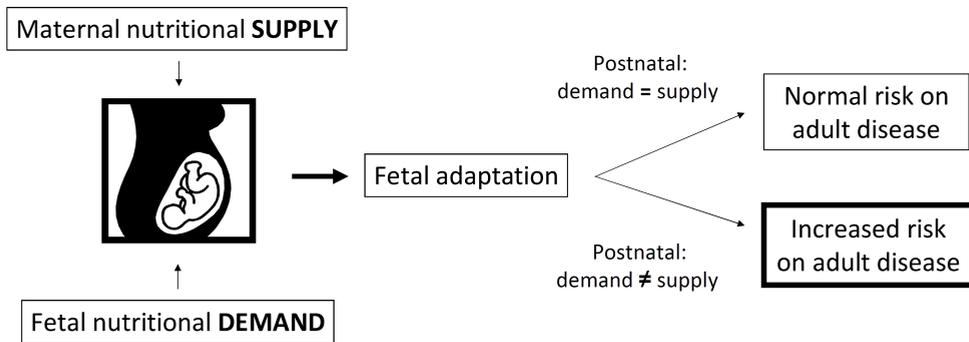
Figure 3. Overview of the concept of fetal programming.
During pregnancy the fetus will adapt according to its nutritional demand and its mother's nutritional supply, making the fetus programmed to the *in utero* nutritional status as it is thought to reflect the nutritional situation it will encounter once born. Indeed, when the postnatal nutritional demand corresponds to the nutritional supply, the adaptations made by the fetus *in utero* will result in a normal development and disease susceptibility. However, if the postnatal nutritional demand does not correspond to the nutritional supply, the risk on adult diseases increases.

Epigenetics: a tool for fetal programming

The first theory comprising the responsiveness of the genome to the environment and its modifying capacity was proposed by Jean-Baptiste Lamarck, in the early 19th century (79). However, his ideas were neglected due to lack of evidence and aberrant conceptualization (79, 80). Conrad Waddington was the first to follow in Lamarck's steps, in the first half of the 20th century. He introduced the word 'epigenetics', which stands for 'epi' meaning 'upon' or 'over' and 'genetics' implying the involvement of genes, to define the study of events over or beyond genes. The term 'epigenetics' was derived from the Aristotelian word 'epigenesis' implying that developmental changes are gradual and qualitative, but that there is a link to heredity (81).

As the phenomena of epigenetic manifestations are gaining increasing appreciation, researchers grow eager, focusing their studies on understanding the control of gene expression and its connection with the underlying processes of human development of diseases. Moreover, they seek the origin of disease in the epigenetic regulation within the placenta (82, 83). Evidently studies regarding the epigenetic alterations within the placenta have provided crucial insights into the fetal origins of the susceptibility to specific diseases later in life. Such epigenetic mechanisms include changes in promoter DNA methylation resulting in hypo- or hyper-methylation of DNA CpG regions; modifications of histone proteins; chromatin conformation as well as micro- and non-coding RNA-mediated control of gene expression within the placenta (82-85). However, considering this thesis, only the epigenetic regulation *via* DNA methylation will be further elaborated.

DNA methylation can be described as the incorporation of a methyl (CH_3) group, obtained from the S-adenylmethionine that serves the purpose of a methyl donor, on a cytosine ring located in CG dinucelotides forming methyl-cytosine, which is catalyzed by DNA methyltransferases (DNMTs) (86). The maintenance and incorporation of these methyl groups is carried out by specific DNMTs, namely DNMT3A, DNMT3B and DNMT1 (82, 86). The finding that this DNA modification occurs predominantly on cytosines that come before guanosine in the sequence and that this occurs mostly in non-coding DNA indicates its importance in global genomic maintenance. Methylation of so-called CpG islands in DNA generally leads to silencing of transcription. CpG islands are mainly located in gene promoter regions, which are mostly unmethylated in order to allow normal gene expression. Therefore, aberrant methylation patterns may be detrimental for the well being and can lead to the development or progression of human diseases.

During fetal development, epigenetic alterations are crucially involved in the orchestration of gene expression; the epigenome cycles through several precisely timed methylation changes to ensure proper development (87). Shortly after fertilization the paternal genome is actively demethylated, while the maternal genome is passively demethylated. Some epigenetic marks are maintained to allow proper expression of imprinted genes (parent-of-origin methylation marks) (87) (about 100 identified in mammals) (88). Subsequently, a new pattern of DNA methylation, which is also referred to as *de novo* methylation, is established predominantly at the blastocyst stage (82, 83, 89). This *de novo* methylation can be considered as a reprogramming of the DNA methylation patterns in the zygote, which are normally retained throughout the organism's life, and are crucial for somatic cell viability (82, 83, 89). The appropriate succession of the resetting and reprogramming phase of methylation is fundamental to post-gestational health and survival. This signifies an important sensitive window within the development of an embryo, where the environment can have profound effects on the expression pattern of certain genes throughout life (83, 90). A clear example of fetal programming by diet induced changes in methylation is given by Dolinoy *et al.* (91). They showed that heterozygous agouti mice exposed to the flavonoid genistein *via* the maternal diet, at levels comparable with humans consuming a high-soy diet, resulted in an altered coat color, namely towards pseudo-agouti. These mice were also protected against obesity later in life, which is associated with the agouti phenotype. These changes were caused by hypermethylation of transposable repetitive elements, namely intracisternal A particles (IAP), upstream of the transcription start site of the *Agouti* gene which remained unaltered throughout life.

Aims and outlines of this thesis

Many studies on human diseases, including many types of cancers, suggest that the *in utero* period plays a role in the occurrence of adult diseases (92-94). Therefore, in this thesis we aimed to provide more insight in how dietary flavonoids could alter cancer susceptibility of the individual through direct effects on genomic stability (Chapter 2) and *via* fetal programming (Chapter 3-6).

Effects on genetic stability and formation of translocations

In Chapter 2 the effect of *in utero* exposure to the flavonoids genistein and quercetin on the onset of *Mll* translocations, is assessed using a mouse model with an impaired DNA repair for double-strand breaks. As translocations in *MLL* may not directly result in the onset of infant leukemia, they do increase the risk on developing leukemia later in life (Figure 4).
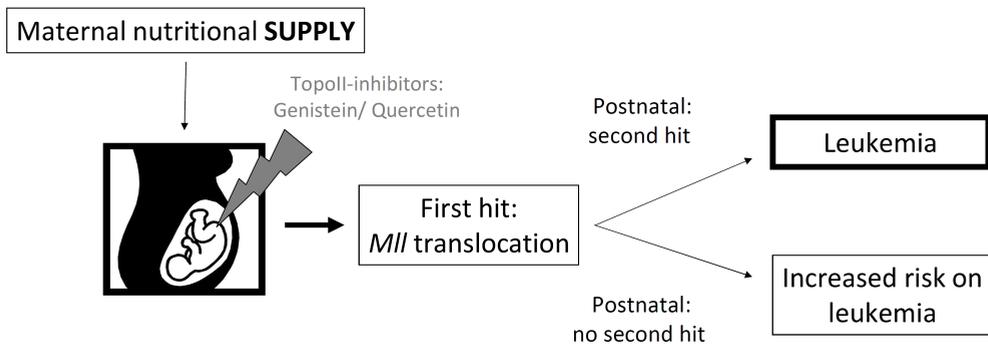
Figure 4. Assumed outcome of increased maternal intake of topoII-inhibitors during pregnancy on infant leukemia. *Via* the maternal diet the fetus will be exposed to increased levels of the topoII-inhibitors genistein or quercetin. This could result in *Mll* translocation ('first hit') as fetal cells are rapidly dividing cells and therefore more sensitive towards topoII inhibitors. Throughout life, the offspring has an increased risk on developing leukemia. Whether or not the offspring will develop leukemia depends on following events ('second hit' or more) it will encounter throughout life.

Health effects later in life by persistent changes; examples studied in this thesis

Persistent changes in gene expression

It has been shown that prenatal exposure to genistein results in long-lasting alterations in gene expression of agouti mice. Therefore the long-lasting effects of prenatal exposure to genistein on bone marrow cell gene expression, concerning genes involved in hematopoiesis and estrogen responsive genes (as genistein is a know phytoestrogen) are investigated as described in Chapter 3.

Oxidative stress related processes

Flavonoids are mostly known for their protective antioxidant effects in adults. However, there is no unambiguous hypothesis as some studies show that flavonoids act as antioxidants, while other studies attribute a pro-oxidant function to these compounds at high concentrations (95-97). Breinholt *et al.* (98) showed that female rats exposed to genistein or quercetin *via* the diet had reduced enzymatic antioxidant capacity of red blood cells. They hypothesized that the antioxidant enzymes in red blood cells were down-regulated by the flavonoids in response to an improved antioxidant status of the red blood cells due to the presence of flavonoids. Furthermore, it has been demonstrated that quercetin up-regulates the gene expression of *NQO1* and *GSTP1*, as well as the gene expression of *Nrf2* in Caco-2 cells (99). This can be considered as a pro-oxidant effect, because when oxidative stress occurs, nuclear factor erythroid 2-related factor (Nrf2) pathway is activated to overcome this stress. Nrf2, a cytosolic protein, is associated with its chaperon Kelch-like ECH-associated protein (Keap1) *via* protein-protein interactions resulting in its proteasomal degradation. However, in the presence of oxidative stress, Keap1-Nrf2 interactions are disrupted and Nrf2 translocates into the nucleus were it binds to antioxidant response elements (ARE), resulting in the transcription of phase II enzymes, including *NQO*, *GST* and *UGT* (100). Other important antioxidant enzymes are superoxide dismutase (SOD) which scavenges $O_2^{\cdot-}$ and catalase and glutathione peroxidase (GPX) which scavenges hydrogen peroxide (H_2O_2). The body's innate antioxidant capacity can be augmented by antioxidants delivered through the diet, for instance vitamin C and E, β-carotene, but also for these dietary antioxidants it is known that they may promote oxidative stress under certain conditions (101), leading to damage to proteins and membranes (18, 102). Damage to membranes caused by free radicals (lipid peroxidation) results in the generation of malondialdehyde that can react with DNA resulting in the formation of M_1dG-adducts. However, M_1dG-adducts can also be generated by hydroxyl radicals, independently from lipid peroxidation by the removal of deoxyribose 4'-hydrogen in DNA, generating a base propenal. M_1dG-adducts are normally repaired *via* the nucleotide excision repair pathway, but malfunctioning of this pathway can result in mutations and eventually cancer (103). Oxidized proteins can be repaired by ascorbate or glutathione (GSH). If not, for instance if the levels of ascorbate and GSH are depleted, then these proteins are recycled by proteases. When irreversibly modified proteins cannot be processed by proteases and accumulate in the cell, making it impossible for the cell to properly function resulting in its death (102). ROS can also directly oxidize DNA resulting in the formation of 8-oxo-7,8-dihydro-2'deoxyguanosine (8-oxo-dG) (103).

As CYP450 activity also results in the formation of ROS (18, 103), a link between the aryl hydrocarbon receptor (AhR) and Nrf2-pathway is suggested (104, 105). This has been confirmed by the finding of a xenobiotic binding site of AhR in the regulatory region of the *Nrf2* gene (106). For this interaction, see section 'Quercetin as AhR agonist modifies xenobiotic metabolism' below.

Therefore, the effect of prenatal exposure to quercetin, but also to genistein on the antioxidant capacity at adult age is investigated to elucidate whether a pre-emptive trigger of the antioxidant system during fetal development will result in adaptations at adulthood (Chapter 4).

Iron chelating properties of quercetin as programming trigger

In addition, as quercetin is a powerful iron chelator, the effects of prenatal exposure to quercetin on iron homeostasis and eryhtropoiesis was determined at adulthood but also during the fetal development, as iron is an essential micronutrient for a normal development (Chapter 5). Iron plays an essential role in the generation of ROS. Its redox cycling promotes the formation of the potent oxidating hydroxyl radical (Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$) (107). However, iron is also an essential micronutrient that besides the oxidative metabolism is also required for erythropoiesis and optimal functioning of immune responses. About 1-2 mg of iron is daily absorbed from the diet and this absorption is tightly regulated due to the fact that the body has no effective means of excreting iron. Once iron is released into the circulation it binds to transferrin and is transported to sites of use or to be stored. About 65 % of the body's iron is used for the production of hemoglobin (108, 109). Hemoglobin consists out of a porphyrin ring containing an iron ion (heme part of hemoglobin) and 4 globin chains (2 α-globin and 2 β-globin chains). The production of the globin chains is dependent on the availability of heme and because heme production is limited by iron supply it is the amount of available iron that limits the hemoglobin production (110).

Depending on the phase of life, different forms of hemoglobin are produced, namely embryonic hemoglobin and adult hemoglobin. In case of mice the first form of hemoglobin, that is the embryonic hemoglobin, consists out of embryonic globins, namely ζ -globin, which is the embryonic form of α-globin and βH1- and εy-globins the embryonic forms of the β-globin (111, 112). This form of hemoglobin is produced solely by primitive erythrocytes, which are generated by the yolk sac and consist of large erythroblasts which are present in the bloodstream from embryonic day (E) 7.5 (111-113). Adult hemoglobin consists out of the adult α- and β-globins (111, 112) and is produced by erythrocytes from the definitive adult lineage, but also by the primitive erythrocytes, though the amount of adult globins expressed in the primitive erythrocytes is negligible (111, 112). At E11.5 erythrocytes from the definitive adult lineage appear in the bloodstream. These cells are first formed by the fetal liver and from birth onwards, primarily by the bone marrow (111-113). After E16.5 the definitive erythrocytes are the predominant cell type in the fetal circulation and ultimately become the exclusive erythrocyte lineage in the adult (111-113). One main difference between both lineages is the place they undergo terminal maturation. Primitive erythroblasts enucleate in the bloodstream, resulting in the formation of a reticulocyte and a pyrenocyte (this is the extruded nucleus). Definitive adult erythrocytes on the other hand mature and enucleate in the fetal liver and later on in life in the bone marrow after which the reticulocyte is released into the bloodstream (111, 112, 114). During erythroid ontogeny in humans, a third form of hemoglobin is produced, namely the fetal hemoglobin, which appears between the embryonic and the adult hemoglobin (112).

For both humans and mice, the switch from embryonic to adult globins is thought to be triggered by difference in oxygen environment at the different stages of pregnancy. During early development, in the uterus, the embryo resides in a low oxygen environment. This hypoxic state is necessary for the early embryogenesis and organogenesis, because at this time point the embryo is the most vulnerable for oxidative stress, which could lead to damage or disruption of the embryo (115, 116). In mice, the utero-placental circulation is

well established between E9.5 and E11. This leads to a higher concentration of oxygen and therefore to an increase in ROS, but it also leads to an enhancement of the antioxidant defense system (116). Because of the low oxygen supply during early development, cells from the primitive erythroid lineage are expressed. Since they consist of embryonic globins which have a high affinity to oxygen they can permanently remove oxygen from the adult globins expressed in the maternal erythrocytes through the placenta (117, 118). Later, when the oxygen supplies increases between E9.5 and E11 (116), the switch from primitive to definitive erythroid lineage takes place (111-113).

During pregnancy the maternal iron absorption raises to fulfill the increasing iron requirement of the fetus However, the increase in iron absorption by the mother during pregnancy is normally not sufficient and therefore maternal iron stores are implemented what could result in iron deficiency anemia (119, 120). Iron deficiency represents the most common nutritional deficit worldwide (121) and has serious consequences for both the mother and the unborn child as it increases the risk of maternal mortality and the risk on cardiovascular disease for the child later in life (119, 120).

Iron homeostasis is highly regulated in adults to prevent the generation of ROS. Therefore, iron that is not used is mainly stored in the liver, where it is bound to ferittin. For an optimal erythropoiesis, internal turnover of iron is needed. Senescent erythrocytes are cleared by macrophages in the liver but primarily in the spleen. The heme part of hemoglobin is metabolized by hemoxygenase-1 (HO1) and the released iron is then recycled for the subsequent hemoglobin production (108, 109). In iron overload diseases the liver is the main organ suffering from injury. In mice, dietary quercetin supplementation decreased the hepatic iron storage and increased the excretion of iron through the feces, thereby lowering the risk on ROS induced damage (122). Previous studies have shown that quercetin can penetrate the cytoplasm of erythrocytes to interact with the heme iron in hemoglobin. This results in the oxidation of heme iron from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state and consequentially to the formation of methemoglobin, which is inactive and incapable of transporting oxygen. However, it also decreases the risk on ROS induced hemolysis (24, 25, 107).

Quercetin as AhR agonist modifies xenobiotic metabolism

Quercetin is also a know aryl hydrocarbon receptor (AhR) ligand an could therefore protect against DNA damage induced by compounds that need metabolism *via* the expression of phase I and II enzymes, including polycyclic aromatic hydrocarbons (PAH). Therefore, the effect of prenatal exposure to quercetin on detoxifying enzymes is described in Chapter 6. Throughout their life, humans are exposed to drugs, environmental contaminants and other foreign compounds. To prevent that these substances cause any damage, they are usually neutralized by xenobiotic metabolism and excreted. Environmental genotoxicants and mutagens cause about 80 % of human malignancies (4). PAHs are widespread environmental contaminants to which we are exposed to through inhalation of smoke from coal, wood, diesel fuel and tobacco; but also through ingestion of roasted, smoked or charbroiled foods. Benzo[a]pyrene (B[a]P) is the best studied PAH and is found to be a mutagenic and carcinogenic environmental pollutant. B[a]P needs to be metabolically activated to become genotoxic. This is mainly performed by the heme-containing phase I enzymes, cytochrome P450 (CYP) 1A1 and CYP1B1 (4, 123, 124). The initial step in B[a]P metabolism is performed by

these phase I enzymes and results in the formation of epoxides, which are in turn the substrate for epoxide hydrolase, resulting in the formation of diols. Next the phase I enzymes will convert the diols into diol epoxides (125). The formed diol epoxide of B[a]P, namely B[a]P-7,8-dihydrodiol-9,10-epoxide (BPDE), is a reactive intermediate that can bind covalently to nucleic acids and proteins. In addition, B[a]P can also be converted into phenols and subsequently to quinones by phase I enzymes (125). However B[a]P can also be detoxified by phase I and especially by phase II enzymes (glutathione-S-transferases (GST), UDP-glucurosyltransferases (UGT) and sulfotransferases (SULT)), converting it into a water-soluble derivate facilitating its excretion (see Figure 5). Activation of these enzymes is regulated through the activation of AhR. This cytosolic protein is activated by B[a]P, whereupon it transfers into the nucleus. Here it dimerizes with AhR nuclear translocator (ARNT) after which it can bind to xenobiotic responsive elements to induce phase I and phase II enzymes (124).
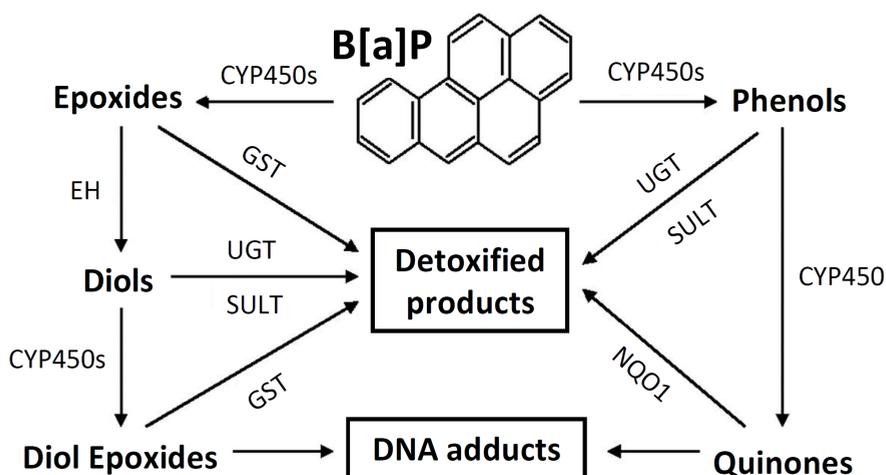
Figure 5. Simplified overview of B[a]P metabolism.
Simplified scheme of B[a]P metabolization, activation and detoxification performed by phase I (CYP450s) and phase II enzymes (EH: epoxide dehydrolase, GST: glutathione-S-transferases, UGT: UDP-glucurosyltransferases, SULT: sulfotransferase, and NQO1: NAD(P)H:quinine oxidereductase 1) (from Ambrosone *et al.* (126)).

Inter-individual variation in drug metabolism and associated cancer susceptibility can partly be explained by different expression and/ or activity of phase I and II enzymes induced by genetic polymorphisms in these genes (127-129), and therefore reduce or increase the exposure to harmful compounds. Other studies also suggest that there is a gender-dependent expression of phase I and II enzymes (130-133). This is thought to be the result of endocrine factors, namely estrogen, which is the female hormone and is also metabolized by CYPs. Metabolism of estrogen by CYPs can also result in reactive metabolites that can damage the DNA (134). Moreover, males and females have a differential susceptibility for B[a]P induced carcinogenicity as males seem to have more protection due to higher levels of GST isoenzymes compared to females (135). In addition, PAHs or their metabolites could have estrogenic effects, which could contribute to their carcinogenicity (136). Though, up till now epidemiological studies are inconsistent regarding the differences in carcinogen metabolism between genders (137, 138).

Another important factor explaining inter-individual differences in xenobiotic metabolism are environmental factors, especially the diet (139, 140). It is estimated that the diet can reduce cancer death rates by 35 % (139). For instance, flavonoids can bind and therefore activate AhR, resulting in an altered expression of phase I enzymes (100, 141, 142). Different studies have also shown that flavonoids can induce phase II gene expression preventing DNA damage (100, 143). Moreover, maternal quercetin intake leads to long-term alterations of CYP activity in female rat offspring (144). Besides altering phase I and phase II gene expression, quercetin also enhances the excretion of B[a]P metabolites from the body (145). When quercetin was co-incubated with B[a]P, it influenced the B[a]P induced effects by increasing the B[a]P induced expression of *CYP1A1* and *1A2*. It also resulted in a diminishing B[a]P induced up-regulation of *Nrf2* and some of its target genes and in an up-regulation of the AhR repressor, which was suppressed by B[a]P (99).

Summary: overall aim of the thesis

Taken together, Chapter 2 describes the possibility of *in utero* exposure to genistein and quercetin to affect genetic stability, namely by assessing the onset of *Mll* translocations in a mouse model with an impaired DNA repair for double-strand breaks. Chapter 3 describes the long-lasting effects of prenatal exposure to genistein on hematopoiesis and on the expression of genes involved in hematopoiesis and estrogen responsive genes (as genistein is a know phytoestrogen). As genistein and quercetin both are potent antioxidants, the effect of prenatal exposure to both flavonoids on the antioxidant capacity at adult age is investigated in Chapter 4 by pre-emptive triggering of the antioxidant system during fetal development. Quercetin is, besides a potent antioxidant, also a powerful iron chelator. Therefore, the effect of prenatal exposure to quercetin on iron homeostasis and eryhtropoiesis was determined during fetal development and at adulthood as described in Chapter 5. Chapter 6 describes the effect of prenatal exposure to quercetin on detoxifying enzymes, as quercetin is also a know aryl hydrocarbon receptor (AhR) ligand and could therefore protect against B[a]P induced DNA damage, as B[a]P needs to be metabolized by AhR induced phase I and II enzymes. As in Chapter 3, 5 and 6 long-lasting changes in gene expression due to *in utero* exposure to genistein or quercetin are investigated, epigenetic mechanisms, namely the induction of changes in methylation status of repetitive elements, are also investigated in these chapters. Overall Chaptor 4-6 describes the effect of prenatal exposure to flavonoids on the overall risk of an individual to develop cancer. Finally, a summary and general discussion on the most important findings of all studies presented in this thesis and the future perspectives are given in Chapter 7.

References

1. Darnton-Hill, I., Nishida, C., and James, W. P. (2004) A life course approach to diet, nutrition and the prevention of chronic diseases. *Public Health Nutr* 7, 101-121
2. Collins, F. S., and McKusick, V. A. (2001) Implications of the Human Genome Project for medical science. *JAMA* 285, 540-544
3. Sutandyo, N. (2010) Nutritional carcinogenesis. *Acta Med Indones* 42, 36-42
4. Khan, T. H., Jahangir, T., Prasad, L., and Sultana, S. (2006) Inhibitory effect of apigenin on benzo(a)pyrene-mediated genotoxicity in Swiss albino mice. *J Pharm Pharmacol* 58, 1655-1660
5. Bolanos, J. P., Moro, M. A., Lizasoain, I., and Almeida, A. (2009) Mitochondria and reactive oxygen and nitrogen species in neurological disorders and stroke: Therapeutic implications. *Adv Drug Deliv Rev* 61, 1299-1315
6. Hensley, K., Butterfield, D. A., Hall, N., Cole, P., Subramaniam, R., Mark, R., Mattson, M. P., Markesbery, W. R., Harris, M. E., Aksenov, M., and et al. (1996) Reactive oxygen species as causal agents in the neurotoxicity of the Alzheimer's disease-associated amyloid beta peptide. *Ann N Y Acad Sci* 786, 120-134
7. Multhaup, G., Ruppert, T., Schlicksupp, A., Hesse, L., Beher, D., Masters, C. L., and Beyreuther, K. (1997) Reactive oxygen species and Alzheimer's disease. *Biochem Pharmacol* 54, 533-539
8. Tabner, B. J., Turnbull, S., El-Agnaf, O., and Allsop, D. (2001) Production of reactive oxygen species from aggregating proteins implicated in Alzheimer's disease, Parkinson's disease and other neurodegenerative diseases. *Curr Top Med Chem* 1, 507-517
9. Tieu, K., Ischiropoulos, H., and Przedborski, S. (2003) Nitric oxide and reactive oxygen species in Parkinson's disease. *IUBMB Life* 55, 329-335
10. Touyz, R. M. (2004) Reactive oxygen species and angiotensin II signaling in vascular cells -- implications in cardiovascular disease. *Braz J Med Biol Res* 37, 1263-1273
11. Yoshizumi, M., Tsuchiya, K., and Tamaki, T. (2001) Signal transduction of reactive oxygen species and mitogen-activated protein kinases in cardiovascular disease. *J Med Invest* 48, 11-24
12. Muhammad, S., Bierhaus, A., and Schwaninger, M. (2009) Reactive oxygen species in diabetes-induced vascular damage, stroke, and Alzheimer's disease. *J Alzheimers Dis* 16, 775-785
13. Lau, A. T., Wang, Y., and Chiu, J. F. (2008) Reactive oxygen species: current knowledge and applications in cancer research and therapeutic. *J Cell Biochem* 104, 657-667
14. Renschler, M. F. (2004) The emerging role of reactive oxygen species in cancer therapy. *Eur J Cancer* 40, 1934-1940
15. Weinberg, F., and Chandel, N. S. (2009) Reactive oxygen species-dependent signaling regulates cancer. *Cell Mol Life Sci* 66, 3663-3673
16. Dugan, L. L., and Quick, K. L. (2005) Reactive oxygen species and aging: evolving questions. *Sci Aging Knowledge Environ* 2005, pe20
17. Reuter, S., Gupta, S. C., Chaturvedi, M. M., and Aggarwal, B. B. (2010) Oxidative stress, inflammation, and cancer: how are they linked? *Free Radic Biol Med* 49, 1603-1616
18. Seifried, H. E., Anderson, D. E., Fisher, E. I., and Milner, J. A. (2007) A review of the interaction among dietary antioxidants and reactive oxygen species. *J Nutr Biochem* 18, 567-579
19. Ross, J. A., and Kasum, C. M. (2002) Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annu Rev Nutr* 22, 19-34
20. Aherne, S. A., and O'Brien, N. M. (2002) Dietary flavonols: chemistry, food content, and metabolism. *Nutrition* 18, 75-81
21. Skibola, C. F., and Smith, M. T. (2000) Potential health impacts of excessive flavonoid intake. *Free Radic Biol Med* 29, 375-383
22. Erdman, J. W., Jr., Balentine, D., Arab, L., Beecher, G., Dwyer, J. T., Folts, J., Harnly, J., Hollman, P., Keen, C. L., Mazza, G., Messina, M., Scalbert, A., Vita, J., Williamson, G., and Burrowes, J. (2007) Flavonoids and heart health: proceedings of the ILSI North America Flavonoids Workshop, May 31-June 1, 2005, Washington, DC. *J Nutr* 137, 718S-737S
23. Egert, S., and Rimbach, G. (2011) Which sources of flavonoids: complex diets or dietary supplements? *Adv Nutr* 2, 8-14
24. Ferrali, M., Signorini, C., Caciotti, B., Sugherini, L., Ciccoli, L., Giachetti, D., and Comporti, M. (1997) Protection against oxidative damage of erythrocyte membrane by the flavonoid quercetin and its relation to iron chelating activity. *FEBS Lett* 416, 123-129

25. Kitagawa, S., Sakamoto, H., and Tano, H. (2004) Inhibitory effects of flavonoids on free radical-induced hemolysis and their oxidative effects on hemoglobin. *Chem Pharm Bull (Tokyo)* 52, 999-1001

26. Miodini, P., Fioravanti, L., Di Fronzo, G., and Cappelletti, V. (1999) The two phyto-oestrogens genistein and quercetin exert different effects on oestrogen receptor function. *Br J Cancer* 80, 1150-1155

27. Marik, R., Allu, M., Anchoori, R., Stearns, V., Umbricht, C. B., and Khan, S. (2011) Potent genistein derivatives as inhibitors of estrogen receptor alpha-positive breast cancer. *Cancer Biol Ther* 11, 883-892

28. Manach, C., Scalbert, A., Morand, C., Remesy, C., and Jimenez, L. (2004) Polyphenols: food sources and bioavailability. *Am J Clin Nutr* 79, 727-747

29. Setchell, K. D., Zimmer-Nechemias, L., Cai, J., and Heubi, J. E. (1998) Isoflavone content of infant formulas and the metabolic fate of these phytoestrogens in early life. *Am J Clin Nutr* 68, 1453S-1461S

30. Valsecchi, A. E., Franchi, S., Panerai, A. E., Rossi, A., Sacerdote, P., and Colleoni, M. (2011) The soy isoflavone genistein reverses oxidative and inflammatory state, neuropathic pain, neurotrophic and vasculature deficits in diabetes mouse model. *Eur J Pharmacol* 650, 694-702

31. Boots, A. W., Drent, M., de Boer, V. C., Bast, A., and Haenen, G. R. (2011) Quercetin reduces markers of oxidative stress and inflammation in sarcoidosis. *Clin Nutr* 30, 506-512

32. Mahn, K., Borras, C., Knock, G. A., Taylor, P., Khan, I. Y., Sugden, D., Poston, L., Ward, J. P., Sharpe, R. M., Vina, J., Aaronson, P. I., and Mann, G. E. (2005) Dietary soy isoflavone induced increases in antioxidant and eNOS gene expression lead to improved endothelial function and reduced blood pressure in vivo. *FASEB J* 19, 1755-1757

33. Loke, W. M., Proudfoot, J. M., Hodgson, J. M., McKinley, A. J., Hime, N., Magat, M., Stocker, R., and Croft, K. D. (2010) Specific dietary polyphenols attenuate atherosclerosis in apolipoprotein E-knockout mice by alleviating inflammation and endothelial dysfunction. *Arterioscler Thromb Vasc Biol* 30, 749-757

34. Heo, H. J., and Lee, C. Y. (2004) Protective effects of quercetin and vitamin C against oxidative stress-induced neurodegeneration. *J Agric Food Chem* 52, 7514-7517

35. Mercer, L. D., Kelly, B. L., Horne, M. K., and Beart, P. M. (2005) Dietary polyphenols protect dopamine neurons from oxidative insults and apoptosis: investigations in primary rat mesencephalic cultures. *Biochem Pharmacol* 69, 339-345

36. Ganesan, S., Faris, A. N., Comstock, A. T., Chattoraj, S. S., Chattoraj, A., Burgess, J. R., Curtis, J. L., Martinez, F. J., Zick, S., Hershenson, M. B., and Sajjan, U. S. (2010) Quercetin prevents progression of disease in elastase/LPS-exposed mice by negatively regulating MMP expression. *Respir Res* 11, 131

37. Hirayama, F., Lee, A. H., Binns, C. W., Hiramatsu, N., Mori, M., and Nishimura, K. (2010) Dietary intake of isoflavones and polyunsaturated fatty acids associated with lung function, breathlessness and the prevalence of chronic obstructive pulmonary disease: possible protective effect of traditional Japanese diet. *Mol Nutr Food Res* 54, 909-917

38. Miyamoto, N., Izumi, H., Miyamoto, R., Kondo, H., Tawara, A., Sasaguri, Y., and Kohno, K. (2011) Quercetin induces the expression of peroxiredoxins 3 and 5 via the Nrf2/NRF1 transcription pathway. *Invest Ophthalmol Vis Sci* 52, 1055-1063

39. Cornish, K. M., Williamson, G., and Sanderson, J. (2002) Quercetin metabolism in the lens: role in inhibition of hydrogen peroxide induced cataract. *Free Radic Biol Med* 33, 63-70

40. Arts, I. C., and Hollman, P. C. (2005) Polyphenols and disease risk in epidemiologic studies. *Am J Clin Nutr* 81, 317S-325S

41. Hollman, P. C., and Katan, M. B. (1999) Dietary flavonoids: intake, health effects and bioavailability. *Food Chem Toxicol* 37, 937-942

42. Yao, L. H., Jiang, Y. M., Shi, J., Tomas-Barberan, F. A., Datta, N., Singanusong, R., and Chen, S. S. (2004) Flavonoids in food and their health benefits. *Plant Foods Hum Nutr* 59, 113-122

43. Espin, J. C., Garcia-Conesa, M. T., and Tomas-Barberan, F. A. (2007) Nutraceuticals: facts and fiction. *Phytochemistry* 68, 2986-3008

44. Harwood, M., Danielewska-Nikiel, B., Borzelleca, J. F., Flamm, G. W., Williams, G. M., and Lines, T. C. (2007) A critical review of the data related to the safety of quercetin and lack of evidence of in vivo toxicity, including lack of genotoxic/carcinogenic properties. *Food Chem Toxicol* 45, 2179-2205

45. Sullivan, K. M., Ford, E. S., Azrak, M. F., and Mokdad, A. H. (2009) Multivitamin use in pregnant and nonpregnant women: results from the Behavioral Risk Factor Surveillance System. *Public Health Rep* 124, 384-390

46. Schroder-van der Elst, J. P., van der Heide, D., Rokos, H., Morreale de Escobar, G., and Kohrle, J. (1998) Synthetic flavonoids cross the placenta in the rat and are found in fetal brain. *Am J Physiol* 274, E253-256

47. Bonacasa, B., Siow, R. C., and Mann, G. E. (2011) Impact of dietary soy isoflavones in pregnancy on fetal programming of endothelial function in offspring. *Microcirculation* 18, 270-285

48. Barjesteh van Waalwijk van Doorn-Khosrovani, S., Janssen, J., Maas, L. M., Godschalk, R. W., Nijhuis, J. G., and van Schooten, F. J. (2007) Dietary flavonoids induce MLL translocations in primary human CD34+ cells. *Carcinogenesis* 28, 1703-1709

49. Strick, R., Strissel, P. L., Borgers, S., Smith, S. L., and Rowley, J. D. (2000) Dietary bioflavonoids induce cleavage in the MLL gene and may contribute to infant leukemia. *Proc Natl Acad Sci U S A* 97, 4790-4795

50. Eden, T. (2010) Aetiology of childhood leukaemia. *Cancer Treat Rev* 36, 286-297

51. Emerenciano, M., Koifman, S., and Pombo-de-Oliveira, M. S. (2007) Acute leukemia in early childhood. *Braz J Med Biol Res* 40, 749-760

52. Aplan, P. D. (2006) Chromosomal translocations involving the MLL gene: molecular mechanisms. *DNA Repair (Amst)* 5, 1265-1272

53. Zandvliet, D. W., Hanby, A. M., Austin, C. A., Marsh, K. L., Clark, I. B., Wright, N. A., and Poulsom, R. (1996) Analysis of foetal expression sites of human type II DNA topoisomerase alpha and beta mRNAs by in situ hybridisation. *Biochim Biophys Acta* 1307, 239-247

54. Gilliland, D. G., Jordan, C. T., and Felix, C. A. (2004) The molecular basis of leukemia. *Hematology Am Soc Hematol Educ Program*, 80-97

55. Nitiss, J. L. (2009) Targeting DNA topoisomerase II in cancer chemotherapy. *Nat Rev Cancer* 9, 338-350

56. Spector, L. G., Xie, Y., Robison, L. L., Heerema, N. A., Hilden, J. M., Lange, B., Felix, C. A., Davies, S. M., Slavin, J., Potter, J. D., Blair, C. K., Reaman, G. H., and Ross, J. A. (2005) Maternal diet and infant leukemia: the DNA topoisomerase II inhibitor hypothesis: a report from the children's oncology group. *Cancer Epidemiol Biomarkers Prev* 14, 651-655

57. Ross, J. A. (2000) Dietary flavonoids and the MLL gene: A pathway to infant leukemia? *Proc Natl Acad Sci U S A* 97, 4411-4413

58. Greaves, M. (2005) In utero origins of childhood leukaemia. *Early Hum Dev* 81, 123-129

59. Khanna, K. K. (2000) Cancer risk and the ATM gene: a continuing debate. *J Natl Cancer Inst* 92, 795-802

60. Barlow, C., Hirotsune, S., Paylor, R., Liyanage, M., Eckhaus, M., Collins, F., Shiloh, Y., Crawley, J. N., Ried, T., Tagle, D., and Wynshaw-Boris, A. (1996) Atm-deficient mice: a paradigm of ataxia telangiectasia. *Cell* 86, 159-171

61. Meier, M., den Boer, M. L., Hall, A. G., Irving, J. A., Passier, M., Minto, L., van Wering, E. R., Janka-Schaub, G. E., and Pieters, R. (2005) Relation between genetic variants of the ataxia telangiectasia-mutated (ATM) gene, drug resistance, clinical outcome and predisposition to childhood T-lineage acute lymphoblastic leukaemia. *Leukemia* 19, 1887-1895

62. Oguchi, K., Takagi, M., Tsuchida, R., Taya, Y., Ito, E., Isoyama, K., Ishii, E., Zannini, L., Delia, D., and Mizutani, S. (2003) Missense mutation and defective function of ATM in a childhood acute leukemia patient with MLL gene rearrangement. *Blood* 101, 3622-3627

63. Gumy Pause, F., Wacker, P., Maillet, P., Betts, D., and Sappino, A. P. (2003) ATM gene alterations in childhood acute lymphoblastic leukemias. *Hum Mutat* 21, 554

64. Spring, K., Ahangari, F., Scott, S. P., Waring, P., Purdie, D. M., Chen, P. C., Hourigan, K., Ramsay, J., McKinnon, P. J., Swift, M., and Lavin, M. F. (2002) Mice heterozygous for mutation in Atm, the gene involved in ataxia-telangiectasia, have heightened susceptibility to cancer. *Nat Genet* 32, 185-190

65. Spring, K., Cross, S., Li, C., Watters, D., Ben-Senior, L., Waring, P., Ahangari, F., Lu, S. L., Chen, P., Misko, I., Paterson, C., Kay, G., Smorodinsky, N. I., Shiloh, Y., and Lavin, M. F. (2001) Atm knock-in mice harboring an in-frame deletion corresponding to the human ATM 7636del9 common mutation exhibit a variant phenotype. *Cancer Res* 61, 4561-4568

66. Barker, D. J. (1991) The intrauterine environment and adult cardiovascular disease. *Ciba Found Symp* 156, 3-10; discussion 10-16

67. Barker, D. J., Osmond, C., and Law, C. M. (1989) The intrauterine and early postnatal origins of cardiovascular disease and chronic bronchitis. *J Epidemiol Community Health* 43, 237-240

68. McMillen, I. C., MacLaughlin, S. M., Muhlhausler, B. S., Gentili, S., Duffield, J. L., and Morrison, J. L. (2008) Developmental origins of adult health and disease: the role of periconceptional and foetal nutrition. *Basic Clin Pharmacol Toxicol* 102, 82-89

69. Vickers, M. H., Breier, B. H., Cutfield, W. S., Hofman, P. L., and Gluckman, P. D. (2000) Fetal origins of hyperphagia, obesity, and hypertension and postnatal amplification by hypercaloric nutrition. *Am J Physiol Endocrinol Metab* 279, E83-87

70. Li, Y., Jaddoe, V. W., Qi, L., He, Y., Lai, J., Wang, J., Zhang, J., Hu, Y., Ding, E. L., Yang, X., Hu, F. B., and Ma, G. (2011) Exposure to the Chinese famine in early life and the risk of hypertension in adulthood. *J Hypertens* 29, 1085-1092

71. Ravelli, A. C., van Der Meulen, J. H., Osmond, C., Barker, D. J., and Bleker, O. P. (1999) Obesity at the age of 50 y in men and women exposed to famine prenatally. *Am J Clin Nutr* 70, 811-816

72. Roseboom, T. J., van der Meulen, J. H., Ravelli, A. C., Osmond, C., Barker, D. J., and Bleker, O. P. (2001) Effects of prenatal exposure to the Dutch famine on adult disease in later life: an overview. *Twin Res* 4, 293-298

73. Barker, D. J., and Clark, P. M. (1997) Fetal undernutrition and disease in later life. *Rev Reprod* 2, 105-112

74. Inoue, T., Kido, Y., Asahara, S., Matsuda, T., Shibutani, Y., Koyanagi, M., and Kasuga, M. (2009) Effect of intrauterine undernutrition during late gestation on pancreatic beta cell mass. *Biomed Res* 30, 325-330

75. Prentice, A. M., and Moore, S. E. (2005) Early programming of adult diseases in resource poor countries. *Arch Dis Child* 90, 429-432

76. Dumortier, O., Blondeau, B., Duvillie, B., Reusens, B., Breant, B., and Remacle, C. (2007) Different mechanisms operating during different critical time-windows reduce rat fetal beta cell mass due to a maternal low-protein or low-energy diet. *Diabetologia* 50, 2495-2503

77. Garofano, A., Czernichow, P., and Breant, B. (1997) In utero undernutrition impairs rat beta-cell development. *Diabetologia* 40, 1231-1234

78. Liang, C., Oest, M. E., and Prater, M. R. (2009) Intrauterine exposure to high saturated fat diet elevates risk of adult-onset chronic diseases in C57BL/6 mice. *Birth Defects Res B Dev Reprod Toxicol* 86, 377-384

79. Jablonka, E., and Lamb, M. J. (2007) Precis of Evolution in Four Dimensions. *Behav Brain Sci* 30, 353-365; discusssion 365-389

80. Holliday, R. (2006) Epigenetics: a historical overview. *Epigenetics* 1, 76-80

81. Jablonka, E., and Lamb, M. J. (2002) The changing concept of epigenetics. *Ann N Y Acad Sci* 981, 82-96

82. Nelissen, E. C., van Montfoort, A. P., Dumoulin, J. C., and Evers, J. L. (2011) Epigenetics and the placenta. *Hum Reprod Update* 17, 397-417

83. Maccani, M. A., and Marsit, C. J. (2009) Epigenetics in the placenta. *Am J Reprod Immunol* 62, 78-89

84. Szyf, M. (2009) The early life environment and the epigenome. *Biochim Biophys Acta* 1790, 878-885

85. Atkinson, S. P., and Keith, W. N. (2007) Epigenetic control of cellular senescence in disease: opportunities for therapeutic intervention. *Expert Rev Mol Med* 9, 1-26

86. Herman, J. G., and Baylin, S. B. (2003) Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 349, 2042-2054

87. Bernal, A. J., and Jirtle, R. L. (2010) Epigenomic disruption: the effects of early developmental exposures. *Birth Defects Res A Clin Mol Teratol* 88, 938-944

88. Verona, R. I., Mann, M. R., and Bartolomei, M. S. (2003) Genomic imprinting: intricacies of epigenetic regulation in clusters. *Annu Rev Cell Dev Biol* 19, 237-259

89. Szyf, M. (2007) The dynamic epigenome and its implications in toxicology. *Toxicol Sci* 100, 7-23

90. Selevan, S. G., Kimmel, C. A., and Mendola, P. (2000) Identifying critical windows of exposure for children's health. *Environ Health Perspect* 108 Suppl 3, 451-455

91. Dolinoy, D. C., Weidman, J. R., Waterland, R. A., and Jirtle, R. L. (2006) Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. *Environ Health Perspect* 114, 567-572

92. Xiao, R., Hennings, L. J., Badger, T. M., and Simmen, F. A. (2007) Fetal programming of colon cancer in adult rats: correlations with altered neonatal growth trajectory, circulating IGF-I and IGF binding proteins, and testosterone. *J Endocrinol* 195, 79-87

93. Newbold, R. R., Padilla-Banks, E., and Jefferson, W. N. (2006) Adverse effects of the model environmental estrogen diethylstilbestrol are transmitted to subsequent generations. *Endocrinology* 147, S11-17

94. Ozanne, S. E., Fernandez-Twinn, D., and Hales, C. N. (2004) Fetal growth and adult diseases. *Semin Perinatol* 28, 81-87

95. Choi, E. J., Chee, K. M., and Lee, B. H. (2003) Anti- and prooxidant effects of chronic quercetin administration in rats. *Eur J Pharmacol* 482, 281-285

96. Galati, G., Sabzevari, O., Wilson, J. X., and O'Brien, P. J. (2002) Prooxidant activity and cellular effects of the phenoxyl radicals of dietary flavonoids and other polyphenolics. *Toxicology* 177, 91-104

97. Mennen, L. I., Walker, R., Bennetau-Pelissero, C., and Scalbert, A. (2005) Risks and safety of polyphenol consumption. *Am J Clin Nutr* 81, 326S-329S

98. Breinholt, V., Lauridsen, S. T., and Dragsted, L. O. (1999) Differential effects of dietary flavonoids on drug metabolizing and antioxidant enzymes in female rat. *Xenobiotica* 29, 1227-1240

99. Niestroy, J., Barbara, A., Herbst, K., Rode, S., van Liempt, M., and Roos, P. H. (2011) Single and concerted effects of benzo[a]pyrene and flavonoids on the AhR and Nrf2-pathway in the human colon carcinoma cell line Caco-2. *Toxicol In Vitro* 25, 671-683

100. Moon, Y. J., Wang, X., and Morris, M. E. (2006) Dietary flavonoids: effects on xenobiotic and carcinogen metabolism. *Toxicol In Vitro* 20, 187-210

101. van Helden, Y. G., Keijer, J., Knaapen, A. M., Heil, S. G., Briede, J. J., van Schooten, F. J., and Godschalk, R. W. (2009) Beta-carotene metabolites enhance inflammation-induced oxidative DNA damage in lung epithelial cells. *Free Radic Biol Med* 46, 299-304

102. Gebicki, J. M., Nauser, T., Domazou, A., Steinmann, D., Bounds, P. L., and Koppenol, W. H. (2010) Reduction of protein radicals by GSH and ascorbate: potential biological significance. *Amino Acids* 39, 1131-1137

103. Singh, R., Sram, R. J., Binkova, B., Kalina, I., Popov, T. A., Georgieva, T., Garte, S., Taioli, E., and Farmer, P. B. (2007) The relationship between biomarkers of oxidative DNA damage, polycyclic aromatic hydrocarbon DNA adducts, antioxidant status and genetic susceptibility following exposure to environmental air pollution in humans. *Mutat Res* 620, 83-92

104. Hayes, J. D., Dinkova-Kostova, A. T., and McMahon, M. (2009) Cross-talk between transcription factors AhR and Nrf2: lessons for cancer chemoprevention from dioxin. *Toxicol Sci* 111, 199-201

105. Kohle, C., and Bock, K. W. (2007) Coordinate regulation of Phase I and II xenobiotic metabolisms by the Ah receptor and Nrf2. *Biochem Pharmacol* 73, 1853-1862

106. Miao, W., Hu, L., Scrivens, P. J., and Batist, G. (2005) Transcriptional regulation of NF-E2 p45-related factor (NRF2) expression by the aryl hydrocarbon receptor-xenobiotic response element signaling pathway: direct cross-talk between phase I and II drug-metabolizing enzymes. *J Biol Chem* 280, 20340-20348

107. Tsantes, A. E., Bonovas, S., Travlou, A., and Sitaras, N. M. (2006) Redox imbalance, macrocytosis, and RBC homeostasis. *Antioxid Redox Signal* 8, 1205-1216

108. Munoz, M., Villar, I., and Garcia-Erce, J. A. (2009) An update on iron physiology. *World J Gastroenterol* 15, 4617-4626

109. Zhang, A. S., and Enns, C. A. (2009) Molecular mechanisms of normal iron homeostasis. *Hematology Am Soc Hematol Educ Program*, 207-214

110. Koury, M. J., and Ponka, P. (2004) New insights into erythropoiesis: the roles of folate, vitamin B12, and iron. *Annu Rev Nutr* 24, 105-131

111. Kingsley, P. D., Malik, J., Emerson, R. L., Bushnell, T. P., McGrath, K. E., Bloedorn, L. A., Bulger, M., and Palis, J. (2006) "Maturational" globin switching in primary primitive erythroid cells. *Blood* 107, 1665-1672

112. Palis, J. (2008) Ontogeny of erythropoiesis. *Curr Opin Hematol* 15, 155-161

113. Keller, G., Lacaud, G., and Robertson, S. (1999) Development of the hematopoietic system in the mouse. *Exp Hematol* 27, 777-787

114. McGrath, K. E., Kingsley, P. D., Koniski, A. D., Porter, R. L., Bushnell, T. P., and Palis, J. (2008) Enucleation of primitive erythroid cells generates a transient population of "pyrenocytes" in the mammalian fetus. *Blood* 111, 2409-2417

115. Jauniaux, E., Gulbis, B., and Burton, G. J. (2003) The human first trimester gestational sac limits rather than facilitates oxygen transfer to the foetus--a review. *Placenta* 24 Suppl A, S86-93

116. Dennery, P. A. (2007) Effects of oxidative stress on embryonic development. *Birth Defects Res C Embryo Today* 81, 155-162

117. Amoyal, I., and Fibach, E. (2007) Hemoglobin switch in the newborn: a flow cytometry analysis. *Neonatology* 91, 61-68

118. Purdie, A., Wells, R. M., and Brittain, T. (1983) Molecular aspects of embryonic mouse haemoglobin ontogeny. *Biochem J* 215, 377-383

119. Gambling, L., and McArdle, H. J. (2004) Iron, copper and fetal development. *Proc Nutr Soc* 63, 553-562

120. Andersen, H. S., Gambling, L., Holtrop, G., and McArdle, H. J. (2006) Maternal iron deficiency identifies critical windows for growth and cardiovascular development in the rat postimplantation embryo. *J Nutr* 136, 1171-1177

121. Scholl, T. O. (2005) Iron status during pregnancy: setting the stage for mother and infant. *Am J Clin Nutr* 81, 1218S-1222S

122. Zhang, Y., Li, H., Zhao, Y., and Gao, Z. (2006) Dietary supplementation of baicalin and quercetin attenuates iron overload induced mouse liver injury. *Eur J Pharmacol* 535, 263-269

123. Uno, S., Dalton, T. P., Derkenne, S., Curran, C. P., Miller, M. L., Shertzer, H. G., and Nebert, D. W. (2004) Oral exposure to benzo[a]pyrene in the mouse: detoxication by inducible cytochrome P450 is more important than metabolic activation. *Mol Pharmacol* 65, 1225-1237

124. Tarantini, A., Maitre, A., Lefebvre, E., Marques, M., Rajhi, A., and Douki, T. (2011) Polycyclic aromatic hydrocarbons in binary mixtures modulate the efficiency of benzo[a]pyrene to form DNA adducts in human cells. *Toxicology* 279, 36-44

125. Gelboin, H. V. (1980) Benzo[alpha]pyrene metabolism, activation and carcinogenesis: role and regulation of mixed-function oxidases and related enzymes. *Physiol Rev* 60, 1107-1166

126. Ambrosone, C. B., and Tang, L. (2009) Cruciferous vegetable intake and cancer prevention: role of nutrigenetics. *Cancer Prev Res (Phila)* 2, 298-300

127. Bosch, T. M., Doodeman, V. D., Smits, P. H., Meijerman, I., Schellens, J. H., and Beijnen, J. H. (2006) Pharmacogenetic screening for polymorphisms in drug-metabolizing enzymes and drug transporters in a Dutch population. *Mol Diagn Ther* 10, 175-185

128. Sailaja, K., Surekha, D., Rao, D. N., Rao, D. R., and Vishnupriya, S. (2010) Association of the GSTP1 gene (Ile105Val) polymorphism with chronic myeloid leukemia. *Asian Pac J Cancer Prev* 11, 461-464

129. 130. Catania, V. A., Dannenberg, A. J., Luquita, M. G., Sanchez Pozzi, E. J., Tucker, J. K., Yang, E. K., and Mottino, A. D. (1995) Gender-related differences in the amount and functional state of rat liver UDP-glucuronosyltransferase. *Biochem Pharmacol* 50, 509-514

131. Zhu, B., Liu, Z. Q., Chen, G. L., Chen, X. P., Ou-Yang, D. S., Wang, L. S., Huang, S. L., Tan, Z. R., and Zhou, H. H. (2003) The distribution and gender difference of CYP3A activity in Chinese subjects. *Br J Clin Pharmacol* 55, 264-269

132. Mollerup, S., Berge, G., Baera, R., Skaug, V., Hewer, A., Phillips, D. H., Stangeland, L., and Haugen, A. (2006) Sex differences in risk of lung cancer: Expression of genes in the PAH bioactivation pathway in relation to smoking and bulky DNA adducts. *Int J Cancer* 119, 741-744

133. Uppstad, H., Osnes, G. H., Cole, K. J., Phillips, D. H., Haugen, A., and Mollerup, S. (2011) Sex differences in susceptibility to PAHs is an intrinsic property of human lung adenocarcinoma cells. *Lung Cancer* 71, 264-270

134. Gasperino, J. (2011) Gender is a risk factor for lung cancer. *Med Hypotheses* 76, 328-331

135. Singh, S. V., Benson, P. J., Hu, X., Pal, A., Xia, H., Srivastava, S. K., Awasthi, S., Zaren, H. A., Orchard, J. L., and Awasthi, Y. C. (1998) Gender-related differences in susceptibility of A/J mouse to benzo[a]pyrene-induced pulmonary and forestomach tumorigenesis. *Cancer Lett* 128, 197-204

136. Kummer, V., Maskova, J., Zraly, Z., Neca, J., Simeckova, P., Vondracek, J., and Machala, M. (2008) Estrogenic activity of environmental polycyclic aromatic hydrocarbons in uterus of immature Wistar rats. *Toxicol Lett* 180, 212-221

137. Daniel, C. R., Schwartz, K. L., Colt, J. S., Dong, L. M., Ruterbusch, J. J., Purdue, M. P., Cross, A. J., Rothman, N., Davis, F. G., Wacholder, S., Graubard, B. I., Chow, W. H., and Sinha, R. (2011) Meat-cooking mutagens and risk of renal cell carcinoma. *Br J Cancer* 105, 1096-1104

138. Xu, X., Kelsey, K. T., Wiencke, J. K., Wain, J. C., and Christiani, D. C. (1996) Cytochrome P450 CYP1A1 MspI polymorphism and lung cancer susceptibility. *Cancer Epidemiol Biomarkers Prev* 5, 687-692

139. Doll, R., and Peto, R. (1981) The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J Natl Cancer Inst* 66, 1191-1308

140. Ingelman-Sundberg, M. (2001) Genetic susceptibility to adverse effects of drugs and environmental toxicants. The role of the CYP family of enzymes. *Mutat Res* 482, 11-19

141. Kang, Z. C., Tsai, S. J., and Lee, H. (1999) Quercetin inhibits benzo[a]pyrene-induced DNA adducts in human Hep G2 cells by altering cytochrome P-450 1A1 gene expression. *Nutr Cancer* 35, 175-179

142. Ciolino, H. P., Daschner, P. J., and Yeh, G. C. (1999) Dietary flavonols quercetin and kaempferol are ligands of the aryl hydrocarbon receptor that affect CYP1A1 transcription differentially. *Biochem J* 340 (Pt 3), 715-722

143. Steiner, C., Peters, W. H., Gallagher, E. P., Magee, P., Rowland, I., and Pool-Zobel, B. L. (2007) Genistein protects human mammary epithelial cells from benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide and 4-hydroxy-2-nonenal genotoxicity by modulating the glutathione/glutathione S-transferase system. *Carcinogenesis* 28, 738-748

144. Makaji, E., Ho, S. H., Holloway, A. C., and Crankshaw, D. J. (2011) Effects in rats of maternal exposure to raspberry leaf and its constituents on the activity of cytochrome p450 enzymes in the offspring. *Int J Toxicol* 30, 216-224

145. Ebert, B., Seidel, A., and Lampen, A. (2007) Phytochemicals induce breast cancer resistance protein in Caco-2 cells and enhance the transport of benzo[a]pyrene-3-sulfate. *Toxicol Sci* 96, 227-236

Chapter 2

Prenatal exposure to flavonoids: implication for cancer risk

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Abstract

Flavonoids are potent antioxidants, freely available as high-dose dietary supplements. However, they can induce DNA double-strand breaks (DSB) and rearrangements in the mixed-lineage leukemia (*MLL*) gene, which are frequently observed in childhood leukemia. We hypothesize that a deficient DSB-repair, as a result of an *Atm* mutation, may reinforce the clastogenic effect of dietary flavonoids and increase the frequency of *Mll* rearrangements. Therefore we examined the effects of *in vitro* and transplacental exposure to high, but biological amounts of flavonoids in mice with different genetic capacities for DSB-repair (homozygous/ heterozygous knock-in for human *Atm* mutation (*Atm*-ΔSRI) versus wild-type). *In vitro* exposure to genistein/ quercetin induced higher numbers of *MLL* rearrangements in bone marrow cells of *Atm*-ΔSRI mutant mice compared to wild-type mice. Subsequently, heterozygous *Atm*-ΔSRI mice were placed on either a flavonoid-poor or a genistein-enriched (270 mg/ kg) or quercetin-enriched (302 mg/ kg) feed throughout pregnancy. Prenatal exposure to flavonoids associated with higher frequencies of *Mll* rearrangements and a slight increase in the incidence of malignancies in DNA repair-deficient mice. These data suggest that prenatal exposure to both genistein and quercetin supplements could increase the risk on *Mll* rearrangements especially in the presence of compromised DNA repair.

Introduction

Bioflavonoids are a diverse group of polyphenolic compounds found in fruits, vegetables, soy, tea, coffee and wine. They are most commonly known for their protective effect against cardiovascular diseases, cancer and inflammation, as a result of their antioxidant capacity (1, 2). Because of the presumed health benefits flavonoid supplements are worldwide over-the-counter available in pharmacies and drugstores. Unfortunately, there is a general belief that these herbal supplements are safe because they are labeled as "natural". Reasons for concern are that besides their antioxidant properties, flavonoids are also potent topoisomerase II (topoII)-inhibitors. TopoII is a nuclear enzyme that plays an essential role in controlling the topology of DNA during replication and recombination. TopoII binds to specific sites on both DNA strands and generates a transient double strand break (DSB). These DSBs are normally relegated. However, certain topoII-inhibiting drugs, such as etoposide or flavonoids can stabilize DSBs and increase the risk of chromosomal abnormalities (3-5).

One of the most common loci involved in chromosomal translocations due to double-stranded DNA lesions caused by topoII-inhibitors, is the breakpoint cluster region (BCR) of the mixed-lineage leukemia (*MLL*) gene. This gene is involved in normal hematopoiesis and is located at chromosome 11q23 (6-8). Approximately 80 % of infants which suffer from acute myelogenous leukemia (AML) or acute lymphoblastic leukemia (ALL) have chromosomal translocations involving the *MLL* gene (3-5). These translocations are also identified in patients with secondary leukemia caused by chemotherapy with topoII-inhibitors (e.g. etoposide) (3, 9-11). It has previously been suggested that dietary flavonoids are also capable of introducing these translocations and cause leukemia (12). Although there are some indications that flavonoids might be involved in leukemogenesis (4), the complex nature of human diet made it impossible to elucidate the contribution of flavonoids. Our previous data (13) demonstrates that exposure of human CD34+ hematopoietic progenitor cells to biologically relevant levels of the flavonoids quercetin, genistein and kaempferol can induce *MLL* translocations *in vitro*. Since the concentrations used in this *in vitro* study can also be obtained *in vivo*, and knowing that flavonoids can penetrate the placenta and accumulate in the fetus (2, 14, 15), it is crucial to further examine the safety of flavonoids, especially during pregnancy. Fetal cells are rapidly proliferating cells and thus have high topoII activity (16), which can theoretically make them more sensitive to the topoII inhibiting effect of flavonoids.

As flavonoids execute their clastogenicity through inhibiting the religation of DSBs, one might expect that any defect in DSB-repair may predispose cells to clastogenic effects of these compounds. DSBs are normally detected by ataxia-telangiectasia-mutated (ATM) protein kinase (17-19). Ye *et al.* (20) demonstrated that exposure of human lymphoblastoid cell lines to quercetin or genistein leads to autophosphorylation of ATM on serine 1981 leading to the active form of ATM (21). Hence, ATM phophorylates its downstream targets (e.g. p53, Chk2 and histone H2AX), which based on the severity of the damage will either execute DNA repair or cell-cycle arrest and apoptosis (20, 22-24). Patients with mutations in the *ATM* gene are prone to developing chromosomal abnormalities (17, 19). Heterozygous germ-line mutations in the *ATM* gene are frequent and occur in 0.5-1 % of the general population (25, 26). These mutations are associated with an increased risk for developing different types of cancer, including leukemia (19, 23, 27, 28). Accordingly, missence mutations in the *ATM* gene have been detected in 25 % of cases with childhood ALL (23).

This study examines the effect of both *in vitro* and transplacental exposure to high, but biological amounts of flavonoids in mice with different genetic capacities for DSB-repair (homozygous/heterozygous knock-in for human *Atm* mutation (*Atm-*ΔSRI) *versus* wild-type). We examined whether a decreased capacity in DNA DSB-repair in *Atm-*ΔSRI mutant mice enhances flavonoid-induced clastogenicity.

Material and methods

Exposure of bone marrow cells to genistein, quercetin or etoposide

Bone marrow cells collected from one femur of adult wild-type (wt) and homozygote mutant (mut) *Atm-*ΔSRI mice were isolated by flushing the marrow with Iscove's Modified Dulbecco's Media (IMDM, Invitrogen, Breda, the Netherlands) supplemented with 10 % FCS. Next, cells were washed, incubated in IMDM and treated for one day with 50 μM of etoposide (Sigma-Aldrich, St. Louis, MO, USA), genistein (LC Laboratories, Woburn, MA, USA), quercetin (Sigma) or the vehicle dimethylsulphoxide (0.05 % DMSO). After exposure cells were washed twice, and left to recover for another day before DNA isolation. Cell counts and viability were determined by a hemocytometer and trypan blue exclusion.

Mice and sample collection

Female *Atm-*ΔSRI heterozygous mice (129/SvJ:C57BL/6J background) approximately 8 weeks of age, received either normal chow (low phytoestrogen content complete feed for mice breeding, in which neither soybean nor alfalfa products were used, resulting in minimized concentrations of the phytoestrogens genistein, daidzein and coumestrol, ssniff®, Soest, Germany, n=8) or the same chow (ssniff®) supplemented with genistein (270 mg/ kg feed, n=9) (LC Laboratories) or quercetin (302 mg/ kg feed, n=8) (Sigma) from 3 days before conception until the end of pregnancy. Male *Atm-*ΔSRI heterozygous mice were placed in the cage only for the duration of copulation. Heterozygote female and male mice were mated because this gives the opportunity to study the *in utero* effects of the three expected genotypes in offspring (homozygous/heterozygous *Atm-*ΔSRI and wild-type). After delivery all mothers and pups received the normal chow. At day 5 after birth, pups were weighted and genotype and gender was determined. At 12 weeks of age, offspring were sacrificed by cardiac puncture, checked for tumor development and blood was collected in EDTA-tubes (Terumo, Leuven, Belgium) to determine the blood composition. Bone marrow was isolated by removing the femurs and flushing the marrow with PBS. Offspring that died before sacrificing day were also checked for tumor development.

To study the direct effects of diet on fetal hematopoiesis, wild-type mice (129/SvJ:C57BL/6J background, control n=5, genistein n=4, quercetin n=3) were mated overnight. The presence of a vaginal plug the next morning was defined as 0.5 day post conception. On day 14.5 of pregnancy (E14.5), mice were sacrificed to isolate the fetuses and fetal liver (please note that the liver is the organ for hematopoiesis at this time point).

Blood composition

Blood composition was determined in duplicate, using the ADVIA 120 Hematology System (Siemens AG, Erlangen, Germany) following the manufacturer's protocol. The following blood cell parameters were measured: total number of red blood cells (10^{12}/ L), hemoglobin level (mmol/ L), hematocrit level (L/ L), mean corpuscular volume (fL), mean corpuscular hemoglobin (fmol), mean corpuscular hemoglobin concentration (mmol/ L), red blood cell distribution width (%), hemoglobin distribution width (mmol/ L), total amount of platelets (10^9/ L), mean platelet volume (fl), total amount (10^9/ L) and percentage of reticulocytes, mean corpuscular hemoglobin concentration of reticulocytes (fmol), total number of white blood cells (10^9/ L), total amount (10^9/ L) and percentage of neutrophils, lymphocytes, monocytes, eosinophils and basophils. In case of suspected leukemia by abnormal blood composition, the diagnosis was further confirmed by performing a May-Grünwald staining on blood smear preparations. Briefly, 10 µl of blood was fixed by methanol. Next, smears were stained in May-Grünwald solution (Sigma) for 5 minutes and washed in tap water. Subsequently, the smears were stained with Giemsa solution (Merck, Darmstadt, Germany) for 20 minutes and washed again in tap water. All preparations were judged by an experienced animal pathologist.

Inverse-PCR assay and sequencing

Genomic DNA was isolated from bone marrow cells (*in vitro* culture or *in vivo* from 12 week old mice) and from the fetal liver of E14.5 fetuses, using DNeasy Blood & Tissue Kit (Qiagen, Venlo, the Netherlands). Fetal livers were first homogenized using an Ultra-Turrax homogenizer (IKA, Staufen, Germany). Next, 1 µg of each DNA sample was subjected to SAP digestion (1 unit of shrimp alkaline phosphatase) (Promega, Madison, WI, USA) for 1 hour at 37 °C followed by an inactivation at 65 °C for 20 minutes. Then, the DNA was digested with 3 units of PciI (Biolabs, Leusden, the Netherlands) for 1 hour at 37 °C followed by 20 minutes inactivation at 80 °C. The DNA was circularized overnight at 6 °C followed by 2 hours at 22 °C and 20 minutes of inactivation at 75 °C using 3 units of T4 DNA ligase (Promega) in a final volume of 50 µl. Approximately, 80 ng of the circularized DNA was used for the first PCR, in which the circularized DNA was amplified. Both the first PCR and nested PCR were performed using 0.7 µl expand long template polymerase mix (Roche, Mannheim, Germany), system 2 reaction buffer, 20 pmol of each primer (Eurogentec, Liege, Belgium, Table 1) and dNTPs (500 µM) in a volume of 50 µl. The reaction was performed using a Biometra Tprofessional thermocycler (Biometra, Leusden, the Netherlands) under the following cycling conditions: 94 °C for 2 minutes, 10 cycles: 94 °C for 30 seconds, 56 °C for 45 seconds, 68 °C for 4 minutes; 25 cycles: 94 °C for 30 seconds, 56 °C for 45 seconds, 68 °C for 4 minutes (+ 20 s/cycle); followed by 7 minutes at 68 °C. The amplified fragments from the first PCR were diluted (1/50) and 2 µl was taken for the nested PCR.

The nested PCR products were separated by electrophoresis on a 1 % agarose gel, aberrant sized bands were excised and DNA was isolated using the QIAquick Gel Extraction Kit (Qiagen). The purified PCR products were subsequently sequenced using big dye v3.1 (Applied Biosystems, Foster city, CA, USA) and 5 pmol primer (Table 1) under the following cycling conditions: 1 minute at 96 °C, 25 cycles: 96 °C for 30 seconds, 50 °C for 15 seconds, 60 °C for 4 minutes followed by 10 minutes at 72 °C. After purification by sephadex columns, the products were sequenced by ABI 3730 Automatic DNA Sequencer (Applied

Biosystems). Nucleotide sequences were analyzed using 'Nucleotide blast' tool of the National Center for Biotechnology Information (BLAST NCBI, Bethesda, MD, USA).

Table 1. Overview of used primers

Primer	Forward primer (5'-3')	Reverse primer (5'-3')
First PCR primer	CCA GAG TAG TGT GCT TTC TC	AGG TGG CTT CTC CTG AGA CAG
Nested PCR primer	AGT GGG CAT GTA GAG GTA AG	AAC AAT GAC TTG CCC TCA TAT TG
Sequence PCR primers:		
1	AAA CAG TGT GCA CAG GTA CG	
2	AAC CGC TGA ACC ATC TCT CC	
3	TGG TGG GA GGT CAT TAG CC	

Statistical analysis

Statistical analysis was performed with Statistical Package for Social Sciences (SPSS version 15 for Windows, SPSS Inc., Chicago, IL, USA). The non-parametric Mann-Whitney test was used to compare the distribution of gender, genotype, pup survival, litter sizes, average weight of pups at postnatal day 5 and tumor development between litters of the different diet groups. 2x2x2 multifactorial univariate ANOVA was performed to identify predictors (diet, genotype, gender) of persistent hematological changes in mice.

Results

Bone marrow cells with Atm-ΔSRI mutation are more sensitive to genistein- and quercetin-induced chromosomal aberrations

In order to examine whether dietary exposure to topoII-inhibitors can induce chromosomal aberrations and whether *Atm-ΔSRI* mutations actually increase this risk, an inverse-PCR method was set-up to detect murine *Mll* translocations. The assay was validated by detecting *Mll* translocations in murine bone marrow cells after exposure to the known topoII-inhibitor etoposide (50 µM). In addition, bone marrow cells from homozygous wild-type and mutant *Atm-ΔSRI* mice were isolated and cultured for approximately 24 hours with either genistein (50 µM) or quercetin (50 µM). Cell viability was evaluated by trypan blue exclusion. We observed no significant decrease in cell viability due to exposure to either flavonoids or etoposide or due to genotype of bone marrow cells in comparison with DMSO treated cells (Figure 1).
Cells were allowed to repair the DSBs for 24 hours in a drug-free medium. Subsequently, DNA was extracted and screened for *Mll* aberrations by inverse-PCR. The DNA isolated from DMSO treated samples resulted in amplification of the wild-type (wt) *Mll* (5.6kb) as visualized by agarose gel electrophoresis (Figure 2) and the identity was confirmed by sequence analysis. In contrast, exposure of bone marrow cells to quercetin, genistein or etoposide generated multiple bands of different sizes. The variety in the size of PCR products was determined by both the location of the breakpoint junction in *Mll* and the position of the PciI site in the fusion partner. Smaller sized bands were more likely to be detected by inverse-PCR than the wt *Mll*, because wt *Mll* is more difficult to amplify due to its length. Sequencing of the breakpoint junction of several distinct *Mll* translocations
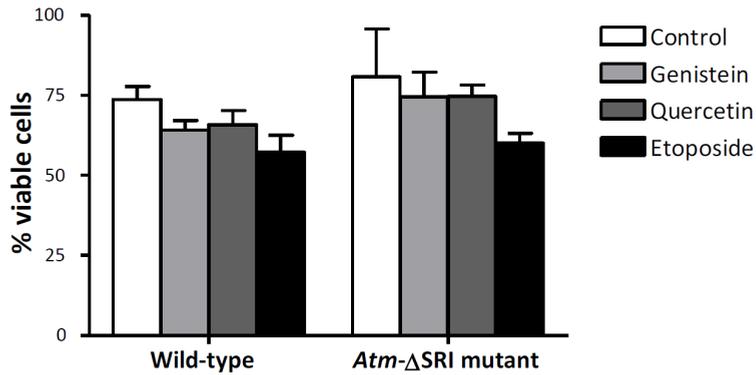
Figure 1. Viability of wt and *Atm-*ΔSRI mutant bone marrow cells was determined after 1 day of exposure to 50 **μ**M of the flavonoids genistein and quercetin, 50 **μ**M etoposide and the vehicle DMSO (0.05 %) by trypan blue exclusion.
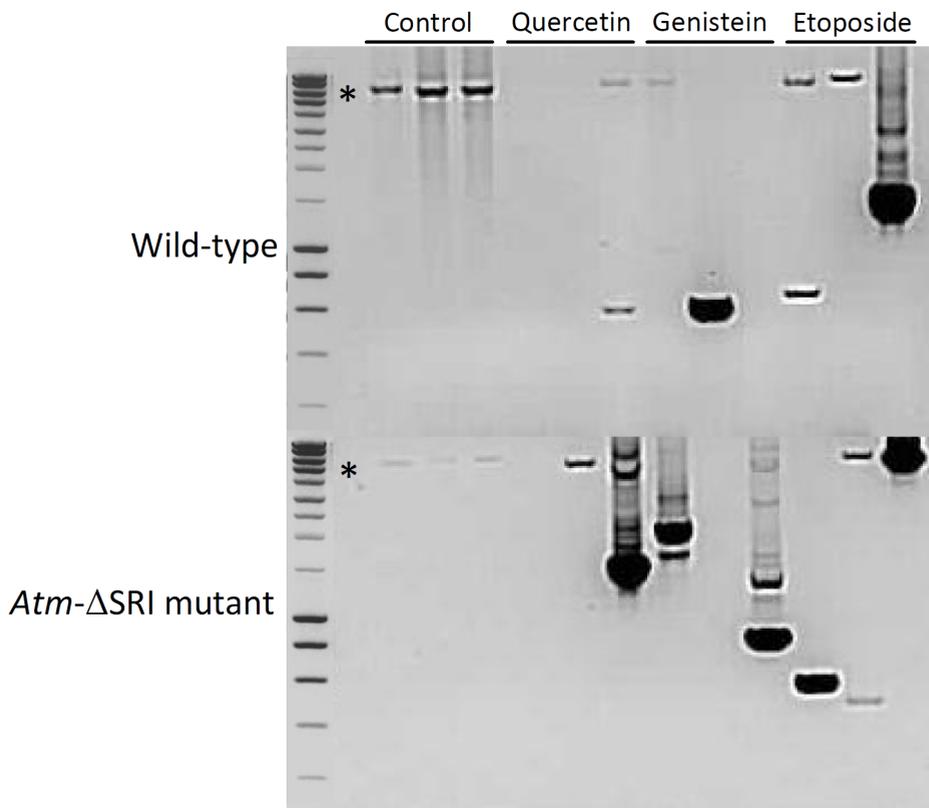
Figure 2. Inverse-PCR products representing *Mll* rearrangements found in bone marrow of wild-type and *Atm-*ΔSRI mutant cells after 24 hours *in vitro* exposure to quercetin (50 **μ**M), genistein (50 **μ**M) or etoposide (50 **μ**M).
Equal aliquots of DNA were used for each inverse-PCR reaction. The samples were visualized by gel electrophoresis. Each band with a size different from the wt 5.6 kb band represents a translocation that results in an abberant size of the PCR product. Wild-type *Mll* is indicated with *.

induced by flavonoids or etoposide confirmed that the bands of abberant size were indeed translocations (Table 4). Although etoposide and both flavonoids induced *Ml* rearrangements in bone marrow cells regardless of the genotype of the mice, the *Atm*-ΔSRI mutant cells (0, 2.1, 5, 1.2 translocations/ 80 ng genomic DNA for the control, quercetin, genistein or etoposide exposed cells, respectively, Figure 2) showed more chromosomal translocations than the wt cells (0.7, 0.2, 0.2, 3 translocations/ 80 ng genomic DNA for the control, quercetin, genistein or etoposide exposed cells, respectively). This indicates that the *Atm* mutation predisposes cells to the clastogenic effects of flavonoids.

Effect of prenatal diet on litter characteristics

Heterozygous *Atm*-ΔSRI mice were exposed to genistein (270 mg/ kg feed, n=9) or quercetin (302 mg/ kg feed, n=8) or control diet (n=8) throughout their pregnancy. There were no differences in gender ratio, average weight of the offspring measured at day 5 after birth or average liter size between control, genistein or quercetin exposed groups (Table 2). Interestingly, the incidence of different genotypes was to some extent affected by prenatal diet. Theoretically, the homozygous wild-type (wt) and mutant (mut) offspring should each count for approximately 25 % of the offspring. Accordingly, wt and mut offspring showed equal prevalence (respectively 28 % and 29 %) in the control group (Table 2). The genistein and quercetin diet groups however, had different prevalences of the genotypes. The number of mut mice born in the quercetin group was lower than in the control or genistein group (P=0.06 and P=0.01, respectively). In the genistein group, the percentage of heterozygote animals seemed to be low, however the distribution of distinct genotypes was not significantly different from the control group.

Changes in blood composition and incidence of malignancies in mice prenatally exposed to flavonoids

A total number of 45 control, 45 genistein- and 40 quercetin-exposed mice reached the age of 12 weeks. The percentage of animals that died before this time point was not significantly different for all three diet groups (Table 2). We were unable to determine the cause of death, but at autopsy no tumors were noted. The mice that reached 12 weeks of age were sacrificed and their internal organs were examined for visual signs of abnormalities and presence of gross tumors (Table 2). In a previous study (29), malignancies were detected in heterozygous *Atm*-ΔSRI mice on average after 18.6 months. In our study, several mice had already developed malignancies at 12 weeks of age. One mouse in the control group (1/45) demonstrated splenomegaly with concomitant erythroleukemia and thymoma. On the other hand, thymoma were detected in three mice (3/45, 7 %) of the genistein exposed group and in one of the 40 mice (2.5 %) that were prenatally exposed to quercetin. Blood count and blood smear examination showed concomitant acute lymphoblastic leukemia in two mice that were exposed to genistein. In general, leukemia and thymoma were only detected in mice that were homozygous for the *Atm* mutation. The only heterozygous mouse that was diagnosed with malignancy (fallopian tube tumor) belonged to the genistein group. Altogether, mutant mice prenatally exposed to genistein showed a slightly higher risk for developing tumors and leukemia compared with wt control mice.

Table 2 Characteristics of the litters and malignancies detected in offspring within different diet groups

	Control	Genistein	Quercetin
Average litter size	6.38 ± 2.07	5.22 ± 2.28	5.13 ± 2.30
Genotype (%)			
Total numbers of litters	8	9	8
wt	28	31	38
hetero	43	41	53
mut	29	28[**c]	8[#, **c]
Average pup weight[a]	3.18 ± 0.71	3.32 ± 0.58	3.13 ± 0.26
Percentage of males	47	57	51
% of deceased pups[b]	10	7	2.5
	Malignancies detected in offspring at 12 weeks of age		
Number of animals with malignancies/ total analyzed (%)	1/45 (2 %)	4/45 (9 %)	1/40 (2 %)
Tumor type	erythroleukemia	acute lymphoblastic leukemia, thymoma, fallopian tube cancer	thymoma
Tissue involved	bone marrow, thymus, spleen	bone marrow, thymus, ovarian ducts	thymus
Genotype	Mut	mut, hetero	mut

General characteristics of the litters prenatally exposed to genistein-, quercetin-supplemented or normal diet. Results represent the mean ± the standard deviation. # P=0.06, ** P≤0.01. [a] Average pup weight (grams) at day 5. [b] Average number of deceased pups after birth and before sacrifice day (week 12). [c] Significant difference between quercetin and genistein group.

The complete blood count at 12 weeks of age was successfully carried out in duplicate for 41 control, 41 genistein and 39 quercetin exposed mice. The three mice that suffered from leukemia (as assessed by ADVIA 1200 Hematology System and subsequent May-Grünwald staining of blood smears) were excluded from the analysis due to the extreme outlying measurements. As shown in Table 3, 2x2x2 multifactorial univariate analysis was used to adjust for confounding variables (gender and genotype). Analysis of the red blood cell fraction demonstrated an increase in the mean corpuscular volume (MCV) for both genistein and quercetin group in comparison to the control (mean ± SE respectively 46.31 ± 0.34 fL, P=0.01; 46.33 ± 0.50 fL, P=0.4 and 44.96 ± 0.45 fL for control,). The red blood cell distribution width (RDW) was only significantly increased in the genistein group (13.92 ± 0.19 % *versus* 13.35 ± 0.12 % for control, P=0.03). A combined high MCV and RDW could be an indication of a higher reticulocyte count. Indeed, the total amount of reticulocytes was elevated in the genistein group (157.57 ± 17.51 x 10^9/ L *versus* 106.79 ± 15.88 x 10^9/ L in control, P=0.4). Although prenatal quercetin exposure was not associated with an increased reticulocyte count, the mean hemoglobin content of reticulocytes (MCHR) was higher (1.03 ± 0.01 fmol) compared to the control group (0.96 ± 0.01 fmol, P=0.07). Prenatal exposure to genistein on the contrary significantly decreased MCHR (0.94 ± 0.03 fmol, P=0.02).
In the genistein group, the total number of red blood cells (7.44 ± 0.12 x 10^{12}/ L *versus* 7.03 ± 0.13 x 10^{12}/ L for control, P=0.07) and consequently the hemoglobin (7.25 ± 0.09 mmol *versus* 6.78 ± 0.11 mmol for control, P=0.009) and hematocrit levels (0.35 ± 0.01 L/ L *versus* 0.32 ± 0.01 L/ L for control, P=0.02) levels were significantly elevated.

Table 3. Multifactorial univariate analysis of the long term effects of genistein and quercetin on the blood composition of 12 week old mice

	Control	Genistein		Quercetin	
	Mean ± SE	Mean ± SE	P-value[a]	Mean ± SE	P-value[a]
Red blood cells (10^{12}/L)	7.03 ± 0.13	7.44 ± 0.12	0.07	7.17 ± 0.13	0.5
Hemoglobin (mmol/L)	6.78 ± 0.11	7.25 ± 0.09	0.009	6.97 ± 0.14	0.5
Mean corpuscular volume (fL)	44.96 ± 0.45	46.31 ± 0.34	0.01	46.33 ± 0.50	0.4
Hematocrit (L/L)	0.32 ± 0.01	0.35 ± 0.01	0.02	0.33 ± 0.01	0.4
Mean corpuscular hemoglobin reticulocytes (fmol)	0.96 ± 0.01	0.94 ± 0.03	0.02	1.03 ± 0.01	0.07
Absolute amount of reticulocytes (10^{9}/L)	106.79 ± 15.88	157.57 ± 17.51	0.4	108.15 ± 11.32	0.9
Red blood cell distribution width (%)	13.35 ± 0.12	13.92 ± 0.19	0.03	13.19 ± 0.14	0.6

[a] P-value calculated for dietary effects adjusted for genotype and gender

Table 4. Selection of MII translocations detected in fetal liver of E14.5 fetuses or in bone marrow cells in vitro exposed or of mice prenatally exposed to topoII-inhibitors

	Exp[a]	Genotype[b]	MII[c]	Fusion partner	Sequence of the translocation junction	# base pair homology
In vitro exp.	Q	mut	44 648 590	MII-chrom 4	CGTTAATAATTCTTCATCTT....TACCTGATGACTTCCAGTAA	131
Adult mice	C	mut	44 648 573	MII-chrom 13	AAAAATAAGCCAGGTGTCGGTGCTTCTTGATCTCA	6
	G	wt	44 648 365	MII-chrom 12	TAATAAAATTTTACTGTTTAᴛAAAATAAAAACCTGCTTACT	1
	G	mut	44 648 168	MII-chrom 17	GACAATCAGAGCTGCAGAGACACTTTTTCCTTCTGCCAT	
	G	mut	44 648 870	MII-chrom 8	CGCAGTCTAGAGTAATGCCCACCAACACTCGGTGGTACAC	
	Q	mut	44 648 162	MII-chrom 17	CAGAGCTGCAGAGAGGAGACCCTGTCTCAAAAAATAAAAAT	
	Q	mut	44 648 565	MII-chrom 3	GCCAGGTGTGGTGGCACATG...ATACAAAGCTACTATAGGTT	119
	Q	mut	44 648 585	MII-chrom 17	ATTAAGAAGTAGAAAAATAA....AGATGCTGAAGAGGACAGAC	143
Fetuses	C	wt	44 648 577	MII-chrom 6	CATCTTTTTATTCGGTCCAC...AGAACTTTTTGGTTCTTATT	101
	C	wt	44 648 853	MII-chrom 8	CCCACCAACACTCGGTGGTGGTACACCATACATACTATATAGACT	3
	Q	wt	44 648 870	MII-chrom 8	CGCAGTCTAGAGTAATGCCCACCAACACTCGGTGGTACAC	
	Q	Wt	44 648 942	MII-chrom 2	AAAAAAAAATTTCTAAAGAA...CGGACACGTACTCACAAAAT	162

The nucleotide sequence of the MII gene and its fusion partners at the breakpoint junction are respectively indicated in normal and italic. The nucleotide sequence homology between MII and the fusion partner is underlined. The nucleotide sequence homology of the translocations which showed an extensive homology could not be demonstrated in this table. [a] Exposure of murine bone marrow cells in vitro to quercetin (Q), genistein (G) or etoposide (E) or diet condition during prenatal period: quercetin (Q), genistein (G) or control diet (C). [b] Genotype of examined mice: wild-type (wt) or Atm-ΔSRI mutant (mut). [c] The nucleotide numbering at the breakpoint junction is based on the MII genomic sequence NCBI database NC_000075.

Detection of Mll translocations in mice prenatally exposed to flavonoids

In order to investigate the clastogenic effects of prenatal genistein and quercetin exposure we studied the occurrence of *Mll* translocations in the fetal liver of E14.5 wt fetuses by inverse-PCR. Genomic DNA obtained from the fetal liver was screened for chromosomal aberrations (Figure 3).
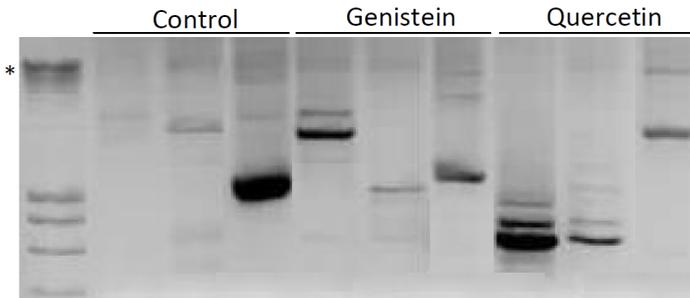

Figure 3. Inverse-PCR products representing *Mll* rearrangements found in fetal liver cells of fetuses exposed to genistein or quercetin.
Equal aliquots of DNA were used for each inverse-PCR reaction. The samples were visualized by gel electrophoresis. Each band with a size different from the wt 5.6 kb band represents a translocation that results in an abberant size of the PCR product. Wild-type *Mll* is indicated with *.

Interestingly, regardless of the maternal diet *Mll* rearrangements could be detected in all the fetuses. The rate of translocations between the control group and both exposure groups showed no differences (control: 4.3 translocations/ 80 ng genomic DNA *versus* 2.5 translocations/ 80 ng genomic DNA and 6.5 translocations/ 80 ng genomic DNA for the genistein and quercetin exposed fetuses, respectively). The average number of translocations is calculated as average number of alternative sized band seen in one inverse-PCR reaction/ DNA input in an inverse-PCR reaction. However, when both flavonoid exposed groups were compared, the quercetin exposed fetuses showed more chromosomal translocations compared to the genistein exposed fetuses. In order to confirm the mutant sized bands as genuine translocations instead of PCR-artifacts, a selection of aberrant *Mll* products were sequenced (Table 4) and all appeared to be real *Mll* translocations with various chromosomes as fusion partners.
Next, bone marrow of 12 week old wt or *Atm*-ΔSRI mut mice prenatally exposed to genistein or quercetin was screened for *Mll* translocations. As shown in Figure 4, prenatal exposure to both genistein and quercetin increased the frequency of *Mll* rearrangements. In wt control mice we identified 0.9 translocations/ 80 ng genomic DNA by inverse-PCR. Prenatal exposure to genistein increased the mutation rate in wt mice to 2.5 translocations/ 80 ng genomic DNA, whereas wt mice prenatally exposed to quercetin showed no change in the frequency of *Mll* rearrangements (1 translocations/ 80 ng genomic DNA).
All three diet groups increased the incidence on *Mll* translocations in mice carrying the *Atm* mutation. In the control group the mutation rate increased up to 2.7 translocations/ 80 ng genomic DNA. This is comparable with the rate seen in wt mice prenatally exposed to genistein. *Atm*-ΔSRI mut mice prenatally exposed to genistein had a 2 fold higher translocation rate (5.6 translocations/ 80 ng genomic DNA) compared with their wt siblings. Although prenatal exposure to quercetin had no effect on the occurrence of *Mll* translocations in wt mice, it increased the mutation rate in *Atm*-ΔSRI mut mice considerably (up to 16

translocations/ 80 ng genomic DNA). This suggests that prenatal exposure to genistein leads to a modest increase in the number of *Mll* rearrangements, while prenatal quercetin exposure considerably elevates the rearrangement rate in *Atm-*ΔSRI mut mice. Again, a selection of aberrant *Mll* products was sequenced to confirm that the alternative PCR products were comprised of genuine translocations (Table 4). No genuine translocations were detected in the wt mice on control diet.
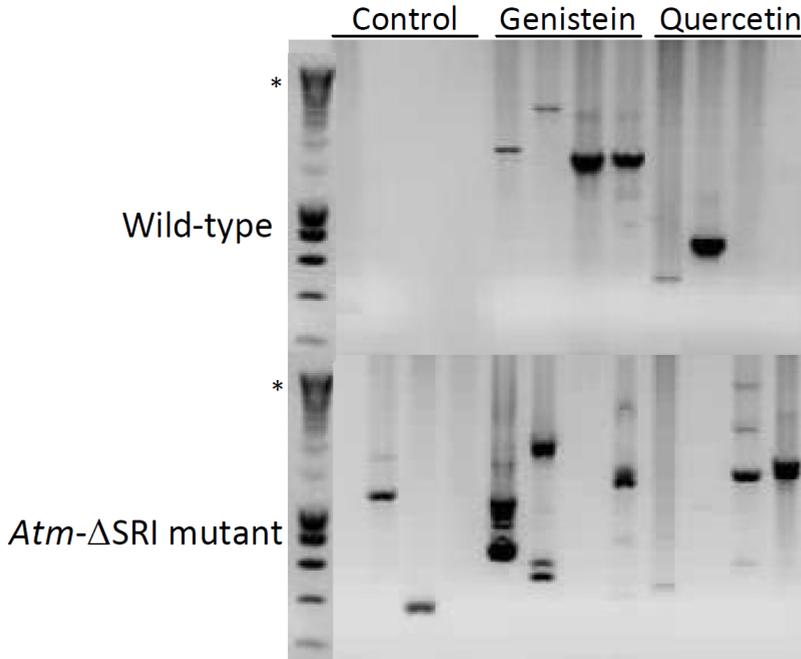
Figure 4. Inverse-PCR products representing *Mll* rearrangements found in bone marrow cells of wild-type and *Atm-*ΔSRI mutant offspring mice at 12 weeks of age, prenatally exposed to genistein or quercetin.
Equal aliquots of DNA were used for each inverse-PCR reaction. The samples were visualized by gel electrophoresis. Each band with a size different from the wt 5.6 kb band represents a translocation that results in an abberant size of the PCR product. Wild-type *Mll* is indicated with *.

Discussion

It has been suggested that high intake of dietary flavonoids contributes to infant leukemia (4, 30). However, no direct experimental evidence has yet been presented. Our previous study (13), together with the study of Strick (5), showed that flavonoids could induce *MLL* translocations in CD34+ cells. However, we are the first to report that prenatal exposure to flavonoids can increase the risk for leukemia onset, as assessed by the frequency of *Mll* translocations in a mouse model prone to develop cancer. To detect potential translocations, an inverse-PCR method was developed. In order to validate this method, murine bone marrow cells of wt and *Atm-*ΔSRI mut mice were exposed to 50 μM of genistein, quercetin and the known topoII-inhibitor etoposide. DNA isolated from these cells was then subjected to inverse-PCR amplification to detect murine *Mll* translocations. Although all exposures induced *Mll*

translocations, *Atm-ΔSRI* mut bone marrow cells that lack adequate DSB-repair were more susceptible for flavonoid and etoposide induced *Mll* rearrangements.

To investigate the effect of prenatal exposure to flavonoids and the predisposing role of deficient DSB-repair, *Atm-ΔSRI* hetero mice were mated to obtain pups with different genetic capacity for DNA repair from a single mother. Bone marrow of 12 week old wt and *Atm-ΔSRI* mut offspring mice that were prenatally exposed to genistein or quercetin, were screened for *Mll* translocations, by using the inverse-PCR method. Both flavonoids were capable of inducing *Mll* translocations in wt and *Atm-ΔSRI* mut mice, but *Atm-ΔSRI* mut mice showed a higher susceptibility to develop these chromosomal aberrations, which was expected from the *in vitro* results. This suggests that exposure to flavonoid supplements can have more severe consequences in individuals with a malfunctioning DNA repair system. Alike flavonoids, radiation induces DNA DSBs. Accordingly, radiation exposure has been shown to induce significantly more cell death and chromosomal aberrations in Atm-ΔSRI mut thymocytes than in wt cells (31).

In order to study the direct effects of flavonoid exposure on the growing fetus, fetal livers were isolated at day 14.5 of pregnancy and screened for *Mll* translocations. These translocations were detectable in all fetuses, regardless of the maternal diet. This indicates that such translocations might be common events during fetal development, which are probably generated due to high topoII activity of the proliferating cells (16). However, the fact that less *Mll* translocations were detected at 12 weeks of age, suggests that the cells carrying these translocations are either eliminated or have restricted expansion capacity.

Prenatal quercetin exposure in this study, led to a minor decrease in average litter size. It seems that quercetin exposure is more lethal to fetuses with homozygous *Atm-ΔSRI* mutations. In fact studying the direct effect of the flavonoids on the fetuses shows that transplacental quercetin exposure induces more chromosomal translocations than genistein. Both prenatal genistein and quercetin exposure had no effect on gender ratio and average birth weight, which suggest that *in utero* dietary supplementation did not affect normal development. Also the number of pups that died spontaneously after birth was not influenced by the diet. Three mice, all *Atm-ΔSRI* mut, developed leukemia (acute lymphoblastic leukemia or erythroleukemia), at 12 weeks of age, confirming our hypothesis that a decrease in DNA DSB-repair (ATM dysfunction) could enhance the leukemia risk. One mouse that developed erythroleukemia was not prenatally exposed to flavonoid supplements, suggesting that in this case the *Atm-ΔSRI* predisposition was responsible for the development of leukemia. Indeed, *Atm-ΔSRI* mut mice are known to have a 50 % chance on developing malignancies (31). The ratio of malignancies between control and genistein exposed mice suggests that prenatal genistein exposure may increase the risk on developing malignancies. All malignancies, except acute lymphoblastic leukemia detected in our mice, were previously described in *Atm-ΔSRI* mice (29, 31). *Atm-ΔSRI* hetero mice develop different categories of tumors at an average age of 18.6 months. However, 44 % of the *Atm-ΔSRI* mut mice die of thymic lymphomas up to 10 months of age. In our study, 1 out of 13 *Atm-ΔSRI* mut mice in the control group had developed malignancy at the age of 3 months. The percentage of malignancies in the genistein and quercetin group was respectively 23 % (3 out of 13 *Atm-ΔSRI* mut mice) and 25 % (1 out of 4 *Atm-ΔSRI* mut mice). The decrease in the number of *Atm-ΔSRI* mut mice born in the quercetin group further suggests the hazards of such exposure in a vulnerable population that lacks an effective DNA DSB-repair.

A complete blood count analysis showed that prenatal exposure to genistein not only influenced the lymphoblastic cell development, but also the myeloblastic cell development and overall

erythropoiesis. Prenatal exposure to genistein associates with an up-regulation of erythropoiesis in adult mice, as seen in the increase in the number of red blood cells, which results in an increase in hemoglobin and hematocrit levels (ratio cells/plasma). The increase in red blood cell distribution width could be the result of an increase in the number of reticulocytes, which could also explain the increase in mean cell volume. However, it could also indicate that there is a coexistence of young and old red blood cells, suggesting a longer survival of these cells. Prenatal exposure to quercetin on the other hand only increased the size of the red blood cells and the amount of hemoglobin in reticulocytes. The increase in red blood cell size could be the result of an increase in the amount of hemoglobin per cell. An increase in the amount of hemoglobin in reticulocytes, would automatically lead to an increase in the amount of hemoglobin in erythrocytes. Knowing that quercetin has iron ion chelating capacities (32) and the fact that the heme part of hemoglobin contains iron, it can be suggested that the effects seen is due to a disturbed iron metabolism in these mice. Further investigation is needed to unravel these aspects of prenatal genistein/ quercetin exposure and the mechanisms behind the long term effects of diet during pregnancy on blood composition.

Due to the fact that flavonoids are present in a variety of food sources, the incidental daily intake can be as high as 1 g, but can increase up to several grams in those supplementing their diet with flavonoids. The average daily dietary intake of quercetin is in the range of 4–68 mg. Daily isoflavone intake in Western countries is approximately 1 to 9 mg. In Asian countries however, the daily intake of isoflavones is much higher, reaching levels of 20-240 mg, due to the high consumption of soy (3). In our study pregnant mice were exposed to approximately 26.7-36.7 mg/ kg bodyweight genistein and 33.3-46.7 mg/ kg bodyweight quercetin per day. Record *et al.* (33) determined that mice fed 20 mg/ kg genistein had an average plasma concentration of 10 μM. In this study, pregnant mice were exposed to a higher dose of genistein and quercetin, probably resulting in even higher plasma concentrations. These concentrations are also higher compared with plasma concentrations seen in humans on both a Western or Asian diet (34). However, both flavonoids are freely available as supplements and the daily dose recommended by manufacturers can be as high as 1-2 g per day. When supplements are taken, plasma concentrations can be 10 to 20 times higher (35) than the levels we tested in mice, indicating that the doses used in this study are biologically relevant. It is also noteworthy that the metabolism of phytoestrogens is different in the fetus and adults. In human, fetal cord blood has a higher genistein level than maternal serum (14, 15, 36). Taken together it is important to establish clear guidelines for the use of flavonoid supplements during pregnancy.

Acknowledgments

References

1. Formica, J. V., and Regelson, W. (1995) Review of the biology of Quercetin and related bioflavonoids. *Food Chem Toxicol* 33, 1061-1080

2. Ross, J. A., and Kasum, C. M. (2002) Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annu Rev Nutr* 22, 19-34

3. Skibola, C. F., and Smith, M. T. (2000) Potential health impacts of excessive flavonoid intake. *Free Radic Biol Med* 29, 375-383

4. Spector, L. G., Xie, Y., Robison, L. L., Heerema, N. A., Hilden, J. M., Lange, B., Felix, C. A., Davies, S. M., Slavin, J., Potter, J. D., Blair, C. K., Reaman, G. H., and Ross, J. A. (2005) Maternal diet and infant leukemia: the DNA topoisomerase II inhibitor hypothesis: a report from the children's oncology group. *Cancer Epidemiol Biomarkers Prev* 14, 651-655

5. Strick, R., Strissel, P. L., Borgers, S., Smith, S. L., and Rowley, J. D. (2000) Dietary bioflavonoids induce cleavage in the MLL gene and may contribute to infant leukemia. *Proc Natl Acad Sci U S A* 97, 4790-4795

6. Felix, C. A., and Lange, B. J. (1999) Leukemia in infants. *Oncologist* 4, 225-240

7. Felix, C. A., Lange, B. J., Hosler, M. R., Fertala, J., and Bjornsti, M. A. (1995) Chromosome band 11q23 translocation breakpoints are DNA topoisomerase II cleavage sites. *Cancer Res* 55, 4287-4292

8. Libura, J., Slater, D. J., Felix, C. A., and Richardson, C. (2005) Therapy-related acute myeloid leukemia-like MLL rearrangements are induced by etoposide in primary human CD34+ cells and remain stable after clonal expansion. *Blood* 105, 2124-2131

9. Blanco, J. G., Edick, M. J., and Relling, M. V. (2004) Etoposide induces chimeric Mll gene fusions. *Faseb J* 18, 173-175

10. Felix, C. A. (1998) Secondary leukemias induced by topoisomerase-targeted drugs. *Biochim Biophys Acta* 1400, 233-255

11. Libura, J., Ward, M., Solecka, J., and Richardson, C. (2008) Etoposide-initiated MLL rearrangements detected at high frequency in human primitive hematopoietic stem cells with in vitro and in vivo long-term repopulating potential. *Eur J Haematol* 81, 185-195

12. Ross, J. A. (2000) Dietary flavonoids and the MLL gene: A pathway to infant leukemia? *Proc Natl Acad Sci U S A* 97, 4411-4413

13. Barjesteh van Waalwijk van Doorn-Khosrovani, S., Janssen, J., Maas, L. M., Godschalk, R. W., Nijhuis, J. G., and van Schooten, F. J. (2007) Dietary flavonoids induce MLL translocations in primary human CD34+ cells. *Carcinogenesis* 28, 1703-1709

14. Adlercreutz, H., Yamada, T., Wahala, K., and Watanabe, S. (1999) Maternal and neonatal phytoestrogens in Japanese women during birth. *Am J Obstet Gynecol* 180, 737-743

15. Schroder-van der Elst, J. P., van der Heide, D., Rokos, H., Morreale de Escobar, G., and Kohrle, J. (1998) Synthetic flavonoids cross the placenta in the rat and are found in fetal brain. *Am J Physiol* 274, E253-256

16. Zandvliet, D. W., Hanby, A. M., Austin, C. A., Marsh, K. L., Clark, I. B., Wright, N. A., and Poulsom, R. (1996) Analysis of foetal expression sites of human type II DNA topoisomerase alpha and beta mRNAs by in situ hybridisation. *Biochim Biophys Acta* 1307, 239-247

17. Khanna, K. K. (2000) Cancer risk and the ATM gene: a continuing debate. *J Natl Cancer Inst* 92, 795-802

18. Montecucco, A., and Biamonti, G. (2007) Cellular response to etoposide treatment. *Cancer Lett* 252, 9-18

19. Oguchi, K., Takagi, M., Tsuchida, R., Taya, Y., Ito, E., Isoyama, K., Ishii, E., Zannini, L., Delia, D., and Mizutani, S. (2003) Missense mutation and defective function of ATM in a childhood acute leukemia patient with MLL gene rearrangement. *Blood* 101, 3622-3627

20. Ye, R., Goodarzi, A. A., Kurz, E. U., Saito, S., Higashimoto, Y., Lavin, M. F., Appella, E., Anderson, C. W., and Lees-Miller, S. P. (2004) The isoflavonoids genistein and quercetin activate different stress signaling pathways as shown by analysis of site-specific phosphorylation of ATM, p53 and histone H2AX. *DNA Repair (Amst)* 3, 235-244

21. Bakkenist, C. J., and Kastan, M. B. (2003) DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* 421, 499-506

22. Golding, S. E., Rosenberg, E., Khalil, A., McEwen, A., Holmes, M., Neill, S., Povirk, L. F., and Valerie, K. (2004) Double strand break repair by homologous recombination is regulated by cell cycle-independent signaling via ATM in human glioma cells. *J Biol Chem* 279, 15402-15410

23. Gumy-Pause, F., Wacker, P., and Sappino, A. P. (2004) ATM gene and lymphoid malignancies. *Leukemia* 18, 238-242

24. van Gent, D. C., Hoeijmakers, J. H., and Kanaar, R. (2001) Chromosomal stability and the DNA double-stranded break connection. *Nat Rev Genet* 2, 196-206

25. Barlow, C., Hirotsune, S., Paylor, R., Liyanage, M., Eckhaus, M., Collins, F., Shiloh, Y., Crawley, J. N., Ried, T., Tagle, D., and Wynshaw-Boris, A. (1996) Atm-deficient mice: a paradigm of ataxia telangiectasia. *Cell* 86, 159-171

26. FitzGerald, M. G., Bean, J. M., Hegde, S. R., Unsal, H., MacDonald, D. J., Harkin, D. P., Finkelstein, D. M., Isselbacher, K. J., and Haber, D. A. (1997) Heterozygous ATM mutations do not contribute to early onset of breast cancer. *Nat Genet* 15, 307-310

27. Stankovic, T., Stewart, G. S., Byrd, P., Fegan, C., Moss, P. A., and Taylor, A. M. (2002) ATM mutations in sporadic lymphoid tumours. *Leuk Lymphoma* 43, 1563-1571

28. Sung, P. A., Libura, J., and Richardson, C. (2006) Etoposide and illegitimate DNA double-strand break repair in the generation of MLL translocations: new insights and new questions. *DNA Repair (Amst)* 5, 1109-1118

29. Spring, K., Ahangari, F., Scott, S. P., Waring, P., Purdie, D. M., Chen, P. C., Hourigan, K., Ramsay, J., McKinnon, P. J., Swift, M., and Lavin, M. F. (2002) Mice heterozygous for mutation in Atm, the gene involved in ataxia-telangiectasia, have heightened susceptibility to cancer. *Nat Genet* 32, 185-190

30. Ross, J. A., Potter, J. D., Reaman, G. H., Pendergrass, T. W., and Robison, L. L. (1996) Maternal exposure to potential inhibitors of DNA topoisomerase II and infant leukemia (United States): a report from the Children's Cancer Group. *Cancer Causes Control* 7, 581-590

Chapter 3

Epigenetics: Prenatal exposure to genistein leaves a permanent signature on the hematopoietic lineage

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Abstract

Recent studies demonstrate that maternal diet during pregnancy results in long-lasting effects on the progeny. Supplementation of maternal diet with genistein, a phytoestrogen ubiquitous in the daily diet, altered coat color of Agouti mice due to epigenetic changes. We studied hematopoiesis of mice prenatally exposed to genistein (270 mg/ kg feed) compared with mice prenatally exposed to phytoestrogen-poor feed and observed a significant increase in granulopoiesis, erythropoiesis and mild macrocytosis at the adult age of 12 weeks. Genistein exposure was associated with hypermethylation of certain repetitive elements, which coincided with a significant down-regulation of estrogen responsive genes and genes involved in hematopoiesis in bone marrow cells of genistein-exposed mice, as assessed by microarray technology. Although, genistein exposure did not affect global methylation in fetal liver of fetuses at embryonic day 14.5, it accelerated the switch from primitive to definitive erythroid lineage. Altogether, our data demonstrate that prenatal exposure to genistein affects fetal erythropoiesis and exerts life-long alterations in gene expression and DNA methylation of hematopoietic cells.

Introduction

Genistein is a naturally occurring phytoestrogen found in soy products. There is an increasing interest in this flavonoid due to its antioxidant properties, but also because there is a general believe that phytoestrogen intake has beneficial effects (1, 2) .

However, recent studies have revealed that genistein exposure through maternal diet during pregnancy can result in long-lasting effects on the progeny. Prenatal exposure of heterozygous yellow agouti mice to genistein, through maternal diet, altered both coat color and body weight. These changes were caused by hypermethylation of transposable repetitive elements (IAP) upstream of the transcription start site of the *Agouti* gene (3). Other studies have shown that transplacental exposure of rats to genistein exerts long-lasting effects on the endocrine and immune system and altered stress response and post-weaning growth (4, 5). Recently, Möller *et al.* (6) demonstrated that genistein exposure during development induces long-term effects on the uterine gene expression profile.

Nutritional deficiencies (including vitamin B12 and folate) and drug exposure can affect blood composition; for instance macrocytosis can have different causes, ranging from primary bone marrow disorders to nutritional deficiencies (7). Prolonged use of estrogenic compounds such as oral contraceptives is also reported to induce macrocytosis, presumably by interfering with folate cofactor interactions and affecting DNA synthesis (8).

In the present study we observed significant changes in blood composition of mice that were prenatally exposed to genistein. Prenatal genistein exposure was associated with increased granulopoiesis and erythropoiesis together with moderate macrocytosis at adult age. The macrocytosis observed in the genistein exposed animals may be attributed to estrogenic properties of this compound, due to its structural similarity to 17β-estradiol (9). However, it is remarkable that such effects are observed at adult age, while genistein exposure has taken place only during the prenatal period. We therefore assume that the past exposure to genistein has left a permanent signature on the hematopoietic lineage of these mice. As genistein exposure is known to alter both global DNA methylation (3) and gene expression (6), we hypothesize that prenatal exposure to genistein leads to adaptation of hematopoiesis by altering both DNA methylation as well as gene expression. In order to find out whether genistein leaves permanent transcriptomic/epigenetic signatures in hematopoietic cells, we compared both DNA methylation of repetitive elements and gene expression profiles in hematopoietic cells of mice prenatally exposed to genistein with the control group at adult age. The methylation status and fetal blood development was also assessed during the fetal period, to study the direct effects of the genistein supplementation.

Material and methods

Mice and sample collection

Mice (129/SvJ:C57BL/6J background) approximately 8 weeks of age, received either normal chow (n=8, low phytoestrogen content complete feed for mice breeding, in which neither soybean nor alfalfa products are used, resulting in minimized concentrations of the phytoestrogens genistein, daidzein and coumestrol, ssniff®,

Soest, Germany) or the same chow (ssniff®) supplemented with genistein (270 mg/ kg feed, n=9) (LC Laboratories, Woburn, MA, USA) from 3 days before conception until the end of pregnancy. Male mice were placed in the cage only for the duration of copulation. After delivery litters were not equalized and all mothers and pups received normal (low phytoestrogen) diet, making the prenatal period the only window of exposure. Offspring were sacrificed at 12 weeks of age by cardiac puncture (Figure 1). Blood was collected in EDTA-tubes (Terumo, Leuven, Belgium). Bone marrow was isolated by removing the femurs and flushing the marrow with PBS.

To study the direct effects of diet on fetal hematopoiesis, mice were mated overnight. The presence of a vaginal plug the next morning was defined as 0.5 day post conception. On day 14.5 of pregnancy (E14.5), mice were sacrificed to isolate the fetuses, fetal blood and fetal liver.

The mouse experiments were conducted in accordance with Dutch animal protection laws by the guidelines of the local veterinary authorities.
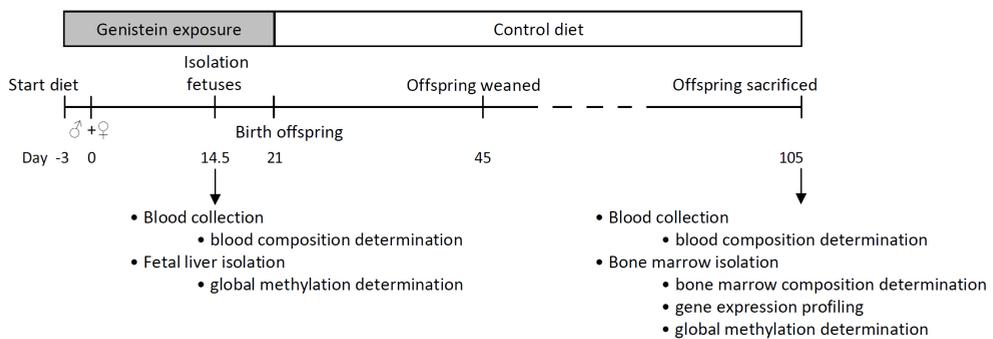
Figure 1. Study design

Blood composition of adult mice

Blood composition was determined in duplicate, using ADVIA 120 Hematology System (Siemens AG, Erlangen, Germany) following user's manual. The following parameters were measured: total number of red blood cells (10^{12}/L), hemoglobin level (mmol/ L), hematocrit level (L/ L), mean corpuscular volume (fL), mean corpuscular hemoglobin (fmol), mean corpuscular hemoglobin concentration (mmol/ L), red blood cell distribution width (%), hemoglobin distribution width (mmol/ L), total amount of platelets (10^9/ L), mean platelet volume (fl), total amount (10^9/ L) and percentage of reticulocytes, mean corpuscular hemoglobin concentration of reticulocytes (fmol), total number of white blood cells (10^9/ L), total amount (10^9/ L) and percentage of neutrophils, lymphocytes, monocytes, eosinophils and basophils.

Blood (fetuses) and bone marrow (adult mice) cytology

After initial fixation of blood (10 μl) or bone marrow smears (isolated from one of both femurs) by methanol, smears were stained in May-Grünwald solution (Sigma-Aldrich, Steinham, Germany) for 5 minutes and washed in tap water. Subsequently, the smears were stained with Giemsa solution (Merck, Darmstadt, Germany) for 20

minutes and washed again in tap water. The blood and bone marrow smears were examined by two independent, experienced examiners, counting approximately 500 cells per slide, using a Leica DMRB microscope (Leica Microsystems B.V., Rijswijk, the Netherlands).

Genome-wide gene expression analysis of adult mice

RNA from bone marrow cells isolated from one femur was isolated with TRIzol Reagent (Invitrogen, Breda, the Netherlands) according to the manufacturer's instructions. Genome-wide gene expression was determined using whole mouse genome (4X44K) oligonucleotide microarrays (Agilent, Santa Clara, CA, USA) according to the manufacturer's instructions. Using the Low RNA Input Linear Amplification Kit (Agilent), cyanine-5-labeled cRNA was generated from RNA of each sample (a total of 6 bone marrow samples: 3 controls and 3 genistein-exposed male mice). Next, samples were hybridized together with cyanine-3-labeled reference cRNA produced from the RNA pool of the 3 control mice (reference sample). Image analysis and initial quality control were performed using the Agilent Feature Extraction (FE) Software v10.5 and Spotfire DesicionSite v9.1 (TIBCO Spotfire, Somerville, MA, USA). Thereafter, microarray data analysis was performed by importing the text-files generated by the Agilent FE Software into R2.8.1 using the Bioconductor 'limma' package 2.8.1 (10). The values for control spots and spots that did not meet the quality control criteria were flagged. The flagged spots were not used in the statistical analysis. In addition, the background-corrected intensities of all microarrays were normalized using the locally weighted scatterplot smoothing (LOESS) algorithm. Finally, a linear model was fitted to the intensities for each reporter, and the genistein-treated mice were compared to controls using an unpaired moderated t-test using the 'limma' functions lmfit and eBayes (10). Benjamini-Hochberg adjusted P-values were used to correct for multiple testing. All Agilent reporters were re-annotated to ensure reporter specificity and to optimize recognition by the pathway analysis procedures (11).

Methylation-sensitive McrBC-real-time polymerase chain reaction (PCR) assay of fetuses and adult mice

Analysis of methylation pattern of the repetitive elements, i.e. long interspersed nucleotide elements (LINEs), short interspersed nucleotide elements (SINEs), intracisternal A particle (IAP), major and minor satellites, was performed using the methylation-sensitive McrBC-real-time PCR assay (12). 1 µg of genomic DNA was digested overnight at 37 °C using 10 U of McrBC (New England Biolabs, Beverly, MA, USA), an endonuclease which cleaves DNA containing 5-methylcytosine, but will not cleave unmethylated DNA. The DNA strand breaks prevent amplification of methylated DNA in the quantitative real-time PCR assay. Two-step quantitative real-time PCR was performed using IQ SYBR Green Supermix (Bio-Rad Laboratories, Veenendaal, the Netherlands) with 4 ng of McrBC-digested DNA and 25 pmol of each primer (Eurogentec, Maastricht, the Netherlands) in a reaction volume of 25 µl. The forward and reverse primers sequence for the different repetitive elements and endogenous reference were: LINE: 5`-TTTGGGACACAATGAAAGCA-3` and 5`-CTGCCGTCTACTCCTCTTGG-3`; SINEB1: 5`-GTGGCGCACGCCTTTAATC-3` and 5`-GACAGGGTTTCTCTGTGTAG-3`; SINEB2: 5`- GAGATGGCTCAGTGGTTAAG-3` and 5`-CTGTCTTCAGACACTCCAG-3`; IAP-GAG: 5`-AGCAGGTGAAGCCACTG-3` and 5`-

CTTGCCACACTTAGAGC-3; Major satellite: 5`-GACGACTTGAAAAATGACGAAATC-3` and 5`-CATATTCCAGGTCCTTCAGTGTGC-3`; and Minor satellite: 5`-CATGGAAAATGATAAAAACC-3` and 5`-CATCTAATATGTTCTACAGTGTGG-3`. The cycling conditions consisted of an initial denaturation at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 45 seconds, 58 °C for 90 seconds, with an exception for the major and minor satellites for which this step was performed at 60 °C, using the iCycler (Bio-Rad). Data were analyzed by MyiQ Software system (Bio-rad), Ct values were normalized for endogenous reference (HPRT), compared with the calibrator (i.e. average Ct value of 4 control samples for the adult mice and of 3 control samples for the fetuses) and expressed as relative expression ($2^{-\Delta\Delta Ct}$). An increase in PCR amplification products is indicative of hypomethylation, whereas a decrease in PCR amplification products indicates hypermethylation.

Results

Prenatal exposure to genistein influences hematopoiesis in adulthood

A comparison of the complete blood count of 41 male and female adult mice (12 weeks of age) from 9 different litters (with an average size of 6.4 ± 2.1) that were prenatally exposed to genistein through maternal diet (supplemented with 270 mg genistein/ kg diet from 3 days before conception throughout pregnancy), with the same number of age matched male and female control mice from 8 different litters (with an average litter size of 5.2 ± 2.3), demonstrated significant differences in blood composition (Table 1). Prenatal exposure to genistein was associated with higher number of red blood cells (mean ± SD, 7.44 ± 0.71 x 10^{12} cells *versus* 7.03 ± 0.80 x 10^{12} cells, P=0.03) and reticulocytes (157.57 ± 52.54 x 10^9 cells *versus* 106.79 ± 42.00 x 10^9 cells, P=0.06). In accordance, the mean corpuscular volume (46.31 ± 2.10 fL *versus* 44.96 ± 2.80 fL, P=0.02), red blood cell distribution width (13.92 ± 1.12 *versus* 13.35 ± 0.75, P=0.01), hemoglobin (7.25 ± 0.53 mmol/ L *versus* 6.78 ± 0.67 mmol/ L, P=0.001) and hematocrit levels (0.35 ± 0.04 L/ L *versus* 0.32 ± 0.05 L/ L, P=0.004) were elevated in the genistein group. Examination of the bone marrow cellularity of adult mice (n=3), which were prenatally exposed to genistein, showed no significant difference in the number of early and late erythroid progenitors with the control group.

Table 1. Persistent effects of prenatal genistein exposure on blood composition in 12 week old mice

	Control (n=41)	Genistein (n=41)	
	Mean ± SE	Mean ± SE	P-value
Red blood cells (10^{12}/ L)	7.03 ± 0.13	7.44 ± 0.12	0.026
Hemoglobin (mmol / L)	6.78 ± 0.11	7.25 ± 0.09	0.001
Mean corpuscular volume (fL)	44.96 ± 0.45	46.31 ± 0.34	0.018
Hematocrit (L/ L)	0.32 ± 0.008	0.35 ± 0.007	0.004
Absolute amount of reticulocytes (10^9/ L)	106.79 ± 15.88	157.57 ± 17.51	*0.056*
Red blood cell distribution width (%)	13.35 ± 0.12	13.92 ± 0.19	0.013
White blood cells (10^9/ L)	1.45 ± 0.9	1.79 ± 0.16	*0.064*
Absolute amount of neutrophils (10^9/ L)	0.23 ± 0.14	0.32 ± 0.03	0.021
Absolute amount of eosinophils (10^9/ L)	0.06 ± 0.005	0.09 ± 0.02	0.041

However, the number of pyrenocytes was significantly elevated in the genistein exposed animals, indicating an increased enucleation of the erythroid cells (10.47 ± 5.39 % *versus* 1.28 ± 1.49 %, P=0.047) (Figure 2). Furthermore, we observed a higher number of granulocytic cells in the genistein exposed animals (39.57 ± 4.93 % *versus* 24.91 ± 9.58 %, P=0.08), which is in accordance with the significantly higher number of neutrophils (0.32 ± 0.20 x 10^9 cells *versus* 0.23 ± 0.09 x 10^9 cells, P=0.02) and eosinophils (0.09 ± 0.09 x 10^9 cells *versus* 0.06 ± 0.03 x 10^9 cells, P=0.04) in the peripheral blood.
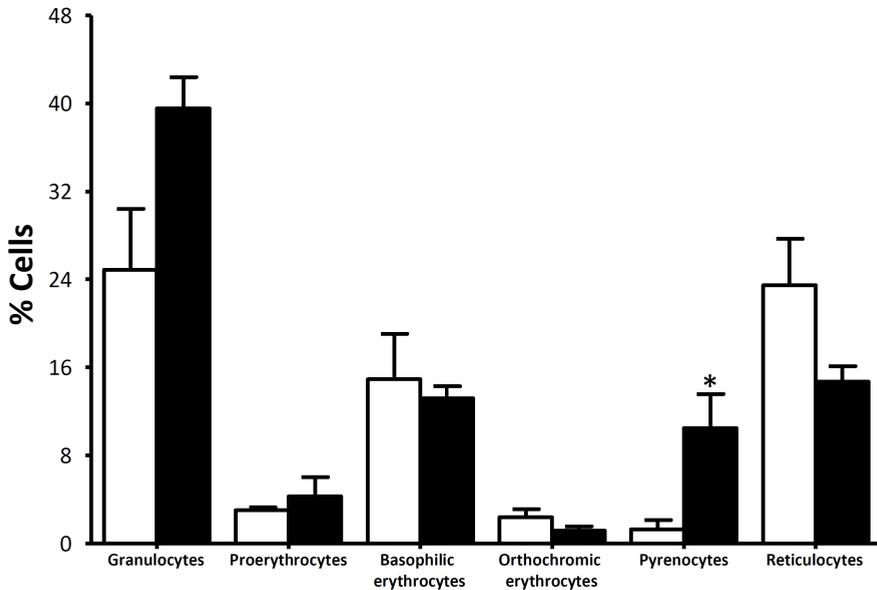
Figure 2. Long-term effect of prenatal exposure to genistein on bone marrow cellularity.
Bone marrow smears of 12 week old mice from the control (n=3; white bars) and genistein exposed (n=3; black bars) group were analyzed to determine the number of pyrenocytes, early and late (proerythroblast, basophilic erythroblast and ortochromic erythroblast) erythroid progenitors and granulocytic cells. Bars represent the percentage of cells. Error bars represent the standard error. Statistically significant differences were analyzed using One-way ANOVA: *: P<0.05.

Prenatal genistein exposure modifies gene expression in adulthood

As prenatal exposure to genistein had induced long-term effects on the blood and bone marrow composition, we hypothesized that the effects of this past exposure to genistein would still be detectable in the gene expression profile of the hematopoietic cells. Therefore, we compared the gene expression profile of bone marrow cells of animals which were prenatally exposed to genistein (n=3) with that of the control group (n=3), using whole mouse genome (4X44K) oligonucleotide microarrays (Agilent). The animals selected for the microarray analysis were all male mice and belonged to different litters to minimize possible litter effects. Approximately 20 % of the 21,742 annotated gene products was differentially expressed between the genistein and the control group (n=4352, 1.2<fold change or fold change <-1.2, P<0.05). 83 % and 36 % of the gene products belonging respectively to the estrogen receptor signaling and hematopoiesis pathway (according to GO terms and literature)

were differentially expressed. Unsupervised hierarchical clustering of differentially expressed genes with a high degree of statistical significance (P<0.001) demonstrates overall differences in gene expression between groups. It is noteworthy that the gene products that were significantly up-regulated in the genistein group (fold change>1.2, P<0.001) represent only 10 % of the total number of differentially expressed genes.

Previously, it has been demonstrated that *in vitro* exposure of XG-1 myeloma cell line to genistein results in down-regulation of *NF-κB* and NF-κB-regulated gene products, including *Bcl2l1*, *Cyclin D1* and intercellular adhesion molecule-1 (*Icam-1*) (13). In our study, genistein exposure resulted in a down-regulation of *NF-κB* and NF-κB-regulated gene products, including *Bcl2l1*, *Cyclin D1* and intercellular adhesion molecule-1 (*Icam-1*) in the hematopoietic cells of mice prenatally exposed to genistein. In addition to Icam-*1*, prenatal genistein exposure attenuated the expression of several other adhesion molecules, such as neural cell adhesion molecule 1 (*Ncam1*), E-cadherin (*Cdh1*), integrin beta 1 (*Intgb1*), integrin alpha 1 (*Itga1*) and chemoattractant protein 1 (*MCP-1*), *P-selectin* and selectin P ligand (*Selplg*). Also *Nocht1*, *Mdm2* and *Pdkcd* showed a significantly lower mRNA expression, 12 weeks after withdrawal from genistein exposure. Likewise, the expression levels of certain genes, such as *Cdkn1b* (*p27kip1*) and retinoblastoma binding protein 8 (*Rbbp8*) were elevated in the genistein exposed animals. Interestingly, some other genes that were previously reported to be up-regulated in reaction to direct genistein exposure at adulthood (*Stat3*, *Pten* and *component C3*) (14, 15) were significantly suppressed in mice that were exposed to genistein during *in utero* development.

Although some of the differences in gene expression can partly be attributed to differences in bone marrow cellularity, persistent effects of genistein exposure on gene expression were still observed 12 weeks after withdrawal of the genistein exposure.

Significant down-regulation of estrogen responsive genes

Because it is known that genistein is a phytoestrogen, and thus has estrogenic properties (16), we studied the expression of different estrogen responsive genes. Notably, several estrogen responsive genes (*Grin2d*, *ApoE*, *Cdkn1a*, *Macrod*, *Vegfa*, *Hdac6*, *Tacc1*, *Abcc5*, *Ctsd*, *Ccnd1*, *Pcna*, *Igf2*) were suppressed in the hematopoietic cells of adult mice prenatally exposed to genistein (Table 2). It is known that activation of the aryl hydrocarbon receptor (AhR) by its ligands, e.g. benzo[a]pyrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), promotes the expression of the estrogen-responsive genes (17). It is remarkable that the *AhR* expression level was indeed significantly elevated (fold change=1.49, P-value=0.006) in the bone marrow of mice that were prenatally exposed to genistein.

Changes in expression of genes involved in hematopoiesis

AhR is known to regulate mRNA expression of several genes with important roles in hematopoiesis, including *Cdkn1b* (*p27Kip1*), *Cdkn1a* (*p21 waf1*/cip1), *Cdk2*, *Myc* and genes encoding for CCAAT/enhancer-binding protein (18). Strikingly, the expression of *Cdkn1b*, *Cebpa*, *Cebpb* was up-regulated, while the expression of *Myc* was down-regulated, indicating that granulocyte differentiation was stimulated. *Cdk2*, up-

regulated during primitive erythropoiesis, was significantly repressed in the genistein group, suggesting an increase in adult erythropoiesis (Table 3). AhR normally binds its chaperon Arnt to associate with the estrogen receptors and to activate transcription of estrogen-responsive genes (19). However, *Arnt* mRNA expression was significantly down-regulated (fold change=-3.06, P-value=6.4 x 10^{-6}) in the genistein group. In addition to the genes regulated by AhR, we encountered several other hematopoiesis-related genes that were differentially expressed in bone marrow of the genistein group. A deficiency in *Cdkn1a*, *Egln1*, *Stat3*, *Dok1* and *Pten* gene expression is known to increase proliferation of hematopoietic stem cells. All these genes were significantly repressed in the genistein group; while *Bmi1 and Angpt1*, which are required respectively for the self-renewal and protection of hematopoietic stem cells, were up-regulated. A down-regulation of *Myc* in the genistein group was also associated with lower expression of *Thbs1* and *Vegf*, resulting in a lower expression of the cell-surface receptor of Vegf (*Kdr*). In general, the expression of several genes that regulate erythroid cell function and development (*Bcl2l1*, *Stat5a*, *Gata1*, *Mapk14*, *Epb4.2*, *Maea*, *Slc12a4*, *Dhfr*, *Flvcr1*, *Thpo*, E*pb4.1* and *Ptprc*) were significantly altered in the genistein group, resulting in an up-regulation of erythrocyte proliferation. Moreover, genistein exposed mice showed an altered expression of genes contributing to an enhanced granulocytic differentiation/proliferation (*Lrg1*, *Cebpa*, *Icam1*, *JunB*, *Thbs1*, *Cdk2* and *Cdkn1b*).

Table 2. Down-regulation of estrogen responsive genes in the bone marrow of adult mice prenatally exposed to genistein

Gene	Genebank accession number	Description	Fold change	P-value
Cdkn1a (p21 waf1/cip1)	NM_007669	Cyclin-dependent kinase inhibitor 1A	-1.78	0.006
Ctsd	NM_009983	Cathepsin D	-2.05	0.0002
Ccnd1	NM_008796	Cyclin D1	-2.04	0.0002
Pcna	NM_011045	Proliferating cell nuclear antigen	-1.99	0.0007
ApoE	NM_009696	Apolipoprotein E	-2.14	0.002
C3	NM_009778	Complement component 3	-1.50	0.009
Igf2	NM_010514	Insulin-like growth factor 2	-2.69	0.02
Grin2d	NM_008172	Glutamate receptor, ionotropic, N-methyl-D-aspartate subunit 2d	-1.50	0.02
Macrod1	NM_134147	Macro domain-containing 1	-1.73	0.003
Vegfa	NM_001025250	Vascular endothelial growth factor	-1.93	0.004
Hdac6	NM_010413	Histone deacetylase 6	-1.89	0.006
Abcc5	NM_013790	ATP-binding cassette subfamily C member 5	-2.50	0.00008
Tacc1	NM_177089	Transforming, acidic, coiled-coil-containing protein	-2.52	0.0003

Table 3. Changes in mRNA expression of genes related to hematopoiesis in the bone marrow of adult mice prenatally exposed to genistein

Down-regulated genes	Genebank accession number	Description	Function	Fold change	P-value
Cdkn1a (p21 waf1/cip1)	NM_007669	Cyclin-dependent kinase inhibitor 1A	Cdkn1a -/- mice show an increase in human stem cells proliferation which eventually leads to stem cell exhaustion (37)	-1.78	0.006
Egln1	NM_053207	C. Elegans, homolog 1	Egln1 plays a critical role in regulating HIF levels. Mutation results in erythrocytosis (38)	-1.67	0.01
Stat3	NM_213659	Signal transducer and activator	Deletion of STAT3 results in a significant increase in proliferation of the myeloid lineage of transcription (14)	-2.32	4.15×10^{-5}
Dok1	NM_010070	Docking protein 1	Myeloid Cells of Dok1 -/- mice show a greater proliferative response to cytokines (39)	-1.41	0.03
Etv6	NM_007961	Ets variant gene 6	Etv6 is required for hematopoiesis within the bone marrow (40)	-1.98	0.0009
Kit	NM_013598	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	Kit is the tyrosine kinase receptor which is critical to pluripotent hematopoietic stem cells (41)	-1.62	0.0009
Pten	NM_008960	Phosphatase and tensin homolog	Pten deficiency promotes human proliferation and can lead to myeloproliferative disease (15)	-1.90	0.01
Myc	NM_010849	V-myc avian myelocytomatosis viral oncogene homolog	Myc -/- embryos show defects in primitive erythropoiesis. Myc expression is required for the expression of Thbs1 and Vegf (42)	-2.33	2.29×10^{-5}
Thbs1	NM_011580	Thrombospondin I	Thbs1 -/- mice have an increased number of circulating white blood cells (especially monocytes and eosinophils) (43)	-4.35	0.004
Vegf	NM_001025250	Vascular endothelial growth factor	Inhibition of Vegf by diverse methods increases hematocrit in both mouse and primate (26)	-1.93	0.004
Kdr	NM_010612	Kinase insert domain receptor	Kdr is the cell-surface receptor of Vegf (tyrosine kinase receptor). Kdr -/- embryos die as the result of an early defect in the development of hematopoietic and endothelial cells (44)	-2.63	0.01

Gene	Accession	Name	Description	Fold	p-value
Bcl2l1	NM_009743	Bcl2-like 1	Bcl2l1 is required for the normal maturation and survival of erythroid cells, especially at the late stage of differentiation (45)	-1.50	0.004
Stat5a	NM_011488	Signal transducer and activator of transcription 5a	Stat5 induces Bcl2l1 gene by direct binding to the promoter. Stat5 -/- embryos have fewer erythroid colonies and showed a significant increase in apoptosis (46)	-2.07	0.02
Gata1	NM_008089	Gata-binding protein 1	Gata1 essential for erythroid and megakaryocyte development and survival. Gata1 -/- embryos die due to proerythroblast arrest and apoptosis of primitive erythroid precursors (47)	-2.07	0.0008
Epb4.2	NM_013513	Erythocytic protein 4.2	Epb4.2 regulates the association of protein 3 with ankyrin on red cell membrane (48)	-1.84	0.01
Mapk14	NM_011951	Mitogen-activated protein kinase 14	MAPK14 plays a critical role in stress-induced and developmental erythropoiesis. Mapk14 -/- embryos failed definitive erythropoiesis (49)	-1.85	0.002
Maea	NM_021500	Macrophage erythroblast attacher	MAEA mediates attachment of erythroblasts to macrophages (50)	-2.30	0.0003
Slc12a4	NM_009195	Solute carrier family 12, member 4	Potassium-chloride co-transporters with high mRNA level during early stages of erythroid differentiation and very low levels in later stages (51)	-1.59	0.02
Dhfr	NM_010049	Dihydrofolate reductase	Deficiency of dihydrofolate reductase prevented conversion of folic acid to tetrahydrofolic acid and leads to megaloblastic anemia (24)	-1.59	0.004
Flvcr1	NM_001081259	Feline leukemia virus subgroup c receptor	Flvcr1 -/- mice lack definitive erythropoiesis. Deletion of the gene during neonatal period leads to severe macrocytic anemia (25). Flvcr1 protects erythroid cells from heme toxicity by exporting of cytoplasmic heme (52)	-1.77	0.002
Lrg1	NM_029796	Leucine-riche alpha-2-glycoprotein 1	Lrg1 up-regulation is an early event in neutrophilic granulocyte differentiation glycoprotein 1 (53)	-2.53	0.0001
Cdk2	NM_016756	Cyclin dependent kinase 2	Cdk2 down-regulates at the end-stage of neutrophil granulocytes differentiation (30)	-1.64	0.007

	Genebank accession number	Description	Function	Fold change	P-value
Cebpa	NM_007678	CCAAT/enhancer-binding protein alpha	Cebpa is specifically up-regulated during granulocytic differentiation. Cebpa -/- mice have no mature granulocytes (27)	-1.67	0.006
Cebpb	NM_009883	CCAAT/enhancer binding protein beta	Cebpb up-regulated during infections to 'ramp up' the number of granulocytes. Cebpb deficient mice have normal granulocyte numbers (54)	-1.50	0.03
Icam1	NM_010493	Intercellular adhesion molecule 1	Icam1 -/- mice exhibit moderate granulocytosis (29)	-1.73	0.03
JunB	NM_008416	Oncogene Jun-b	JunB -/- mice show increased numbers of granulocyte with changes in the expression levels of the antiapoptotic proteins Bcl2 and Bcl2l1 (28)	-1.64	0.03

Up-regulated genes	Genebank accession number	Description	Function	Fold change	P-value
Bmi1	NM_007552	Leukemia viral Bmi-1 oncogene	Bmi1 is required for the self-renewal of murine adult hematopoietic stem cells (55)	1.42	0.04
Thpo	NM_00937	Thrombopoietin	Thpo enhances erythropoiesis (56)	1.44	0.03
Epb4.1	NM_183428	Erythrocyte membrane protein band 4.1	Epb4.1 is a key component of the erythroid membrane skeleton (57)	1.66	0.005
Angpt1	NM_009640	Angiopoietin 1	Angpt1 protects human stem cells from myelosuppressive stress by increasing their ability to become quiescent and bind to the bone (58)	2.77	0.04
Ptprc	NM_011210	Protein-tyrosine phosphatase receptor type C	Ptprc negatively regulates interleukin-3-mediated cellular proliferation and erythropoietin-dependent hematopoiesis (59)	2.09	0.008
Cdkn1b (p27Kip1)	NM_009875	Cyclin-dependent kinase inhibitor 1B	Cdkn1b up-regulates during granulocytic differentiation (60)	2.27	0.0003

Prenatal genistein exposure leads to long-term epigenetic changes

As genistein exposure, early in development, exerts long-term changes in gene expression, we assumed that these effects might be mediated through epigenetic modification.

To examine whether prenatal genistein exposure could lead to long-term epigenetic modifications, we employed an assay in which the methylation-sensitive endonuclease McrBC cleaves methylated DNA but leaves unmethylated CpGs intact. By subsequent PCR amplification, one could quantify the unmethylated repetitive elements (12).

Using this assay, we determined the methylation status of major and minor satellites, intracisternal A particle (IAP-GAG), long interspersed nucleotide elements (LINE1) and short interspersed nucleotide elements (SINEB1 and SINEB2) in hematopoietic cells of both the control and the genistein group.
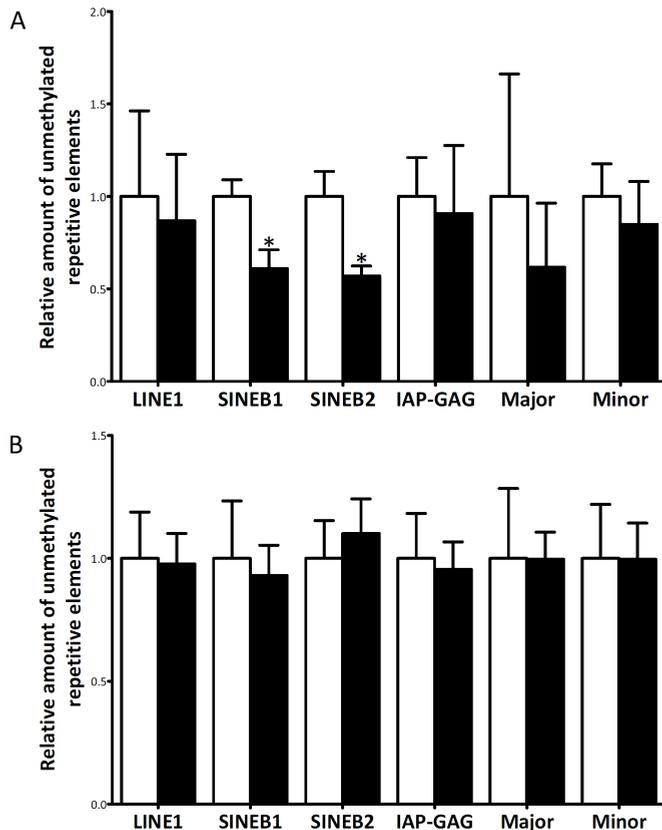
Figure 3. The effect of genistein on DNA methylation of repetitive elements.
A Long-term effects of prenatal exposure to genistein on DNA methylation of repetitive elements in bone marrow cells of mice prenatally exposed to genistein (n=3) compared with control mice (n=3). B Short-term effects of prenatal exposure to genistein on DNA methylation of repetitive elements in fetal liver at E14.5 (genistein exposed fetuses n=4 *versus* control fetuses n=4). The degree of methylation of repetitive elements: long interspersed nucleotide elements (LINE1), short interspersed nucleotide elements (SINEB1 and SINEB2), intracisternal A particle (IAP-GAG), and Major and Minor satellites were determined by methylation-sensitive McrBC-real-time PCR assay. Bars represent the normalized average expression level of the unmethylated repetitive elements between the genistein-exposed (black bars) and the control group (white bars). Error bars represent standard error. Statistical significance was determined by One-way ANOVA: *: P<0.05.

These elements constitute almost 37.5 % of the mouse genome (20). Overall, bone marrow cells of the genistein group (n=3) showed moderate hypermethylation of the repetitive elements observed as lower level of PCR products after McrBC digestion compared with the control group (n=3, Figure 3A). Especially, the SINEB1 and SINEB2 elements were significantly hypermethylated (P=0.045 and P=0.042, respectively). In order to investigate whether these epigenetic modifications had already occurred *in utero*, we studied the methylation status of the repetitive DNA elements in the fetal liver (E14.5) of the control group (n=4) and compared with genistein exposed fetuses (n=4). We did not, however, observed any difference in the methylation status of repetitive elements between the diet groups at that particular time point (Figure 3B).

Genistein exposure affects fetal blood cell development

Although methylation status of the repetitive elements in fetuses which were exposed to genistein through maternal diet was not significantly different from that of the control group, their blood composition already showed substantial differences. Normally the switch from primitive to definitive erythroid lineage starts at embryonic day 11.5 (21).
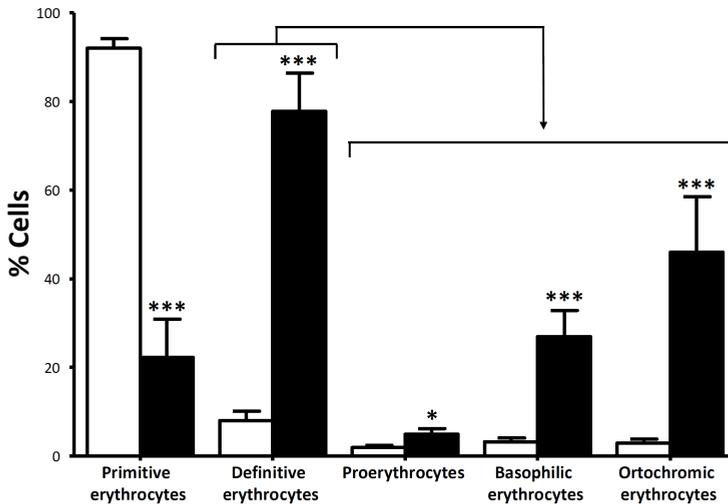
Figure 4. Effect of genistein exposure on the primitive and definitive erythroid lineage.
Blood smears of fetuses (E14.5) from the control (n=15; white bars) and genistein exposed (n=9; black bars) group were analyzed. Bars represent the percentage of cells from the primary and different definitive erythroid (i.e. proerythroblast, basophilic erythroblast and ortochromic erythroblast) lineage. Error bars represent standard error. Statistical significance was determined by One-way ANOVA: *: P<0.05; ***: P<0.001.

A comparison of fetal blood composition (Figure 4) between the genistein (9 mice from 2 litters, n=9) and the control animals (15 mice from 2 litters, n=15) at E14.5, demonstrated an accelerated switch from primitive erythroid lineage to definitive lineage in the fetuses that were exposed to genistein. The percentage of primary erythrocytes was significantly decreased due to genistein exposure (22.23 ± 26.02 % *versus* 92.05 ± 8.31 %, P<0.001) while the percentage of definitive erythrocytes was increased (77.77 ± 26.01 % *versus* 7.96 ± 8.31 %, P<0.001). This increase reflected an overall expansion of definitive progenitors at various stages. The percentage of proerythroblasts (4.92 ± 3.86 % *versus* 1.92 ± 1.75 %,

P=0.02), and especially of basophilic (26.89 ± 17.88 % *versus* 3.17 ± 3.27 %, P<0.001) and orthochromic erythroblasts (45.97 ± 37.57 % *versus* 2.86 ± 3.82 %, P<0.001) were significantly different between the diet groups.

Discussion

In this study, we demonstrate that prenatal exposure of mice to genistein affects fetal red blood cell development and leaves a permanent signature on the hematopoietic lineage. Microarray analysis of hematopoietic cells of mice at 12 weeks of age revealed that prenatal genistein exposure had induced a distinct gene expression profile. Strikingly, some persistent effects of genistein exposure on gene expression could be observed (e.g. down-regulation of *NF-κB*, NF-κB-regulated gene products and several adhesion molecules), while genistein was no longer provided through diet. In general, prenatal genistein exposure was associated with pronounced down-regulation of gene expression. This down-regulation might be partly due to hypermethylation of CpG motifs in the regulatory regions of certain genes. In fact, we demonstrate that prenatal genistein exposure induced hypermethylation of some CpG-rich repetitive elements. However there is no straightforward relation between the hypermethylation seen in the repetitive elements and the change in gene expression. Pogribny *et al.* (22) previously reported that DNA hypermethylation and macrocytosis coincided, as observed in our study. This is presumably by interfering with folate cofactor interactions what affects DNA synthesis and points to the involvement of one-carbon metabolism pathway. It is noteworthy that several genes involved in this pathway, e.g. dihydrofolate reductase (*Dhfr*; fold change=-1.59; P-value=0.004), serine hydroxymethyltransferase 2 (*Shmt2*; fold change=-1.76; P-value=0.005), methylenetetrahydrofolate dehydrogenase 2 (*Mthfd2*; fold change=-2.98; P-value=8.93 x 10^{-5}), 5,10-methenyl-tetrahydrofolate synthetase (*Mthfs*; fold change=-1.58; P-value=0.05) and S-adenosylhomocysteine hydrolase-like 2 (*Ahcyl2*; fold change=-3.31; P-value=0.0008), were significantly down-regulated in the genistein exposed animals.

Prenatal genistein exposure in mice resulted in significant alterations in blood and bone marrow composition later in life, manifested as increased granulopoiesis and erythropoiesis. It is noteworthy that genistein exposed mice exhibited macrocytosis (3 % increase in mean corpuscular volume). The increase in mean corpuscular volume is partly due to the higher number of reticulocytes. On the other hand, the increased erythropoiesis may demand recruitment of more primitive erythroid cells and the progenitor cells may differentiate without prior division. All these phenomena can lead to macrocytosis (23). Remarkably, both *Dhfr* (24) and *Flvcr1* (25), whose deficiencies associate with macrocytosis were down-regulated in the genistein exposed mice. Furthermore, these mice showed down-regulation of *Vegf*. Inhibition of Vegf is reported to increase the hematocrit level (26). Hence the hematocrit level was significantly increased (P=0.004) in mice prenatally exposed to genistein.

Mice in the genistein group had significantly higher numbers of neutrophils (P=0.02) and eosinophils (P=0.04) in the peripheral blood, which was probably a result of the increased granulopoiesis in their bone marrow as seen in Figure 2. Microarray analysis showed that several genes, whose lack of expression associates with defects in granulocytic lineage, were down-regulated in the genistein group (e.g. *Cebpa* (27), *JunB* (28) and *Icam1* (29)).

Furthermore, *Cdk2* expression was down-regulated in the bone marrow, while *Cdkn1b* mRNA was increased. This gene expression pattern relates to end-stage differentiation of neutrophil granulocytes (30). Thus indicating that prenatal exposure to genistein results in an up-regulation of the granlulocytic differentiation, what also could be seen in the increased level of granulocytes detected in both blood and bone marrow of these mice.

As it is know that genistein can stimulate bone marrow formation (31), we assume that most alterations in gene expression are related to genistein-induced changes in bone marrow cellularity. However there are certain changes that do not seem to be directly related to differences in marrow cellularity, for instance changes in the expression of genes that are directly related to genistein exposure or changes in estrogen responsive genes.

Genistein is a well known endocrine-disrupting compound that, due to its structural similarity to 17β-estradiol, exerts its effect through estrogen receptors (9). Several estrogen responsive genes were suppressed in the bone marrow of adult mice that were prenatally exposed to genistein. Down-regulation of estrogen responsive genes has also been reported after exposure to certain other endocrine-disrupting chemicals, known as AhR agonists (e.g. benzo[a]pyrene and TCDD) (17). It is therefore possible that AhR is involved in the genistein-induced gene expression pattern. In fact Zhang *et al.* (32) demonstrated that genistein is a ligand of AhR. It has previously been shown that exposure to AhR ligands can affect hematopoietic blood composition. Exposure of mice to TCDD, skewed differentiation of bone marrow cells in favor of granulocytic and myeloid lineage and altered transcription of several genes (e.g. *Myc*, *Hes1*, *Cdkn1a*, *Cdkn1b*, *Cebp*, *Per1* and *Per2*). Likewise, culturing CD34+ progenitors with the polyaromatic hydrocarbon benzo[a]pyrene, another AhR ligand, skews differentiation toward myeloid cells (30). Therefore, not only the distinct gene expression profile, but also cellular composition of the blood and bone marrow of the genistein exposed mice show signs of AhR involvement. Future studies need to explore the effects of prenatal exposure to other AhR-ligands on hematopoiesis.

In our study, direct exposure of mice fetus to genistein accelerated the switch from primitive erythroid lineage to definitive erythroid lineage. Normally, this switch starts at embryonic day 11.5 when blood circulation has been fully established (21). Examination of fetal blood composition at E14.5 revealed that the percentage of definitive erythrocytes were significantly higher in the genistein group (P<0.001). It is therefore evident that genistein exposure during development has both direct- and long-term effects on hematopoiesis.

Prenatal genistein exposure leads to long-term epigenetic changes. Exposure of heterozygous yellow agouti mice to genistein *via* maternal diet shifted the coat color by hypermethylation of transposable repetitive elements upstream of the transcription start site of the *Agouti* gene. Although genistein exposure terminated after weaning, the altered coat color remained unchanged throughout life (3). Our results show that prenatal genistein exposure associates with hypermethylation of the SINEB1 (P=0.045) and SINEB2 (P=0.042) repetitive elements in bone marrow, at adult age. SINE transcripts are normally up-regulated in response to cell stress. Both the SINEB1 and SINEB2 classes are strongly up-regulated in mice after prolonged exposure to gamma radiation or DNA-damaging compounds, e.g. cisplatin, etoposide (33). Genistein is also a potent DNA-damaging agent, due to its topoisomerase-II inhibiting property (34). It is also interesting to study the direct effects of genistein exposure on SINE repetitive elements. The results presented here demonstrate that despite of the long-term effects of genistein on SINEB1 and SINEB2, the

methylation status of these repeats is not affected directly by genistein exposure during fetal development, but occurs at later time points.

In our study pregnant mice were exposed to approximately 0.8 mg genistein per day. In human, adults consuming modest amounts of soy-containing foods have a total dialy isoflavone intake of approximately 1 to 9 mg. In Asian countries however, the daily intake of isoflavones is much higher, reaching levels of 20-240 mg, due to the high consumption of soy (35). Also vegetarians, who consume large amounts of soy products, have a high intake of genistein. Due to potential health benefits associated with genistein intake, observed as decreased risk for developing breast and prostate cancer in Asian countries (36), genistein supplements are becoming increasingly popular. The manufacturers recommend a daily dose of up to 6 tablets of 25-100 mg of these supplements. It is noteworthy that the metabolism of phytoestrogens is different in the fetus. In human, fetal cord blood has a higher genistein level than maternal serum (16). As genistein accumulates in the fetus and affects hematopoiesis, it is important to establish clear guidelines for fetal exposure to soy derivatives.

Acknowledgments

References

1. Kurzer, M. S., and Xu, X. (1997) Dietary phytoestrogens. *Annu Rev Nutr* 17, 353-381
2. Scalbert, A., and Williamson, G. (2000) Dietary intake and bioavailability of polyphenols. *J Nutr* 130, 2073S-2085S
3. Dolinoy, D. C., Weidman, J. R., Waterland, R. A., and Jirtle, R. L. (2006) Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. *Environ Health Perspect* 114, 567-572
4. Klein, S. L., Wisniewski, A. B., Marson, A. L., Glass, G. E., and Gearhart, J. P. (2002) Early exposure to genistein exerts long-lasting effects on the endocrine and immune systems in rats. *Mol Med* 8, 742-749
5. Ohta, R., Shirota, M., Kanazawa, Y., Shindo, T., Furuya, M., Seki, T., Ono, H., Kojima, K., Asai, S., Watanabe, G., and Taya, K. (2009) Effects of transmaternal exposure to genistein in Hatano high- and low-avoidance rats. *Exp Anim* 58, 471-479
6. Moller, F. J., Zierau, O., Hertrampf, T., Bliedtner, A., Diel, P., and Vollmer, G. (2009) Long-term effects of dietary isoflavones on uterine gene expression profiles. *J Steroid Biochem Mol Biol* 113, 296-303
7. Aslinia, F., Mazza, J. J., and Yale, S. H. (2006) Megaloblastic anemia and other causes of macrocytosis. *Clin Med Res* 4, 236-241
8. Scott, J. M., and Weir, D. G. (1980) Drug-induced megaloblastic change. *Clin Haematol* 9, 587-606
9. Miodini, P., Fioravanti, L., Di Fronzo, G., and Cappelletti, V. (1999) The two phyto-oestrogens genistein and quercetin exert different effects on oestrogen receptor function. *Br J Cancer* 80, 1150-1155
10. Smyth, G. K., Michaud, J., and Scott, H. S. (2005) Use of within-array replicate spots for assessing differential expression in microarray experiments. *Bioinformatics* 21, 2067-2075
11. Gaj, S., van Erk, A., van Haaften, R. I., and Evelo, C. T. (2007) Linking microarray reporters with protein functions. *BMC Bioinformatics* 8, 360
12. Pogribny, I. P., Ross, S. A., Tryndyak, V. P., Pogribna, M., Poirier, L. A., and Karpinets, T. V. (2006) Histone H3 lysine 9 and H4 lysine 20 trimethylation and the expression of Suv4-20h2 and Suv-39h1 histone methyltransferases in hepatocarcinogenesis induced by methyl deficiency in rats. *Carcinogenesis* 27, 1180-1186
13. He, H., Chen, L., Zhai, M., and Chen, J. Z. (2009) Genistein down-regulates the constitutive activation of nuclear factor-kappaB in human multiple myeloma cells, leading to suppression of proliferation and induction of apoptosis. *Phytother Res* 23, 868-873
14. Welte, T., Zhang, S. S., Wang, T., Zhang, Z., Hesslein, D. G., Yin, Z., Kano, A., Iwamoto, Y., Li, E., Craft, J. E., Bothwell, A. L., Fikrig, E., Koni, P. A., Flavell, R. A., and Fu, X. Y. (2003) STAT3 deletion during hematopoiesis causes Crohn's disease-like pathogenesis and lethality: a critical role of STAT3 in innate immunity. *Proc Natl Acad Sci U S A* 100, 1879-1884
15. Yilmaz, O. H., Valdez, R., Theisen, B. K., Guo, W., Ferguson, D. O., Wu, H., and Morrison, S. J. (2006) Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. *Nature* 441, 475-482
16. Adlercreutz, H., Yamada, T., Wahala, K., and Watanabe, S. (1999) Maternal and neonatal phytoestrogens in Japanese women during birth. *Am J Obstet Gynecol* 180, 737-743
17. Tanaka, J., Yonemoto, J., Zaha, H., Kiyama, R., and Sone, H. (2007) Estrogen-responsive genes newly found to be modified by TCDD exposure in human cell lines and mouse systems. *Mol Cell Endocrinol* 272, 38-49
18. Singh, K. P., Casado, F. L., Opanashuk, L. A., and Gasiewicz, T. A. (2009) The aryl hydrocarbon receptor has a normal function in the regulation of hematopoietic and other stem/progenitor cell populations. *Biochem Pharmacol* 77, 577-587
19. Ohtake, F., Takeyama, K., Matsumoto, T., Kitagawa, H., Yamamoto, Y., Nohara, K., Tohyama, C., Krust, A., Mimura, J., Chambon, P., Yanagisawa, J., Fujii-Kuriyama, Y., and Kato, S. (2003) Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. *Nature* 423, 545-550
20. Waterston, R. H., Lindblad-Toh, K., Birney, E., Rogers, J., Abril, J. F., Agarwal, P., Agarwala, R., Ainscough, R., Alexandersson, M., An, P., Antonarakis, S. E., Attwood, J., Baertsch, R., Bailey, J., Barlow, K., Beck, S., Berry, E., Birren, B., Bloom, T., Bork, P., Botcherby, M., Bray, N., Brent, M. R., Brown, D. G., Brown, S. D., Bult, C., Burton, J., Butler, J., Campbell, R. D., Carninci, P., Cawley, S., Chiaromonte, F., Chinwalla, A. T., Church, D. M., Clamp, M., Clee, C., Collins, F. S., Cook, L. L., Copley, R. R., Coulson, A., Couronne, O., Cuff, J., Curwen, V., Cutts, T., Daly, M., David, R., Davies, J., Delehaunty, K. D., Deri, J., Dermitzakis, E. T., Dewey, C., Dickens, N. J., Diekhans, M., Dodge, S., Dubchak, I., Dunn, D. M., Eddy, S. R., Elnitski, L., Emes, R. D., Eswara, P., Eyras, E., Felsenfeld, A., Fewell, G. A., Flicek, P., Foley, K., Frankel, W. N., Fulton, L. A., Fulton, R. S., Furey, T. S., Gage,

D., Gibbs, R. A., Glusman, G., Gnerre, S., Goldman, N., Goodstadt, L., Grafham, D., Graves, T. A., Green, E. D., Gregory, S., Guigo, R., Guyer, M., Hardison, R. C., Haussler, D., Hayashizaki, Y., Hillier, L. W., Hinrichs, A., Hlavina, W., Holzer, T., Hsu, F., Hua, A., Hubbard, T., Hunt, A., Jackson, I., Jaffe, D. B., Johnson, L. S., Jones, M., Jones, T. A., Joy, A., Kamal, M., Karlsson, E. K., Karolchik, D., Kasprzyk, A., Kawai, J., Keibler, E., Kells, C., Kent, W. J., Kirby, A., Kolbe, D. L., Korf, I., Kucherlapati, R. S., Kulbokas, E. J., Kulp, D., Landers, T., Leger, J. P., Leonard, S., Letunic, I., Levine, R., Li, J., Li, M., Lloyd, C., Lucas, S., Ma, B., Maglott, D. R., Mardis, E. R., Matthews, L., Mauceli, E., Mayer, J. H., McCarthy, M., McCombie, W. R., McLaren, S., McLay, K., McPherson, J. D., Meldrim, J., Meredith, B., Mesirov, J. P., Miller, W., Miner, T. L., Mongin, E., Montgomery, K. T., Morgan, M., Mott, R., Mullikin, J. C., Muzny, D. M., Nash, W. E., Nelson, J. O., Nhan, M. N., Nicol, R., Ning, Z., Nusbaum, C., O'Connor, M. J., Okazaki, Y., Oliver, K., Overton-Larty, E., Pachter, L., Parra, G., Pepin, K. H., Peterson, J., Pevzner, P., Plumb, R., Pohl, C. S., Poliakov, A., Ponce, T. C., Ponting, C. P., Potter, S., Quail, M., Reymond, A., Roe, B. A., Roskin, K. M., Rubin, E. M., Rust, A. G., Santos, R., Sapojnikov, V., Schultz, B., Schultz, J., Schwartz, M. S., Schwartz, S., Scott, C., Seaman, S., Searle, S., Sharpe, T., Sheridan, A., Shownkeen, R., Sims, S., Singer, J. B., Slater, G., Smit, A., Smith, D. R., Spencer, B., Stabenau, A., Stange-Thomann, N., Sugnet, C., Suyama, M., Tesler, G., Thompson, J., Torrents, D., Trevaskis, E., Tromp, J., Ucla, C., Ureta-Vidal, A., Vinson, J. P., Von Niederhausern, A. C., Wade, C. M., Wall, M., Weber, R. J., Weiss, R. B., Wendl, M. C., West, A. P., Wetterstrand, K., Wheeler, R., Whelan, S., Wierzbowski, J., Willey, D., Williams, S., Wilson, R. K., Winter, E., Worley, K. C., Wyman, D., Yang, S., Yang, S. P., Zdobnov, E. M., Zody, M. C., and Lander, E. S. (2002) Initial sequencing and comparative analysis of the mouse genome. *Nature* 420, 520-562

21. Kingsley, P. D., Malik, J., Emerson, R. L., Bushnell, T. P., McGrath, K. E., Bloedorn, L. A., Bulger, M., and Palis, J. (2006) "Maturational" globin switching in primary primitive erythroid cells. *Blood* 107, 1665-1672

22. Pogribny, I. P., Karpf, A. R., James, S. R., Melnyk, S., Han, T., and Tryndyak, V. P. (2008) Epigenetic alterations in the brains of Fisher 344 rats induced by long-term administration of folate/methyl-deficient diet. *Brain Res* 1237, 25-34

23. Tsantes, A. E., Bonovas, S., Travlou, A., and Sitaras, N. M. (2006) Redox imbalance, macrocytosis, and RBC homeostasis. *Antioxid Redox Signal* 8, 1205-1216

24. Tauro, G. P., Danks, D. M., Rowe, P. B., Van der Weyden, M. B., Schwarz, M. A., Collins, V. L., and Neal, B. W. (1976) Dihydrofolate reductase deficiency causing megaloblastic anemia in two families. *N Engl J Med* 294, 466-470

25. Keel, S. B., Doty, R. T., Yang, Z., Quigley, J. G., Chen, J., Knoblaugh, S., Kingsley, P. D., De Domenico, I., Vaughn, M. B., Kaplan, J., Palis, J., and Abkowitz, J. L. (2008) A heme export protein is required for red blood cell differentiation and iron homeostasis. *Science* 319, 825-828

26. Tam, B. Y., Wei, K., Rudge, J. S., Hoffman, J., Holash, J., Park, S. K., Yuan, J., Hefner, C., Chartier, C., Lee, J. S., Jiang, S., Nayak, N. R., Kuypers, F. A., Ma, L., Sundram, U., Wu, G., Garcia, J. A., Schrier, S. L., Maher, J. J., Johnson, R. S., Yancopoulos, G. D., Mulligan, R. C., and Kuo, C. J. (2006) VEGF modulates erythropoiesis through regulation of adult hepatic erythropoietin synthesis. *Nat Med* 12, 793-800

27. Pabst, T., Mueller, B. U., Zhang, P., Radomska, H. S., Narravula, S., Schnittger, S., Behre, G., Hiddemann, W., and Tenen, D. G. (2001) Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. *Nat Genet* 27, 263-270

28. Passegue, E., Jochum, W., Schorpp-Kistner, M., Mohle-Steinlein, U., and Wagner, E. F. (2001) Chronic myeloid leukemia with increased granulocyte progenitors in mice lacking junB expression in the myeloid lineage. *Cell* 104, 21-32

29. Sligh, J. E., Jr., Ballantyne, C. M., Rich, S. S., Hawkins, H. K., Smith, C. W., Bradley, A., and Beaudet, A. L. (1993) Inflammatory and immune responses are impaired in mice deficient in intercellular adhesion molecule 1. *Proc Natl Acad Sci U S A* 90, 8529-8533

30. Singh, K. P., Wyman, A., Casado, F. L., Garrett, R. W., and Gasiewicz, T. A. (2009) Treatment of mice with the Ah receptor agonist and human carcinogen dioxin results in altered numbers and function of hematopoietic stem cells. *Carcinogenesis* 30, 11-19

31. Hertrampf, T., Schleipen, B., Offermanns, C., Velders, M., Laudenbach, U., and Diel, P. (2009) Comparison of the bone protective effects of an isoflavone-rich diet with dietary and subcutaneous administrations of genistein in ovariectomized rats. *Toxicol Lett* 184, 198-203

32. Zhang, S., Qin, C., and Safe, S. H. (2003) Flavonoids as aryl hydrocarbon receptor agonists/antagonists: effects of structure and cell context. *Environ Health Perspect* 111, 1877-1882

33. Williams, W. P., Tamburic, L., and Astell, C. R. (2004) Increased levels of B1 and B2 SINE transcripts in mouse fibroblast cells due to minute virus of mice infection. *Virology* 327, 233-241

34. Barjesteh van Waalwijk van Doorn-Khosrovani, S., Janssen, J., Maas, L. M., Godschalk, R. W., Nijhuis, J. G., and van Schooten, F. J. (2007) Dietary flavonoids induce MLL translocations in primary human CD34+ cells. *Carcinogenesis* 28, 1703-1709

35. Skibola, C. F., and Smith, M. T. (2000) Potential health impacts of excessive flavonoid intake. *Free Radic Biol Med* 29, 375-383

36. Coward, L., Barnes, N., Setchell, K., and Barnes, S. (1993) Genistein, daidzein, and their .beta.-glycoside conjugates: antitumor isoflavones in soybean foods from American and Asian diets. *J Agric Food Chem* 41, 1961–1967

37. Cheng, T., Rodrigues, N., Shen, H., Yang, Y., Dombkowski, D., Sykes, M., and Scadden, D. T. (2000) Hematopoietic stem cell quiescence maintained by p21cip1/waf1. *Science* 287, 1804-1808

38. Percy, M. J., Zhao, Q., Flores, A., Harrison, C., Lappin, T. R., Maxwell, P. H., McMullin, M. F., and Lee, F. S. (2006) A family with erythrocytosis establishes a role for prolyl hydroxylase domain protein 2 in oxygen homeostasis. *Proc Natl Acad Sci U S A* 103, 654-659

39. Yasuda, T., Shirakata, M., Iwama, A., Ishii, A., Ebihara, Y., Osawa, M., Honda, K., Shinohara, H., Sudo, K., Tsuji, K., Nakauchi, H., Iwakura, Y., Hirai, H., Oda, H., Yamamoto, T., and Yamanashi, Y. (2004) Role of Dok-1 and Dok-2 in myeloid homeostasis and suppression of leukemia. *J Exp Med* 200, 1681-1687

40. Wang, L. C., Swat, W., Fujiwara, Y., Davidson, L., Visvader, J., Kuo, F., Alt, F. W., Gilliland, D. G., Golub, T. R., and Orkin, S. H. (1998) The TEL/ETV6 gene is required specifically for hematopoiesis in the bone marrow. *Genes Dev* 12, 2392-2402

41. Geissler, E. N., Ryan, M. A., and Housman, D. E. (1988) The dominant-white spotting (W) locus of the mouse encodes the c-kit proto-oncogene. *Cell* 55, 185-192

42. Baudino, T. A., McKay, C., Pendeville-Samain, H., Nilsson, J. A., Maclean, K. H., White, E. L., Davis, A. C., Ihle, J. N., and Cleveland, J. L. (2002) c-Myc is essential for vasculogenesis and angiogenesis during development and tumor progression. *Genes Dev* 16, 2530-2543

43. Lawler, J., Sunday, M., Thibert, V., Duquette, M., George, E. L., Rayburn, H., and Hynes, R. O. (1998) Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. *J Clin Invest* 101, 982-992

44. Shalaby, M. R., Carter, P., Maneval, D., Giltinan, D., and Kotts, C. (1995) Bispecific HER2 x CD3 antibodies enhance T-cell cytotoxicity in vitro and localize to HER2-overexpressing xenografts in nude mice. *Clin Immunol Immunopathol* 74, 185-192

45. Gregoli, P. A., and Bondurant, M. C. (1997) The roles of Bcl-X(L) and apopain in the control of erythropoiesis by erythropoietin. *Blood* 90, 630-640

46. Socolovsky, M., Fallon, A. E., Wang, S., Brugnara, C., and Lodish, H. F. (1999) Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. *Cell* 98, 181-191

47. Fujiwara, Y., Browne, C. P., Cunniff, K., Goff, S. C., and Orkin, S. H. (1996) Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-1. *Proc Natl Acad Sci U S A* 93, 12355-12358

48. Davies, K. A., and Lux, S. E. (1989) Hereditary disorders of the red cell membrane skeleton. *Trends Genet* 5, 222-227

49. Tamura, K., Sudo, T., Senftleben, U., Dadak, A. M., Johnson, R., and Karin, M. (2000) Requirement for p38alpha in erythropoietin expression: a role for stress kinases in erythropoiesis. *Cell* 102, 221-231

50. Hanspal, M., Smockova, Y., and Uong, Q. (1998) Molecular identification and functional characterization of a novel protein that mediates the attachment of erythroblasts to macrophages. *Blood* 92, 2940-2950

51. Pellegrino, C. M., Rybicki, A. C., Musto, S., Nagel, R. L., and Schwartz, R. S. (1998) Molecular identification and expression of erythroid K:Cl cotransporter in human and mouse erythroleukemic cells. *Blood Cells Mol Dis* 24, 31-40

52. Quigley, J. G., Yang, Z., Worthington, M. T., Phillips, J. D., Sabo, K. M., Sabath, D. E., Berg, C. L., Sassa, S., Wood, B. L., and Abkowitz, J. L. (2004) Identification of a human heme exporter that is essential for erythropoiesis. *Cell* 118, 757-766

53. O'Donnell, L. C., Druhan, L. J., and Avalos, B. R. (2002) Molecular characterization and expression analysis of leucine-rich alpha2-glycoprotein, a novel marker of granulocytic differentiation. *J Leukoc Biol* 72, 478-485

54. Kincade, P. W. (2006) Supplying the demand for granulocytes. *Nat Immunol* 7, 701-702

55. Park, I. K., Qian, D., Kiel, M., Becker, M. W., Pihalja, M., Weissman, I. L., Morrison, S. J., and Clarke, M. F. (2003) Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature* 423, 302-305

56. Kaushansky, K., Broudy, V. C., Grossmann, A., Humes, J., Lin, N., Ren, H. P., Bailey, M. C., Papayannopoulou, T., Forstrom, J. W., and Sprugel, K. H. (1995) Thrombopoietin expands erythroid progenitors, increases red cell production, and enhances erythroid recovery after myelosuppressive therapy. *J Clin Invest* 96, 1683-1687

57. Takakuwa, Y. (2001) Regulation of red cell membrane protein interactions: implications for red cell function. *Curr Opin Hematol* 8, 80-84

58. Arai, F., Hirao, A., Ohmura, M., Sato, H., Matsuoka, S., Takubo, K., Ito, K., Koh, G. Y., and Suda, T. (2004) Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* 118, 149-161

59. Irie-Sasaki, J., Sasaki, T., Matsumoto, W., Opavsky, A., Cheng, M., Welstead, G., Griffiths, E., Krawczyk, C., Richardson, C. D., Aitken, K., Iscove, N., Koretzky, G., Johnson, P., Liu, P., Rothstein, D. M., and Penninger, J. M. (2001) CD45 is a JAK phosphatase and negatively regulates cytokine receptor signalling. *Nature* 409, 349-354

60. Klausen, P., Bjerregaard, M. D., Borregaard, N., and Cowland, J. B. (2004) End-stage differentiation of neutrophil granulocytes in vivo is accompanied by up-regulation of p27kip1 and down-regulation of CDK2, CDK4, and CDK6. *J Leukoc Biol* 75, 569-578

Chapter 4

Intrauterine exposure to flavonoids modifies antioxidant status at adulthood and protects against oxidative stress induced DNA damage

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Abstract

The *in utero* environment is predominantly determined by the maternal diet and may alter the risk for several diseases at adult age. Flavonoids are polyphenolic compounds in our diet that are known for their antioxidant properties and their ability to up-regulate the enzymatic antioxidant capacity of cells. Therefore, we investigated whether maternal intake of genistein (1 mmol/ kg feed) or quercetin (1 mmol/ kg feed) could modulate antioxidant capacity and subsequent oxidative stress induced DNA damage in liver and lung tissue of 12 week old offspring mice. At day 14.5 of gestation, intake of these flavonoids increased gene expression of several antioxidant genes in the liver of fetuses. This effect persisted till adult age, because 12 week old mice prenatally exposed to flavonoids still had enhanced expression of some of these antioxidant genes in liver and lung. In liver, relative expression of hemeoxygenase-1 (*Ho1*) was >6-fold enhanced by maternal quercetin intake, which indicates Nrf2-pathway activation. This is in line with the observation that prenatal exposure to genistein, but especially to quercetin resulted in lower levels of oxidative stress induced DNA lesions in liver, while no effects were found in lung. Prenatal diet had mild effect on the non-enzymatic antioxidant capacity of the liver at adult age, which was vitamin C independent. Prenatal exposure to genistein increased the non-enzymatic antioxidant capacity of the lung, though no changes in the amount of oxidative stress induced DNA lesions were found. These results suggest that prenatal exposure to flavonoids protects against oxidative stress induced DNA damage in the liver by a persistent pre-emptive trigger of the enzymatic antioxidant system.

Introduction

Studies in humans and animals have shown that the *in utero* environment is determinant for the risk of several diseases at older age, including cancer (1, 2) as well as metabolic (3-5) and cardiovascular disorders (3, 6). Since reactive oxygen species (ROS) are thought to play an important role in the development and progression of all these chronic diseases (7) and because the *in utero* environment is predominantly determined by the maternal diet, one could speculate that intake of dietary antioxidants during pregnancy may provide an *in utero* environment that may prevent the development of chronic diseases later in life. One major source of antioxidants in our diet are the polyphenolic flavonoids (8). The daily dietary intake of mixed flavonoids in the human population ranges from 65 to 250 mg/ day (9). The most predominant flavonoid in the human diet is quercetin, which is mainly found in onions, apples, tea and red wine (8, 10). Another widely consumed flavonoid is the phytoestrogen genistein, which is mainly found in soy products (11, 12). Flavonoids are thought to be health beneficial since in epidemiological studies they were associated with protection against cardiovascular diseases (13, 14) and neurodegenerative diseases (15, 16) and they acted anti-inflammatory (17, 18). These effects are mainly thought to be the result of their antioxidant property (8, 19) as ROS play a major role in disease development and progression (20-26). However, pro-oxidant effects have also been reported (27-30).

Besides antioxidant nutrients, cells also possess enzymatic antioxidants to protect them against endogenous and exogenous sources of oxidative stress, like superoxide dismutase (Sod) which scavenges superoxide ($O_2^{\bullet-}$) and catalase (Cat) and glutathione peroxidase (Gpx) which scavenge hydrogen peroxide (H_2O_2) (31, 32). An important transcription factor that regulates gene expression to counteract oxidative stress is the nuclear erythroid 2-related factor 2 (Nrf2). This transcription factor is normally targeted for proteasomal degradation by binding to its chaperon Kelch-like ECH-associated protein (Keap1). However, ROS and electrophiles can alter the conformation of the Nrf2-Keap1 complex resulting in the nuclear translocation of Nrf2. Here, it can bind to antioxidant response elements (ARE) in the promoter region of target genes (for instance hemeoxygenase-1, *Ho1*) resulting in up-regulation of their gene expression (33, 34). The flavonoid quercetin was found to regulate the Nrf2-pathway and as such enhance the antioxidant potential of cells (35, 36). Hur *et al.* (34) stated that a pre-emptive activation of the Nrf2 pathway potentiates the level of a wide range of protective enzymes that counteract oxidative and environmental stresses, and confers resistance to subsequent challenges of cellular stress. On the other hand, Breinholt *et al.* (37) showed that female mice exposed for 2 weeks to high doses of several flavonoids, including genistein and quercetin, had reduced activity of glutathione reductase, Cat and Gpx in red blood cells. Still, these rats were protected from 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP)-induced oxidative stress as seen in the decreased levels of plasma malondialdehyde.

Overall, these data indicate that there is a close relation between dietary and enzymatic antioxidants and this relation can potentially be affected by the presence of antioxidants in the *in utero* environment. Therefore, we investigated whether prenatal exposure to genistein and quercetin could affect the antioxidant capacity at adult age. To this end, we exposed female mice from 3 days before and throughout the gestation to genistein (1 mmol/ kg feed) or quercetin (1 mmol/ kg feed). The gene expression of genes involved in the Nrf2-pathway and of several other antioxidant genes (*Cat, Sod2* and Gpx3) was determined in liver and lung of adult offspring mice, and also in the liver of fetuses (at day 14.5 of gestation) exposed to

genistein or quercetin. To study the non-enzymatic antioxidant capacity of liver and lung the trolox equivalent antioxidant capacity (TEAC), vitamin C levels and the presence of vitamin C radicals in 12 week old mice prenatally exposed to genistein or quercetin was determined. ROS induced DNA damage was determined in adult mice by measuring 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) and malondialdehyde-deoxyguanosine (M_1dG) levels in DNA from liver and lung.

Material and methods

Mice and sample collection

Female mice (129/SvJ:C57BL/6J background) approximately 8 weeks of age, received either normal chow (low phytoestrogen content complete feed for mice breeding, ssniff®, Soest, Germany) or the same chow supplemented with genistein (1 mmol (270 mg)/ kg feed, LC Laboratories, Woburn, MA, USA) or with quercetin (1 mmol (302 mg)/ kg feed, Sigma, Zwijndrecht, the Netherlands) from 3 days before conception until the end of gestation. After delivery all mothers and pups, regardless of their prenatal diet, received normal chow and the offspring were sacrificed by cardiac puncture after anesthesia at 12 weeks of age. Liver and lung tissue of 5 male mice per diet were isolated.

To determine the direct effects of genistein and quercetin exposure on antioxidant gene expression, female mice (129/SvJ:C57BL/6J background) were mated overnight and conception day was determined by recognition of a vaginal plug the next morning (considered as day 0.5 of gestation). Female mice were placed on the same control or genistein- or quercetin-enriched chow as described above, starting three days before conception. On day 14.5 of gestation (E14.5), mice were sacrificed to isolate fetal livers.

Quantitative real-time PCR

RNA was isolated from liver of E14.5 fetuses and from the lung and liver of 12 week old mice by homogenizing the tissue using TRIzol Reagent (Invitrogen, Breda, the Netherlands) according to manufacturer's instructions and using the Ultra-Turrax homogenizer (IKA, Staufen, Germany). Quantity and purity control of the RNA was spectrophotometrically assessed by using the Nanodrop 1000 (Thermo Scientific, Wilmington, USA). Next, 1 µg of RNA was used to synthesize cDNA using the iScript cDNA synthesis kit (Biorad, Hercules, USA) following manufacturer's instructions. The reaction was performed using a Biometra Tprofessional thermocycler (Biometra, Leusden, the Netherlands).

An aliquot of one 10th of the resulting cDNA was applied for quantitative PCR amplification together with 12.5 µl SYBR Green (Qiagen) and 7.5 pmol of each primer (see Table 1, Eurogentec, Maastricht, the Netherlands) in a volume of 25 µl. The reactions were carried out using a MyiQ Single Color RT-PCR detection system (Bio-rad), under the following conditions: 1 cycle at 95 °C for 3 minutes and then 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute followed by 1 cycle at 95 °C for 1 minute and 1 cycle at 65 °C. Data were analyzed by MyiQ Software system (Bio-rad), Ct values were normalized for an endogenous reference gene (β-actin) and compared with the calibrator (i.e. average Ct value of control samples) and expressed as fold change ($2^{-\Delta\Delta Ct}$).

Table 1: Forward and reverse primers used for quantitative real-time PCR

	Forward primer (5'-3')	Reverse primer (5'-3')
Cat	AGCGACCAGATGAAGCAGTG	TCCGCTCTCTGTCAAAGTGTG
Sod2	CAGACCTGCCTTACGACTATGG	CTCGGTGGCGTTGAGATTGTT
Gpx3	CCTTTTAAGCAGTATGCAGGCA	CAAGCCAAATGGCCCAAGTT
Keap1	CGGGGACGCAGTGATGTATG	TGTGTAGCTGAAGGTTCGGTTA
Nrf2	CTTTAGTCAGCGACAGAAGGAC	AGGCATCTTGTTTGGGAATGTG
Ho1	TCCAGAGTTTCCGCATAC	CGGACTGGGCTAGTTCA
β-actin	CAAGAAGGAAGGCTGGAAAAGA	ACGGCCAGGTCATCACTATTG

Liver and lung tissue homogenates

To determine TEAC, vitamin C level and vitamin C radical formation in liver and lung tissue, liver and lung extracts were obtained by homogenizing the tissue in cold 10 mM Tris 150 mM KCl buffer (pH 7.4), using the Ultra-Turrax homogenizer (IKA), followed by centrifugation at 4 °C for 15 minutes. Protein concentration of the homogenates was determined by the Lowry assay (Bio-Rad) with bovine serum albumin as a standard.

Trolox equivalent antioxidant capacity

The antioxidant capacity (TEAC) was quantified as TEAC according to Fischer *et al.* (38). The samples were deproteinized with a final concentration of 5% trichloroacetic acid (TCA). The samples were than incubated with ABTS radical solution prepared according the procedure described previously (38). After five minute incubation, the absorbance at 734 nm was determined. The assay was calibrated using solutions of the synthetic vitamin E analog trolox. The TEAC is expressed in trolox equivalents.

Vitamin C concentration in liver and lung tissue

To determine the vitamin C level, 250 µl of liver or lung homogenate was deproteinized using 10 % trichloroacetic acid (TCA) (1:4). Ascorbate was oxidized to dehydroascorbate using ascorbate oxidase. Subsequently, o-phenylene diamine was added, to convert dehydroascorbate into a fluorescent product that was quantified using HPLC. HPLC analysis of the samples was performed using a reverse phase C18 column with methanol potassium phosphate buffer (20/80) as eluens, using a fluorescence detector with 355 nm as excitation and 425 nm as emission wavelengths.

Electron spin resonance spectroscopy

To determine the presence of vitamin C radicals, liver and lung homogenates were placed on ice for direct measurements using electron spin resonance (ESR) spectroscopy. ESR spectra were recorded using the settings as previously described by Linschooten *et al.* (39) on a Bruker EMX 1273 spectrometer equipped with an ER 4119HS high sensitivity resonator. Vitamin C radical signals, characterized by a doublet with a splitting constant (AH) of 1.8 G were quantified (in arbitrary units) through peak surface measurements using the WIN-EPR spectrum manipulation program.

8-oxo-dG measurements

Genomic DNA isolation and 8-oxo-dG measurements were performed as described previously (40). Briefly, HPLC with electrochemical detection (ECD) was performed using genomic DNA obtained by grinding frozen liver and lung, followed by standard phenol extraction (41). The DNA-extraction procedure was optimized to minimize artificial induction of 8-oxo-dG, by using radical-free phenol, minimizing exposure to oxygen and by addition of 1 mM deferoxamine mesylate and 20 mM TEMPO (2,2,6,6-tetramethylpiperidine-N-oxyl). DNA concentrations were spectrophotometrically quantified at 260 nm. HPLC-ECD of 8-oxo-dG was performed by digesting 30 μg DNA to deoxyribonucleosides by adding of 6 μl 0.5 M NaAc, 9 μl 10 mM $ZnCl_2$ and 1.5 μl nuclease P1 (stock: 1 U/ μl) and incubation for 90 minutes at 37 °C. Subsequently, 30 μl 0.5 M Tris–HCl (pH 7.4) and 1.5 μl alkaline phosphatase (0.014 U/ μl) were added followed by incubation at 37 °C for 45 minutes. The digest was then analyzed by HPLC-ECD on a Supelcosil™ LC-18S column (250 mm × 4.6 mm) (Supelco Park, Bellefonte, PA, USA) in combination with a DECADE electrochemical detector (Antec, Leiden, the Netherlands). The ECD-signal was first stabilized with mobile phase (94 mM KH_2PO_4, 13 mM K_2HPO_4, 26 mM KCl and 0.5 mM EDTA, 10 % methanol) for approximately 3 hours at a flow rate of 1 ml/ minute. After stabilization, 8-oxo-dG was detected at a potential of 400 mV and dG was simultaneously monitored by UV absorption at 260 nm.

M_1dG level measurements

Genomic DNA was isolated from liver and lung tissue of adult mice using DNAzol Reagent (Invitrogen, Bleiswijk, the Netherlands) following the manufacturer's protocol and by homogenizing the tissue using an Ultra-Turrax homogenizer (IKA, Staufen, Germany). ^{32}P-DNA post-labeling of M_1dG was performed as described previously (42). Reference adduct standards were used for the optimization of the [3]2P-DNA post-labeling procedure. DNA (2 μg) was digested by micrococcal nuclease and spleen phosphodiesterase. Hydrolyzed samples were subsequently treated with 2.5 μg of nuclease P1 for 30 minutes at 37 °C. The nuclease P1-treated samples were incubated with 15–25 μCi of [γ-32P]-ATP (3000 Ci/ mmol) and T4-polynucleotide kinase (0.75 U/ μl) to generate ^{32}P-labeled M_1dG. Samples were applied to the origin of polyethyleneimine-cellulose (PEI) sheets (10 x 20 cm) and developed with 0.35 $MgCl_2$ for 2.0 cm on a filter paper wick. Plates were developed in the opposite direction with 2.1 M lithium formate, 3.75 M urea (pH 3.75), and then run at the right angle to the previous development with 0.24 M sodium phosphate, 2.4 M urea (pH 6.4). Detection and quantification of M_1dG and total nucleotides was obtained by storage phosphor imaging technique employing intensifying screens (Molecular Dynamics, Sunnyvale, CA, USA) for 0.20–48 hour. The screens were scanned using a Typhoon 9210 (Amersham, Buckinghamshire, UK). To process the data ImageQuant (Molecular Dynamics) was used. After background subtraction, the levels of M_1dG-adducts were expressed as relative adduct labeling (RAL=screen pixel in adducted nucleotides/ screen pixel in total normal nucleotides). To calculate the levels of total normal nucleotides, aliquots of hydrolyzed DNA were appropriately diluted and reacted in the same mixtures used for M_1dG labeling. The obtained ^{32}P-labeled total nucleotides were separated on PEI-cellulose-sheets using 280 mM ammonium sulfate and 50 mM sodium phosphate. The values measured for M_1dG-adducts

were corrected across experiments based on the recovery of internal standard after the ^{32}P-DNA postlabeling assay.

Statistical analysis

Statistical analysis was performed with Statistical Package for Social Sciences (SPSS version 17 for Windows, SPSS Inc., Chicago, IL, USA). Nested ANOVA was performed to investigate the effect of genistein or quercetin exposure on genes involved in antioxidant defense system of the fetal liver (day 14.5 of gestation). ANOVA was applied to test differences in antioxidant gene expression, non-enzymatic antioxidant capacity, vitamin C level and radical formation; and oxidative stress induced DNA damage in liver and lung of 12 week old control mice compared to 12 week old mice prenatally exposed to genistein or quercetin. To avoid litter effects no more than two adult mice per litter were used for statistical analysis.

Results

Exposure to quercetin induced Nrf2 and Sod2 expression in the liver of fetuses at day 14.5 of the pregnancy

To investigate whether exposure to antioxidants/ pro-oxidants could directly affect the gene expression of several antioxidant genes including the key regulators of the oxidative stress response pathway, namely *Nrf2*, its negative regulator *Keap1* and down-stream target gene *Ho1,* of the unborn child, female mice were exposed *via* their diet to genistein (1 mmol/ kg feed) or quercetin (1 mmol/ kg feed) starting from three days before conception and female mice were sacrificed at day 14.5 of gestation. The liver of the fetuses were isolated to determine gene expression. Results showed that only exposure to quercetin could induce the gene expression significantly at day 14.5 of the gestation, namely of *Sod2* (P=0.001) and *Nrf2* (P=0.04, Figure 1).

Prenatal exposure to flavonoids induced Nrf2-pathway in liver and antioxidant genes in liver and lung of 12 week old mice

To investigate whether the prenatal diet still had a measurable effect on gene expression of enzymatic antioxidants in offspring at adulthood, expression of genes involved in the antioxidant defense system (*Cat*, *Sod2*, *Gpx3*, *Nrf2*, *Keap1* and *Ho1*) was assessed in liver and lung of adult offspring mice. In the liver of 12 week old mice prenatally exposed to quercetin we found that *Nrf2* (P=0.002), *Ho1* (P=0.02), *Cat* (P=0.04) and *Gpx3* (P=0.05) gene expression was increased compared to offspring mice of non-supplemented mothers (Figure 2A). Interestingly, of these genes only *Nrf2* gene expression was already up-regulated in liver of fetuses at day 14.5 of gestation. Adult mice prenatally exposed to genistein also showed a significant increase in gene expression of *Nrf2* (P=0.01), while induction of *Cat* in the liver did just not reach statistical significance (P=0.06).
In the lung of the genistein exposed mice only *Gpx3* gene expression showed a significant increase (P=0.03) compared to control animals (Figure 2B). In addition, prenatal exposure to genistein also resulted in a modest increase in gene expression of *Sod2* gene expression (P=0.07) in the lung of 12 week old mice whereas prenatal exposure to quercetin induced

Sod2 gene expression significantly (P=0.01). *Keap1* gene expression was also significantly increased (P=0.04) in mice prenatally exposed to quercetin, as was for *Nrf2*, though not statistically significant (P=0.09).
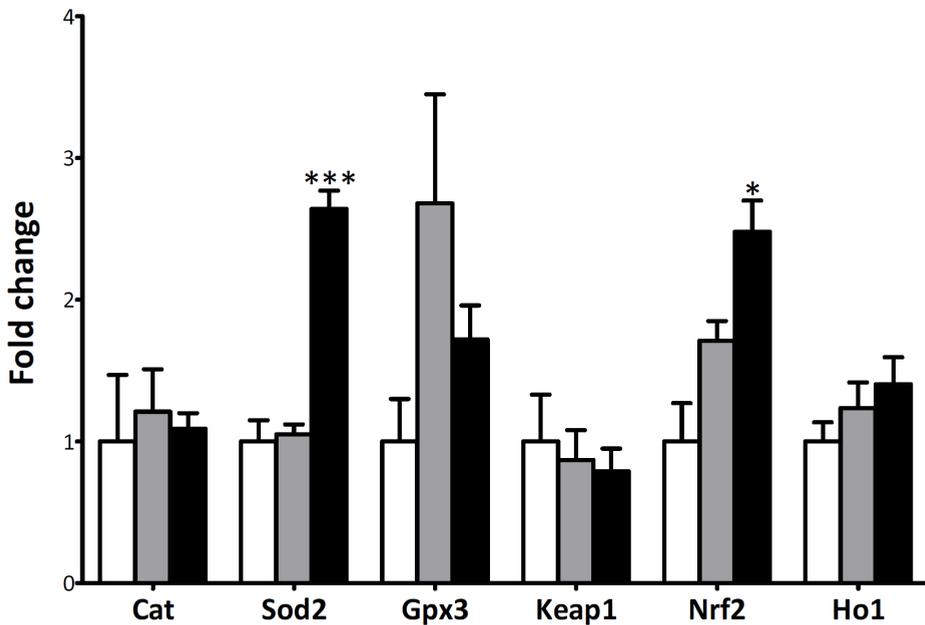
Figure 1. Direct effect of genistein and quercetin exposure on antioxidant, *Keap1*, *Nrf2* and *Ho1* gene expression in the liver of E14.5 fetuses.

mRNA expression level of antioxidant genes (*Cat*, *Sod2* and *Gpx3*), *Keap1*, *Nrf2* and *Ho1* were measured in the liver of E14.5 fetuses. Grey bars represent the genistein exposed group (n=5), black bars the quercetin exposed group (n=5) and white bars the control group (n=5). Bars represent the normalized average fold change (calculated as $2^{-\Delta\Delta CT}$) and error bars represent the standard errors. *: P<0.05, ***: P≤0.001.

Prenatal exposure to genistein increases the non-enzymatic antioxidant capacity of the lung

Although a relation between maternal flavonoid intake during gestation and enzymatic antioxidant expression was observed, one cannot exclude that there might also be a long-lasting effect on the non-enzymatic antioxidant capacity directly induced by the prenatal diet or in response to alterations in the enzymatic antioxidant defense system. Therefore, the non-enzymatic antioxidant capacity of both liver and long tissue of 12 week old mice prenatally exposed to genistein or quercetin was determined. Hence, the trolox equivalent antioxidant capacity (TEAC) was assessed, in combination with vitamin C levels and the presence of vitamin C radicals in tissue extracts. In liver tissue of adult mice prenatally exposed to genistein or quercetin no significant differences in TEAC were noticeable, though it seemed that the liver TEAC levels were slightly decreased, while for the lung prenatal exposure to genistein significantly increased TEAC levels (P<0.05, Table 2). This suggests that only prenatal exposure to genistein could affect the non-enzymatic antioxidant capacity of liver and lung at adult age.
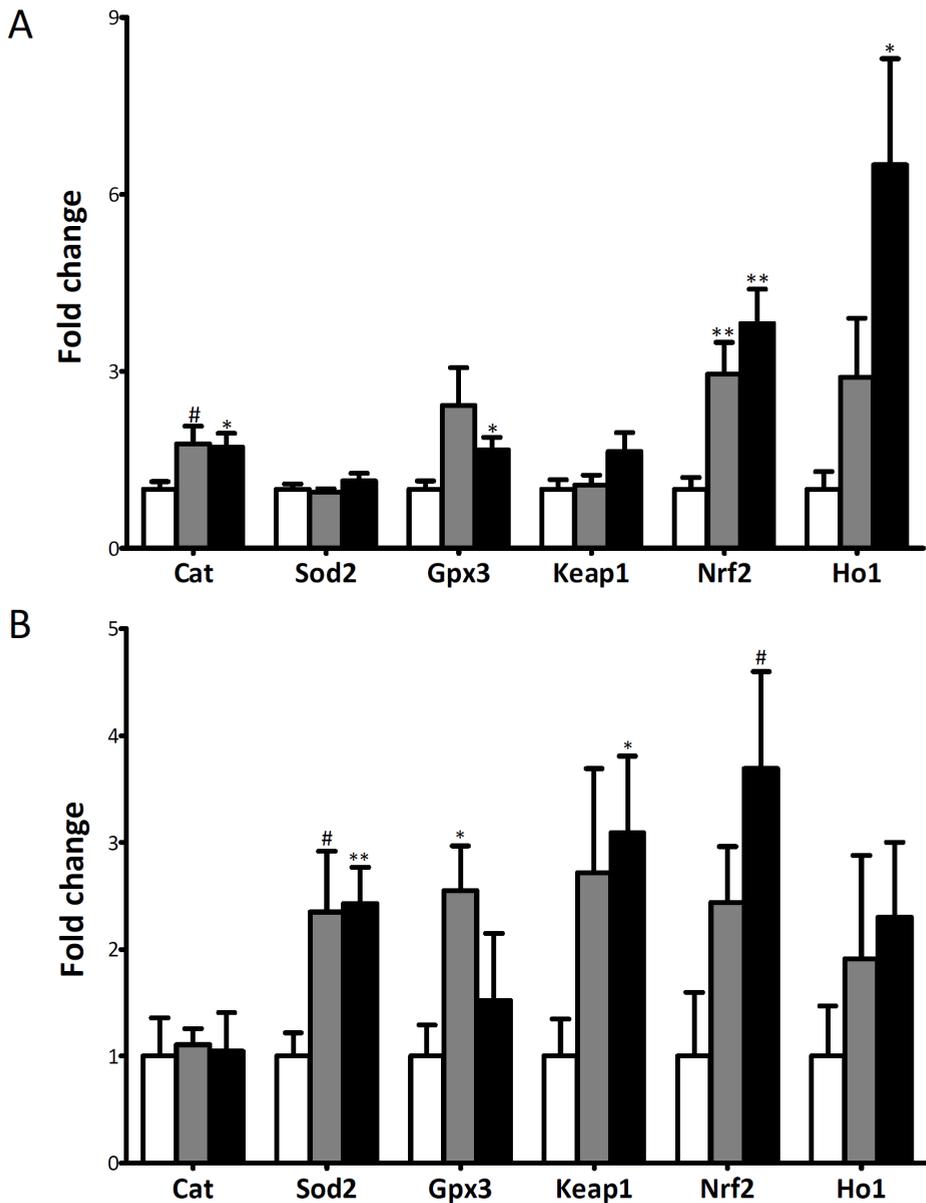
Figure 2. Long-term effect of genistein and quercetin exposure on antioxidant, *Keap1*, *Nrf2* and *Ho1* gene expression in the liver and lung of 12 week old mice.

mRNA expression level of antioxidant genes (*Cat*, *Sod2* and *Gpx3*), *Keap1*, *Nrf2* and *Ho1* were measured in the liver (A) and lung (B) of 12 week old mice prenatally exposed to either genistein (grey bars, n=5) or quercetin (black bars, n=5) compared to the control group (white bars, n=5). Bars represent the normalized average fold change (calculated as $2^{-\Delta\Delta CT}$) and error bars represent the standard errors. #: P≤0.09, *: P<0.05, **: P≤0.01.

Next, vitamin C levels were measured in liver and lung tissues of mice prenatally exposed to either genistein or quercetin. Although it appeared that more vitamin C was present in the lung of adult mice prenatally exposed to genistein compared to quercetin exposed and control mice, no significant differences were found, due to the high variation within the genistein exposed group (Table 2). In addition, when comparing the contribution of vitamin C to the trolox capacity of these organs, there was no difference between control and exposed animals (Table 2).

Table 2: TEAC, vitamin C concentration and vitamin C radical level measured in the liver and lung of 12 week old mice prenatally exposed to genistein or quercetin

		Control	Genistein	Quercetin
Liver	TEAC (µmol/ g)	54. 2 ± 8.9	41.8 ± 4.1	40.9 ± 8.7
	Vitamin C (µmol/ g)	21.5 ± 4.4	20.2 ± 1.7	21.2 ± 1.8
	Vitamin C/ TEAC ratio	0.4	0.5	0.5
	Vitamin C radical (1×10^5 AUC/ g)	0.3 ± 0.2	0.4 ± 0.2	0.3 ± 0.1
Lung	TEAC (µmol/ g)	50.3 ± 4.7	64.6 ± 4.0*	49.3 ± 4.6
	Vitamin C (µmol/ g)	7.5 ± 1.7	19.2 ± 7.2	8.2 ± 4.2
	Vitamin C/ TEAC ratio	0.1	0.3	0.2
	Vitamin C radical (1×10^5 AUC/ g)	8.5 ± 0.6	7.2 ± 0.3[#a]	7.2 ± 0.2[#a]

For each measurement n=5 for all three the diets. AUC: area under the curve. Data represent mean ± SE.
*: P<0.05; [#]: P<0.08; [a]: P=0.05 for both flavonoids compared to control.

Prenatal exposure to flavonoids decreased the in vivo scavenging of ROS by vitamin C in the lung tissue of adult mice

Next, we investigated how these organs coped with oxidative stress *in vivo* by measuring the vitamin C radical level in tissue homogenates using ESR spectroscopy. In the liver of mice prenatally exposed to genistein or quercetin there were no differences seen in the amount of vitamin C radicals compared to control mice (Table 2). In the lung on the contrary, prenatal exposure to flavonoids resulted in a decrease in the amount of vitamin C radicals formed *in vivo* (P=0.05 taken both flavonoids together; P=0.06 for genistein; P= 0.08 for quercetin) compared to control mice.

Prenatal exposure to flavonoids protected the liver against oxidative stress induced DNA damage

In both liver and lung of adult offspring mice, *Nrf2* expression was upregulated by maternal flavonoid intake. However, its downstream regulated gene *Ho1* was only increased in liver, which indicates that an antioxidant response may have been triggered in the liver. In lung, increased *Nrf2* expression coincided with enhanced *Keap1* expression, which may inhibit further Nrf2 signaling. Therefore, it was expected that a persistent antioxidant effect can only be seen in the liver. Indeed, oxidative stress induced DNA damage in liver was decreased in mice prenatally exposed to genistein (P=0.04) or quercetin (P=0.003) as they displayed lower levels of 8-oxo-dG compared to control mice (Figure 3A). In the lung, prenatal exposure to genistein or quercetin had no effect on the amount of 8-oxo-dG levels compared to control mice (Figure 3C).

Another type of DNA damage induced by oxidative stress and lipid peroxidation is M_1dG. Interestingly, these data confirmed the findings observed for 8-oxo-dG, since prenatal exposure to both genistein and quercetin resulted in decreased levels of M_1dG in liver (P=0.008 for genistein and P=0.001 for quercetin; Figure 3B), while no differences in the amount of M_1dG was detected in the lung of 12 week old mice (Figure 3D).
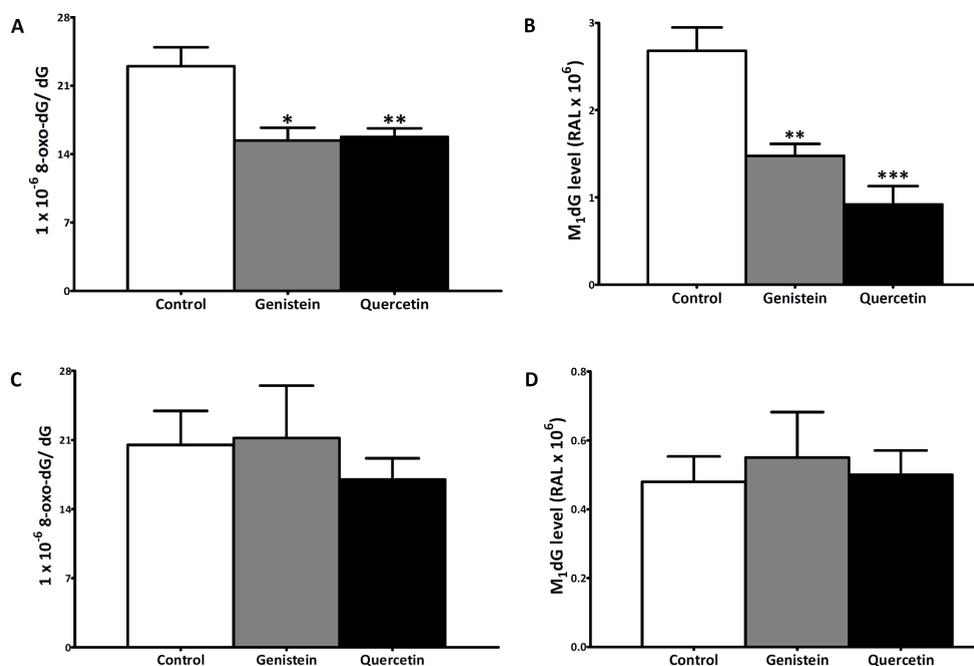
Figure 3. ROS induced DNA damage in liver and lung tissue of 12 week old mice prenatally exposed to genistein or quercetin.
8-oxo-dG (A, C) and M_1dG (B, D) levels were assessed as measure for ROS induced DNA damage in the liver (A, B) and lung (C, D) of 12 week old mice prenatally exposed to either genistein (grey bars) or quercetin (black bars) compared to the control group (white bars). Data represents the mean ± SE. RAL=relative adduct labeling.
*: P<0.05, **: P≤0.01, ***: P≤0.001

Discussion

In this study we showed that prenatal exposure to both genistein and quercetin protected against oxidative stress induced DNA damage in the liver, as shown by lower levels of 8-oxo-dG and M_1dG in 12 week old mice. We assume that this is the result of long-lasting adaptations made in the antioxidant network as seen in the up-regulation of the enzymatic antioxidants.

During development, direct exposure to quercetin resulted in an up-regulation of *Nrf2* gene expression in the fetal liver at day 14.5 of gestation. Nrf2 is the key transcription factor regulating the antioxidant response. It is normally targeted for proteasomal degradation by Keap1. However it translocates into the nucleus when oxidative stress occurs, since this

results in a conformational change of Keap1 and therefore the release of Nrf2 (43). The increase in *Nrf2* gene expression suggests that quercetin acted as a pro-oxidant *in utero*, which has also been shown *in vitro* (35, 44). Fetuses exposed to genistein also had an increase in *Nrf2* gene expression, though not statistically significant. This suggests that genistein also had a pro-oxidant action *in utero*, but milder compared to quercetin. However, the lower induction of *Nrf2* gene expression seen in fetuses exposed to genistein could also be due to the fact that genistein impedes the nuclear export and degradation of Nrf2 after it has activated antioxidant response genes (45). This suggests that an up-regulation of *Nrf2* gene expression is not essential for genistein induced activation of the Nrf2-pathway, which may in our model also result in a different effect in the adult offspring. Although *Nrf2* gene expression was elevated in exposed fetuses compared to control fetuses, expression of a major target gene of Nrf2, namely *Ho1* was only modestly elevated. This indicates that gene expression levels of *Nrf2* does not necessarily result in more functional Nrf2 proteins; or upon an oxidative stress induced trigger, Nrf2 could not translocate into the nucleus to perform its function. Moreover, due to the fact that Nrf2 has to reach a threshold before it can enter the nucleus and induce downstream effects (43), it could be that in our model at gestationa day 14.5 this threshold has not been reached yet. Fetuses exposed to quercetin were also found to have increased gene expression of *Sod2*. Sod2 normally converts $O_2^{-\bullet}$, a byproduct of the mitochondrial electron transport chain into H_2O_2 and O_2. De Marchi *et al.* (46) showed that rat liver mitochondria exposed to quercetin had an increased production of $O_2^{-\bullet}$, explaining the up-regulation of *Sod2* gene expression seen in fetuses exposed to quercetin.

At adult age, mice prenatally exposed to genistein, but especially to quercetin still had increased expression of *Nrf2* in their liver. Moreover, they also showed an increased expression of the Nrf2 target gene *Ho1* in their liver, suggesting that the increased expression of *Nrf2* also resulted in an increased down-stream effect, modulating oxidative stress responses.

Moreover, prenatal exposure to genistein and quercetin resulted in the increased expression of the antioxidant genes *Cat* and *Gpx3* in the liver of adult mice. Both antioxidants are responsible for scavenging H_2O_2 and their mRNAs are shown to accumulate *in utero* to be translated in advance of birth (47), as these enzymes are needed from then onwards for protection against oxidative stress by aerobic respiration. Therefore, it is possible that the effect of genistein and quercetin exposure on the induction of gene expression of these genes could not be observed during mid-gestation, but long-lasting effects are seen in adult offspring.

The lung of mice prenatally exposed to genistein and quercetin showed a totally different enzymatic antioxidant network compared to the liver. Here, *Nrf2* gene expression was also up-regulated in case of both flavonoids. However, *Keap1* gene expression was increased to a comparable extent. Therefore, we assume that the oxidative stress response pathway was activated to a lesser degree, which was also confirmed by the modest increase in *Ho1* gene expression and no effect on DNA damage parameters. In the lung, prenatal exposure to genistein and quercetin also resulted in the up-regulation of *Sod2* and *Gpx3* gene expression, while *Cat* gene expression remained unaffected. Differences in antioxidant gene expression profiles between liver and lung tissue of adult mice prenatally exposed to genistein or quercetin could be explained by the fact that the liver is largely differentiated before birth (48), while the lung develops mainly in the final stages of pregnancy and even continues to

develop after birth (49). Therefore, the different responses of both tissues to the flavonoids could be the result of a difference in maturation state of the organ during exposure.

Although prenatal exposure to genistein and quercetin resulted in the up-regulation of the gene expression of enzymatic antioxidants, it had only minor effects on the non-enzymatic antioxidant capacity. TEAC levels in liver were slightly decreased while in lung they were only increased in case of prenatal exposure to genistein. The contribution of vitamin C to the TEAC did not differ between diet groups, suggesting that prenatal exposure to genistein or quercetin affected other components of the non-enzymatic antioxidant network. Still, lungs of mice prenatally exposed to genistein had increased levels of vitamin C, although not significantly due to the large variation within the genistein exposed group, compared to the control group. Unlike humans, mice synthesize vitamin C from glucose in the liver (50). As genistein exposure can result in an increased uptake of glucose by cells (51) and improves glucose tolerance (52), we assume that mice prenatally exposed to genistein produce more vitamin C and transport it throughout the body, for instance to the lung, as they may be programmed to take up more glucose.

Vitamin C radicals were measured in liver and lung homogenates as they seem to provide a stable and reliable signal for oxidative stress (39). In the liver of mice prenatally exposed to genistein and quercetin, there was no difference in the amount of vitamin C radicals formed *in vivo*. In the lung however, the amount of vitamin C radicals was slightly decreased in mice prenatally exposed to flavonoids, probably as a result of modest increase in both the enzymatic as non-enzymatic antioxidant activity.

In our study pregnant mice were exposed to approximately 26.7-36.7 mg/ kg bodyweight genistein and 33.3-46.7 mg/ kg bodyweight quercetin per day. This is a high, but biological relevant dose, because the average human daily dietary intake of quercetin is in the range of 10–100 mg (53) and the average human daily dietary isoflavone intake lies between 1-40 mg (54). Both flavonoids are freely available as dietary supplements with a daily recommended dose that can be as high as 1-2 gram per day (55, 56), which could correspond to 14-28 mg for a person weighing 70 kg. Moreover, it is noteworthy that the metabolism of flavonoids differs between fetuses and adults. In humans, fetal cord blood has a higher genistein level than maternal serum (57), suggesting that the fetus could be exposed to even higher concentrations.

These data show that prenatal exposure to genistein but especially quercetin results in a pro-oxidant trigger *in utero*, with long-lasting effects on the antioxidant capacity of the liver and lung. These long-lasting effects may be mediated through epigenetic mechanisms and need further study. Mild effects were seen for the non-enzymatic antioxidants, suggesting that prenatal exposure to genistein and quercetin results in an adapted antioxidant defense system in both organs and activated the Nrf2-antioxidant pathway in the liver. This resulted in the protection from oxidative stress induced DNA damage in the liver of adult mice prenatally exposed to genistein and quercetin.

References

1. Vanhees, K., de Bock, L., Godschalk, R. W., van Schooten, F. J., and van Waalwijk van Doorn-Khosrovani, S. B. (2011) Prenatal exposure to flavonoids: implication for cancer risk. *Toxicol Sci* 120, 59-67
2. Ahlgren, M., Sorensen, T., Wohlfahrt, J., Haflidadottir, A., Holst, C., and Melbye, M. (2003) Birth weight and risk of breast cancer in a cohort of 106,504 women. *Int J Cancer* 107, 997-1000
3. Vickers, M. H., Breier, B. H., Cutfield, W. S., Hofman, P. L., and Gluckman, P. D. (2000) Fetal origins of hyperphagia, obesity, and hypertension and postnatal amplification by hypercaloric nutrition. *Am J Physiol Endocrinol Metab* 279, E83-87
4. Ravelli, A. C., van Der Meulen, J. H., Osmond, C., Barker, D. J., and Bleker, O. P. (1999) Obesity at the age of 50 y in men and women exposed to famine prenatally. *Am J Clin Nutr* 70, 811-816
5. Dolinoy, D. C., Weidman, J. R., Waterland, R. A., and Jirtle, R. L. (2006) Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. *Environ Health Perspect* 114, 567-572
6. Barker, D. J. (1991) The intrauterine environment and adult cardiovascular disease. *Ciba Found Symp* 156, 3-10; discussion 10-16
7. Reuter, S., Gupta, S. C., Chaturvedi, M. M., and Aggarwal, B. B. (2010) Oxidative stress, inflammation, and cancer: how are they linked? *Free Radic Biol Med* 49, 1603-1616
8. Ross, J. A., and Kasum, C. M. (2002) Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annu Rev Nutr* 22, 19-34
9. Erdman, J. W., Jr., Balentine, D., Arab, L., Beecher, G., Dwyer, J. T., Folts, J., Harnly, J., Hollman, P., Keen, C. L., Mazza, G., Messina, M., Scalbert, A., Vita, J., Williamson, G., and Burrowes, J. (2007) Flavonoids and heart health: proceedings of the ILSI North America Flavonoids Workshop, May 31-June 1, 2005, Washington, DC. *J Nutr* 137, 718S-737S
10. Aherne, S. A., and O'Brien, N. M. (2002) Dietary flavonols: chemistry, food content, and metabolism. *Nutrition* 18, 75-81
11. Marik, R., Allu, M., Anchoori, R., Stearns, V., Umbricht, C. B., and Khan, S. (2011) Potent genistein derivatives as inhibitors of estrogen receptor alpha-positive breast cancer. *Cancer Biol Ther* 11
12. Miodini, P., Fioravanti, L., Di Fronzo, G., and Cappelletti, V. (1999) The two phyto-oestrogens genistein and quercetin exert different effects on oestrogen receptor function. *Br J Cancer* 80, 1150-1155
13. Loke, W. M., Proudfoot, J. M., Hodgson, J. M., McKinley, A. J., Hime, N., Magat, M., Stocker, R., and Croft, K. D. (2010) Specific dietary polyphenols attenuate atherosclerosis in apolipoprotein E-knockout mice by alleviating inflammation and endothelial dysfunction. *Arterioscler Thromb Vasc Biol* 30, 749-757
14. Mahn, K., Borras, C., Knock, G. A., Taylor, P., Khan, I. Y., Sugden, D., Poston, L., Ward, J. P., Sharpe, R. M., Vina, J., Aaronson, P. I., and Mann, G. E. (2005) Dietary soy isoflavone induced increases in antioxidant and eNOS gene expression lead to improved endothelial function and reduced blood pressure in vivo. *FASEB J* 19, 1755-1757
15. Heo, H. J., and Lee, C. Y. (2004) Protective effects of quercetin and vitamin C against oxidative stress-induced neurodegeneration. *J Agric Food Chem* 52, 7514-7517
16. Mercer, L. D., Kelly, B. L., Horne, M. K., and Beart, P. M. (2005) Dietary polyphenols protect dopamine neurons from oxidative insults and apoptosis: investigations in primary rat mesencephalic cultures. *Biochem Pharmacol* 69, 339-345
17. Boots, A. W., Drent, M., de Boer, V. C., Bast, A., and Haenen, G. R. (2011) Quercetin reduces markers of oxidative stress and inflammation in sarcoidosis. *Clin Nutr*
18. Valsecchi, A. E., Franchi, S., Panerai, A. E., Rossi, A., Sacerdote, P., and Colleoni, M. (2011) The soy isoflavone genistein reverses oxidative and inflammatory state, neuropathic pain, neurotrophic and vasculature deficits in diabetes mouse model. *Eur J Pharmacol* 650, 694-702
19. Formica, J. V., and Regelson, W. (1995) Review of the biology of Quercetin and related bioflavonoids. *Food Chem Toxicol* 33, 1061-1080
20. Bolanos, J. P., Moro, M. A., Lizasoain, I., and Almeida, A. (2009) Mitochondria and reactive oxygen and nitrogen species in neurological disorders and stroke: Therapeutic implications. *Adv Drug Deliv Rev* 61, 1299-1315
21. Hensley, K., Butterfield, D. A., Hall, N., Cole, P., Subramaniam, R., Mark, R., Mattson, M. P., Markesbery, W. R., Harris, M. E., Aksenov, M., and et al. (1996) Reactive oxygen species as causal agents in the neurotoxicity of the Alzheimer's disease-associated amyloid beta peptide. *Ann N Y Acad Sci* 786, 120-134
22. Multhaup, G., Ruppert, T., Schlicksupp, A., Hesse, L., Beher, D., Masters, C. L., and Beyreuther, K. (1997) Reactive oxygen species and Alzheimer's disease. *Biochem Pharmacol* 54, 533-539

23. Tabner, B. J., Turnbull, S., El-Agnaf, O., and Allsop, D. (2001) Production of reactive oxygen species from aggregating proteins implicated in Alzheimer's disease, Parkinson's disease and other neurodegenerative diseases. *Curr Top Med Chem* 1, 507-517

24. Tieu, K., Ischiropoulos, H., and Przedborski, S. (2003) Nitric oxide and reactive oxygen species in Parkinson's disease. *IUBMB Life* 55, 329-335

25. Touyz, R. M. (2004) Reactive oxygen species and angiotensin II signaling in vascular cells -- implications in cardiovascular disease. *Braz J Med Biol Res* 37, 1263-1273

26. Yoshizumi, M., Tsuchiya, K., and Tamaki, T. (2001) Signal transduction of reactive oxygen species and mitogen-activated protein kinases in cardiovascular disease. *J Med Invest* 48, 11-24

27. Boots, A. W., Li, H., Schins, R. P., Duffin, R., Heemskerk, J. W., Bast, A., and Haenen, G. R. (2007) The quercetin paradox. *Toxicol Appl Pharmacol* 222, 89-96

28. Cao, G., Sofic, E., and Prior, R. L. (1997) Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free Radic Biol Med* 22, 749-760

29. Choi, E. J., Chee, K. M., and Lee, B. H. (2003) Anti- and prooxidant effects of chronic quercetin administration in rats. *Eur J Pharmacol* 482, 281-285

30. Ullah, M. F., Shamim, U., Hanif, S., Azmi, A. S., and Hadi, S. M. (2009) Cellular DNA breakage by soy isoflavone genistein and its methylated structural analogue biochanin A. *Mol Nutr Food Res* 53, 1376-1385

31. Gebicki, J. M., Nauser, T., Domazou, A., Steinmann, D., Bounds, P. L., and Koppenol, W. H. (2010) Reduction of protein radicals by GSH and ascorbate: potential biological significance. *Amino Acids* 39, 1131-1137

32. Seifried, H. E., Anderson, D. E., Fisher, E. I., and Milner, J. A. (2007) A review of the interaction among dietary antioxidants and reactive oxygen species. *J Nutr Biochem* 18, 567-579

33. Siow, R. C., and Mann, G. E. (2010) Dietary isoflavones and vascular protection: activation of cellular antioxidant defenses by SERMs or hormesis? *Mol Aspects Med* 31, 468-477

34. Hur, W., and Gray, N. S. (2011) Small molecule modulators of antioxidant response pathway. *Curr Opin Chem Biol* 15, 162-173

35. Niestroy, J., Barbara, A., Herbst, K., Rode, S., van Liempt, M., and Roos, P. H. (2011) Single and concerted effects of benzo[a]pyrene and flavonoids on the AhR and Nrf2-pathway in the human colon carcinoma cell line Caco-2. *Toxicol In Vitro* 25, 671-683

36. Chow, J. M., Shen, S. C., Huan, S. K., Lin, H. Y., and Chen, Y. C. (2005) Quercetin, but not rutin and quercitrin, prevention of H2O2-induced apoptosis via anti-oxidant activity and heme oxygenase 1 gene expression in macrophages. *Biochem Pharmacol* 69, 1839-1851

37. Breinholt, V., Lauridsen, S. T., and Dragsted, L. O. (1999) Differential effects of dietary flavonoids on drug metabolizing and antioxidant enzymes in female rat. *Xenobiotica* 29, 1227-1240

38. Fischer, M. A., Gransier, T. J., Beckers, L. M., Bekers, O., Bast, A., and Haenen, G. R. (2005) Determination of the antioxidant capacity in blood. *Clin Chem Lab Med* 43, 735-740

39. Linschooten, J. O., Laubenthal, J., Cemeli, E., Baumgartner, A., Anderson, D., Sipinen, V. E., Brunborg, G., Haenen, G. R., Fthenou, E., Briede, J. J., van Schooten, F. J., and Godschalk, R. W. (2011) Incomplete protection of genetic integrity of mature spermatozoa against oxidative stress. *Reprod Toxicol*

40. Langie, S. A., Kowalczyk, P., Tudek, B., Zabielski, R., Dziaman, T., Olinski, R., van Schooten, F. J., and Godschalk, R. W. (2010) The effect of oxidative stress on nucleotide-excision repair in colon tissue of newborn piglets. *Mutat Res* 695, 75-80

41. Godschalk, R. W., Maas, L. M., Van Zandwijk, N., van 't Veer, L. J., Breedijk, A., Borm, P. J., Verhaert, J., Kleinjans, J. C., and van Schooten, F. J. (1998) Differences in aromatic-DNA adduct levels between alveolar macrophages and subpopulations of white blood cells from smokers. *Carcinogenesis* 19, 819-825

42. Peluso, M., Srivatanakul, P., Munnia, A., Jedpiyawongse, A., Ceppi, M., Sangrajrang, S., Piro, S., and Boffetta, P. (2010) Malondialdehyde-deoxyguanosine adducts among workers of a Thai industrial estate and nearby residents. *Environ Health Perspect* 118, 55-59

43. Li, W., and Kong, A. N. (2009) Molecular mechanisms of Nrf2-mediated antioxidant response. *Mol Carcinog* 48, 91-104

44. Kimura, S., Warabi, E., Yanagawa, T., Ma, D., Itoh, K., Ishii, Y., Kawachi, Y., and Ishii, T. (2009) Essential role of Nrf2 in keratinocyte protection from UVA by quercetin. *Biochem Biophys Res Commun* 387, 109-114

45. Kaspar, J. W., and Jaiswal, A. K. (2011) Tyrosine phosphorylation controls nuclear export of Fyn, allowing Nrf2 activation of cytoprotective gene expression. *FASEB J* 25, 1076-1087

46. De Marchi, U., Biasutto, L., Garbisa, S., Toninello, A., and Zoratti, M. (2009) Quercetin can act either as an inhibitor or an inducer of the mitochondrial permeability transition pore: A demonstration of the ambivalent redox character of polyphenols. *Biochim Biophys Acta* 1787, 1425-1432

47. el-Hage, S., and Singh, S. M. (1990) Temporal expression of genes encoding free radical-metabolizing enzymes is associated with higher mRNA levels during in utero development in mice. *Dev Genet* 11, 149-159

48. Zaret, K. S. (2002) Regulatory phases of early liver development: paradigms of organogenesis. *Nat Rev Genet* 3, 499-512
49. Ten Have-Opbroek, A. A. (1991) Lung development in the mouse embryo. *Exp Lung Res* 17, 111-130
50. Naidu, K. A. (2003) Vitamin C in human health and disease is still a mystery? An overview. *Nutr J* 2, 7
51. Ha, B. G., Nagaoka, M., Yonezawa, T., Tanabe, R., Woo, J. T., Kato, H., Chung, U. I., and Yagasaki, K. (2011) Regulatory mechanism for the stimulatory action of genistein on glucose uptake in vitro and in vivo. *J Nutr Biochem*
52. Cederroth, C. R., and Nef, S. (2009) Fetal programming of adult glucose homeostasis in mice. *PLoS One* 4, e7281
53. Egert, S., and Rimbach, G. (2011) Which sources of flavonoids: complex diets or dietary supplements? *Adv Nutr* 2, 8-14
54. Manach, C., Scalbert, A., Morand, C., Remesy, C., and Jimenez, L. (2004) Polyphenols: food sources and bioavailability. *Am J Clin Nutr* 79, 727-747
55. Espin, J. C., Garcia-Conesa, M. T., and Tomas-Barberan, F. A. (2007) Nutraceuticals: facts and fiction. *Phytochemistry* 68, 2986-3008
56. Harwood, M., Danielewska-Nikiel, B., Borzelleca, J. F., Flamm, G. W., Williams, G. M., and Lines, T. C. (2007) A critical review of the data related to the safety of quercetin and lack of evidence of in vivo toxicity, including lack of genotoxic/carcinogenic properties. *Food Chem Toxicol* 45, 2179-2205
57. Todaka, E., Sakurai, K., Fukata, H., Miyagawa, H., Uzuki, M., Omori, M., Osada, H., Ikezuki, Y., Tsutsumi, O., Iguchi, T., and Mori, C. (2005) Fetal exposure to phytoestrogens--the difference in phytoestrogen status between mother and fetus. *Environ Res* 99, 195-203

Chapter 5

Maternal quercetin intake during pregnancy results in an adapted iron homeostasis at adulthood

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Abstract

The flavonoid quercetin is a powerful iron chelator, capable of oxidizing heme iron in hemoglobin from Fe^{2+} to Fe^{3+}. Moreover, quercetin crosses the placenta and accumulates in the fetus. Since adaptations made by the fetus to cope with inappropriate nutrition may lead to permanent changes, a relative high intake of quercetin may have detrimental affects later in life. Therefore, we investigated the effects of maternal exposure to quercetin (302 mg/ kg feed), starting from 3 days before conception until the end of gestation, on erythropoiesis and iron homeostasis at embryonic day 14.5 and in 12 week old mice. During fetal development, quercetin exposure had no effect on the erythroid lineage switch and concomitant globin switch. However, adult mice prenatally exposed to quercetin had significant increased iron storage in the liver, by up-regulating iron-associated cytokine expression (*hepcidin*, *IL-18*, *IL-6* and *IL-10*). These long-term changes in gene expression could be mediated through epigenetic modifications, as prenatal quercetin exposure resulted in a modest hypermethylation of repetitive elements. Despite the increased iron levels, oxidative stress was significantly decreased in the liver of these animals as assessed by 8-oxo-dG levels. These data suggest that prenatal quercetin exposure results in increased iron storage, while decreasing oxidative stress induced DNA damage together with a shift towards increased expression of inflammation associated cytokines in the liver at adult age.

Introduction

Flavonoids are polyphenolic compounds found in diverse foods and beverages of plant origin, such as fruits, vegetables, soy products, tea and wine. They are most commonly known for the health benefits they provide against cancer and heart diseases, which is attributed to their antioxidant capacity (1). Quercetin (3,3',4',5,7-pentahydroxyflavone) is the most widely consumed flavonoid in the diet. This polyphenolic compound is a potent free radical scavenger and iron chelator. Previous studies have shown that quercetin can penetrate the cytoplasm of erythrocytes (2, 3). Here, it interacts with the heme iron in hemoglobin and oxidizes it from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state. This latter form of hemoglobin, namely methemoglobin, is inactive and is incapable of transporting oxygen (3). About 65 % of the body's iron is used for the production of hemoglobin. Iron is an essential micronutrient that besides erythropoiesis is also required for oxidative metabolism and immune responses. Iron absorption is tightly regulated due to the fact that the body has no effective means of excreting iron and iron overload leads to toxicity due to increased free radical formation (4). Quercetin can also have pro-oxidant capacities (5), especially in the presence of transition metal iron (6). As it has been shown that several flavonoids can cross the placenta to accumulate in fetal tissue (7), and because we previously showed that prenatal exposure to quercetin resulted in a higher incidence of *Mll* translocations in mice with an impaired DNA repair (8), we assume that quercetin can also cross the placenta. Therefore it is interesting to study the iron chelating effect of quercetin in developing fetuses. Godfrey *et al.* (9) stated that adaptations made by the fetus to cope with inappropriate nutrition may lead to morphological and physiological changes that persist into postnatal life. These changes, although ensuring fetal survival, may have detrimental affects later in life. We therefore investigated whether prenatal exposure to the iron chelator quercetin could result in a permanent change in iron homeostasis and erythropoiesis later in life.

To this end, mice were prenatally exposed to quercetin (302 mg/ kg feed) and erythropoiesis (red blood cell (RBC) progenitor composition, RBC turnover and globin status) was assessed at adult age. Microarray analysis of the bone marrow cells was performed to investigate the expression of genes involved in erythropoiesis and iron homeostasis. Liver and spleen iron levels and gene expression of *hepcidin* and interleukins (IL) involved in iron storage in the liver were investigated. In addition, 8-oxo-dG levels were measured in the liver of 12 week old mice as a measure for iron induced oxidative stress. To investigate whether long-term effects already occurred *in utero*, the effect of quercetin exposure on fetal erythropoiesis was studied in female mice that were exposed to quercetin and sacrificed at gestational day 14.5 as the switch in erythroid lineage occurs at that developmental time point. Subsequently, erythropoiesis in the liver of fetuses was investigated. Also the total amount of iron, bilirubin and erythropoietin (EPO) levels were measured in the amniotic fluid of these fetuses to characterize iron status and homeostasis.

Material and methods

Mice and sample collection

Female mice (129/SvJ:C57BL/6J background) approximately 8 weeks of age, received either normal chow (low phytoestrogen content complete feed for mice breeding, ssniff®, Soest, Germany, n=8) or the same chow supplemented with quercetin (302 mg/ kg feed, n=8,

Sigma, Zwijndrecht, the Netherlands) from 3 days before conception until the end of gestation. After delivery all mothers and pups, regardless of their prenatal diet, received normal chow and the offspring were anesthetized and sacrificed by cardiac puncture at 12 weeks of age. Bone marrow was isolated by removing the femurs and flushing the marrow with PBS; liver and spleen were isolated and stored at -80 °C until further analysis.

To determine the direct effects of quercetin on erythropoiesis in fetuses, female mice (129/SvJ:C57BL/6J background, n=5 for control group and n=3 for quercetin exposed group) were mated overnight and conception day was determined by recognition of a vaginal plug the next morning (E0.5). Female mice were placed on the same control or quercetin enriched chow, as described above, from three days before conception. On day 14.5 of gestation, mice were sacrificed and the amniotic fluid, placentas, fetuses and their blood and liver were isolated. Also the liver of the mothers was isolated for further analysis.

Fetal blood and adult bone marrow cytology

After initial fixation of the fetal blood or adult bone marrow smears by methanol, smears were stained for 5 minutes with May-Grünwald solution (Sigma). After removing the excess May-Grünwald solution by washing the smears in tap water, they were stained with Giemsa solution (Merck, Darmstadt, Germany) for 20 minutes. Afterwards, smears were again washed in tap water. Blood and bone marrow smears were examined using a Leica DMRB microscope (Leica Microsystems B.V., Rijswijk, the Netherlands) under a 620 × oil-immersion magnification and analyzed using the Leica IM50 image manager (Leica). Two independent examiners assessed the blood and bone marrow smears and judged approximately 500 cells on each slide.

Microarray analysis of bone marrow cells

RNA from bone marrow cells isolated from one femur (n=3 for both diet groups) was isolated with TRIzol Reagent (Invitrogen, Breda, the Netherlands) according to the manufacturer's instructions. Genome-wide gene expression was determined using whole mouse genome (4X44K) oligonucleotide microarrays (Agilent, Santa Clara, CA, USA) as described previously (10). More detailed information regarding the analysis of microarray data can be found in the supplementary data[†].

Quantitative real-time PCR analysis

RNA was isolated from the liver of E14.5 fetuses, from the spleen and liver of 12 week old mice and from the liver of maternal mice at gestational day 14.5 by homogenizing the tissue using the Ultra-Turrax homogenizer (IKA, Staufen, Germany), after which TRIzol Reagent (Invitrogen) was added according to the manufacturer's instructions. RNA isolation of the bone marrow samples of 12 week old mice was performed using TRIzol LS Reagent (Invitrogen) according to the manufacturer's instructions. Quantity and purity control of the RNA was spectrophotometrically assessed by using the Nanodrop 1000 (Thermo Scientific, Wilmington, USA). Next, 1 μg of RNA was used to synthesize cDNA using the iScript cDNA synthesis kit (Biorad, Hercules, USA) following manufacturer's instructions. The reaction was performed using a Biometra Tprofessional thermocycler (Biometra, Leusden, the Netherlands).

Table 1. Overview of used primers and probes for quantitative real-time PCR

	Forward primer (5'-3')	Reverse primer (5'-3')	FAM-probe (5'-3')
ζ-globin	GCGAGCTGCATGCCTACAT	GCCATTGTGACCAGCAGACA	TGGATCCGGTCAACTTCAAGCTCCTGT
βH1-globin	AGGCAGCTATCACAAGCATCTG	AACTTGTCAAAGAATCTCTGAGTCCAT	AGAAACTCTGGGAAGGCTCCTGATTGTTACC
εγ-globin	CAAGCTACATGTGGATCCTGAGAA	TGCCGAAGTGACTAGCCAAA	TCAAACTCTTGGGTAATGTGCTGGTGATTG
α-globin	AATATGGAGCTGAAGCCCTGG	ATCAAAGTGAGGGAAGTAGGTCT	AAGGATGTTTGCCAGCTTCCCCACTACT
β-globin	GTGAGCTCCACTGTGACAAGCT	GGTGGCCCAGCACAAATCACGATC	CATGTGGATCCTGAGAACTTCAGGCTCCT
Ho1	TCCAGAGTTTCCGCATAC	CGGACTGGGCTAGTTCA	
Hepcidin	GCTGCCTGTCTCCTGCTTCT	GCTCTGTAGTCTGTCTCATCTGTTGAT	
Il-1β	AAGATGAAGGGCTGCTTCCA	GTGCTGCTGCCGAGATTTGAA	
IL-6	ACAAGTCGGAGGCTTAATTACACAT	AATCAGAATTGCCATTGCACAA	
IL-10	CCAGAGCCACATGCTCCTAGA	GGTCCTTTGTTTGAAAGAAAGTCTTC	
LINE1	TTTGGGACACAATGAAAGCA	CTGCCGTCTACTCCTCTTGG	
SINEB1	GTGGCGCACGCCTTTAATC	GACAGGGTTTCTCTGTGTAG	
SINEB2	GAGATGGCTCAGTGGTTAAG	CTGTCTTCAGACACTCCAG	
IAP-GAG	AGCAGGTGAAGCCACTG	CTTGCCACACTTAGAGC	
Major satellites	GACGACTTGAAAAATGACGAAATC	CATATTCCAGGTCCTTCAGTGTGC	
Minor satellites	CATGGAAAATGATAAAAACC	CATCTAATATGTTCTACAGTGTGG	
GPA	GCCGAATGACAAAGAAAAGTTCA	TCAATAGAACTCAAAGGCACACTGT	TTGACATCCAATCTCCTGAGGGTGGTGA
β-actin	CAAGAAGGAAGGCTGGAAAAGA	ACGGCCAGGTCATCACTATTG	
HPRT	CCTAAGATGAGCGCAAGTTGAA	CCACAGGACTAGAACACCTGCTAA	

For determination of globin mRNA levels a quantitative real time-PCR was carried out using 12.5 µl iQ Supermix (Biorad), 7.5 pmol of each forward/reverse primer (see Table 1, Eurogentec, Maastricht, the Netherlands), 5 pmol FAM-probe (Eurogentec) in a volume of 25 µl. cDNA concentration used depended on the analyzed gene (*ζ-*, *βH1-*, *εγ-globi*n: 667 ng; *α-*, *β-globi*n: 67 ng for adults and 667 ng for fetuses). For determination of hemeoxygenase-1 (*Ho1*), *hepcidin* and interleukin (*IL*)-1β, *IL-6*, *IL-10*, a quantitative real-time PCR mixture comprising of 12.5 µl IQ SYBR Green (Qiagen), 7.5 pmol of each

forward/reverse primer (see Table 1, Eurogentec) in a volume of 25 µl was used. cDNA concentration used depended on the analyzed gene (*hepcidin, IL-1ß, IL-6, IL-10*: 667 ng; *Ho1*: 200 ng). All of the reactions were performed using a MyiQ Single Color RT-PCR detection system (Biorad) under the following cycling conditions: 1 cycle at 95 °C for 3 minutes and then 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute followed by 1 cycle at 95 °C for 1 minute and 1 cycle at 65 °C. Data were analyzed by MyiQ Software system (Biorad), Ct values were normalized for endogenous reference (*GPA* for ζ-, ßH1-, εγ-, α-, ß-globin; and ß-actin for *Ho1, hepcidin, IL-1ß, IL-6* and *IL-10*), compared with the calibrator (i.e. average Ct value of control samples) and expressed as fold change using the $2^{-\Delta Ct}$ method for the globin genes and the $2^{-\Delta\Delta Ct}$ for *Ho1, hepcidin* and IL genes.

Biochemical component determination in amniotic fluid

For the determination of the total amount of iron, bilirubin and EPO levels, the amniotic fluid of all pups from one litter were pooled. The total iron amount and bilirubin levels were determined in the amniotic fluid using the Synchron LX20 system (Beckman Coulter, Fullerton, USA). Briefly, the Synchron LX20 system determined the iron concentration using a fixed-time endpoint method. First, iron was dissociated from the transport protein transferrin and Fe^{3+} was reduced to Fe^{2+}. Hereafter, the total amount of Fe^{2+} was complexed with the Ferrozine iron reagens. The difference in absorption was measured and was proportional to the amount of total iron in the sample.

Bilirubin was determined using a timed-endpoint diazo method to measure the concentration of total bilirubin. In the reaction, bilirubin reacted with diazo reagent in the presence of caffeine, benzoate, and acetate as accelerators to form azobilirubin. The system monitored the change in absorbance at 520 nm at a fixed time interval. This change in absorbance was directly proportional to the concentration of total bilirubin in the sample.

The amount of EPO was detected in the amniotic fluid by IMMULITE 1000 (Siemens), an automated quantitative immunoassay analyzer that uses the immulite chemiluminescent immunoassay system. Sample and ligand-labeled anti-EPO antibody were incubated with the solid phase. EPO in the sample bound to the monoclonal antibody was immobilized onto the solid phase. Alkaline phosphatase labeled polyclonal anti-EPO antibody was then introduced, which bound to the EPO attached to the solid phase, and unbound enzyme conjugate was removed by a wash step. A chemiluminescent substrate was added, and the photon output, which was proportional to the EPO concentration in the sample, was measured by a luminometer.

Total iron levels measured by atomic absorption spectrometry

Total amount of iron was measured in the liver of the mothers during gestation, and liver and spleen of 12 week old offspring by atomic absorption spectrometry. Ground tissues were hydrolyzed overnight in 1 ml of 7 M HNO_3 at 60 °C. After centrifugation, 20 µl of the supernatant was directly injected into a graphite-furnace atomic absorption spectrometer with Zeeman background correction (Varian, Bergen op Zoom, the Netherlands). Fe concentrations were determined at 372 nm. Solutions with known concentrations of Fe were used for calibration. All the glassware was rinsed with 1 % HNO_3 to avoid contamination. Total Fe-concentration in ng Fe per mg tissue was calculated from the calibration curves and the weight of the tissue.

8-oxo-dG measurements

Genomic DNA isolation and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) measurements were performed as described earlier (11). Briefly, to detect the base-oxidation product 8-oxo-dG, HPLC with electrochemical detection (ECD) was performed. Genomic DNA was obtained by grinding frozen liver tissues followed by standard phenol extraction. The DNA-extraction procedure was optimized to minimize artificial induction of 8-oxo-dG, by using radical-free phenol, minimizing exposure to oxygen and by addition of 1 mM deferoxamine mesylate and 20 mM TEMPO (2,2,6,6-tetramethylpiperidine-N-oxyl). DNA concentrations were spectrophotometrically quantified. HPLC-ECD of 8-oxo-dG was performed by digesting 30 µg DNA to deoxyribonucleosides by addition of 6 µl 0.5 M NaAc, 9 µl 10 mM $ZnCl_2$ and 1.5 µl nuclease P1 (stock: 1 U/ µl) and incubation for 90 minutes at 37 °C. Subsequently, 30 µl 0.5 M Tris–HCl (pH 7.4) and 1.5 µl alkaline phosphatase (0.014 U/ µl) were added followed by incubation at 37 °C for 45 minutes. The digest was then analyzed by HPLC-ECD on a Supelcosil™ LC-18S column (250 mm × 4.6 mm) (Supelco Park, Bellefonte, PA, USA) in combination with a DECADE electrochemical detector (Antec, Leiden, the Netherlands). The ECD-signal was first stabilized with mobile phase (94 mM KH_2PO_4, 13 mM K_2HPO_4, 26 mM KCl and 0.5 mM EDTA, 10 % methanol) for approximately 3 hours at a flow rate of 1 ml/ minute. After stabilization, 8-oxo-dG was detected at a potential of 400 mV and dG was simultaneously monitored by UV absorption at 260 nm.

Methylation-sensitive McrBC-real-time PCR assay

Genomic DNA isolation was performed by lysating livers of E14.5 fetuses and bone marrow cells of 12 week old mice using 0.5 ml lysis buffer (25 mM EDTA, 10 mM TRIS-HCl, pH 8.0, 100 mM NaCl, 1% SDS) and 20 µl of proteinase K (Qiagen). Tissue was homogenized, incubated at 56 °C for 1 hour and homogenized again. Samples were then allowed to completely lyse by incubating at 56 °C overnight. DNA was extracted with phenol/chloroform extraction (phenol/chloroform/isoamyl alcohol (25:24:1)) and DNA concentration and purity determined by measuring the OD 260/280. Analysis of methylation pattern of the repetitive elements, i.e. long interspersed nucleotide elements (LINEs), short interspersed nucleotide elements (SINEs), intracisternal A particle (IAP), Major and Minor satellites, was performed as described earlier (10). Briefly, 1 µg of genomic DNA was digested overnight at 37 °C using 10 U of McrBC (New England Biolabs, Beverly, MA, USA). The DNA strand breaks prevent amplification of methylated DNA in the subsequent two-step quantitative real-time PCR, which consisted of IQ SYBR Green Supermix (Biorad) with 4 ng of McrBC-digested DNA and 25 pmol of each primer (See Table 1, Eurogentec) in a reaction volume of 25 µl. The cycling conditions consisted of an initial denaturation at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 45 seconds, 58 °C for 90 seconds, with an exception for the Major and Minor satellites for which this step was performed at 60 °C, using the iCycler (Biorad). Data were analyzed by MyiQ Software system (Biorad), Ct values were normalized for endogenous reference (*HPRT*), compared with the calibrator (i.e. average Ct) and expressed as relative expression ($2^{-\Delta\Delta Ct}$). An increase in PCR amplification products is indicative of hypomethylation, whereas a decrease in PCR amplification products indicates hypermethylation.

Statistical analysis

Statistical analysis was performed with Statistical Package for Social Sciences (SPSS version 17 for Windows, SPSS Inc., Chicago, IL, USA). Nested ANOVA was performed to analyze erythropoietic changes in fetuses prenatally exposed to quercetin to account for litter effects. One-way ANOVA was used to assess the impact of quercetin on: 1. litter sizes at birth and at day 14.5 of gestation, 2. average pup weight at postnatal day 5 or at day 14.5 of gestation, 3. placental weight at day 14.5 of gestation, 4. dietary effects between control and prenatally exposed 12 week old mice and maternal iron homeostasis. To avoid litter effects in the analyses of 12 week old animals, not more than two mice per litter were used for analysis and nests with less than 5 litters were excluded from the analysis. Log transformed data were used for analysis by an independent sample t-test for the biochemical determination of amniotic fluid and the gene expression levels of *IL-1β*, *Il-6*, *IL-10* and *hepcidin* as these results were not normally distributed.

Results

Quercetin exposure during fetal development

To investigate the effect of quercetin exposure on fetal development and fetal erythropoiesis, female mice were exposed to quercetin (302 mg/ kg feed) from 3 days before conception until day 14.5 of gestation, at which the mice were sacrificed and fetuses were isolated. As shown in Table 2, no differences in litter size, fetal weight or placental weight could be observed between control and quercetin exposed fetuses. Furthermore, as quercetin is known to chelate iron *in vitro* (2, 3), the total amount of free iron was measured in the amniotic fluid. Again, no differences between fetuses exposed to quercetin and control fetuses could be detected (4.1 ± 0.6 µmol/ L for quercetin exposed fetuses *versus* 5.9 ± 0.3 µmol/ L for control fetuses, P=0.54, amniotic fluid was pooled per litter, n=3 in case of both diets).

Although we could not directly measure the iron level of fetal livers, levels measured in the liver of their mothers were assessed to gain insight in the fetal situation. Iron levels measured in the liver of maternal mice were similar for both diet groups (84.5 ± 17.0 ng/ mg *versus* 85.2 ± 5.5 ng/ mg, P= 0.97, n=3 for quercetin exposed mothers and n=5 for control mothers). In addition we also measured the gene expression of *hepcidin*, the main iron regulatory hormone (4), in the liver of the mothers and found no differences between both diet groups (0.5 ± 0.2-fold *versus* 1.0 ± 0.2-fold for control animals (n=5), n=3 for quercetin exposed, P=0.11). Interestingly, *Ho1* levels in the maternal spleen were significantly increased in expression in case of the mothers that were exposed to quercetin (3.6 ± 0.9-fold *versus* 1.0 ± 0.3-fold for control animals (n=5), P=0.03, n=3 for quercetin exposed).

As 65 % of the body's iron is used for the production of hemoglobin (4), the globin status was analyzed in the liver of E14.5 fetuses (Supplementary data, Table 1[†]) using a quantitative real-time PCR method   Results showed that fetuses exposed to quercetin (n=8: 8 fetuses from 3 different litters) had no changes in the level of embryonic (*ζ-*, *βH1-*, and *εγ-globins*) and adult (*α-* and *β-globins*) globins compared

with control fetuses (n=9: 9 fetuses from 3 different litters), suggesting that quercetin exposure also had no effect on hemoglobin profile *in utero*.

Since the globin status is expected to change simultaneously with the change in erythroid lineage, the distribution of primitive and adult RBCs in E14.5 fetuses was determined (Supplementary data, Table 2[†]) by blood smear analysis. Here, quercetin exposure had no statistically significant effect on the amount of primitive erythrocytes or definitive erythrocytes (proerythrocytes, basophilic erythrocytes and ortochromic erythrocytes, n=9: 9 fetuses from 2 different quercetin exposed litters and n=15: 15 fetuses from 3 different control litters), suggesting that prenatal exposure to quercetin did not influence the switch in erythroid lineage during fetal development.

The level of bilirubin, a breakdown product of the heme part of hemoglobin (12), was measured in the amniotic fluid (amniotic fluid was pooled per litter, n=3 in case of both diets). This showed a slight elevation in bilirubin levels in the amniotic fluid of fetuses exposed to quercetin (2.7 ± 0.5 µmol/ L for quercetin exposed fetuses *versus* 1.2 ± 0.1 µmol/ L for control fetuses, P=0.05). Therefore, Ho1 gene expression level was measured in the liver of E14.5 fetuses using quantitative real-time PCR to further investigate the possibility of higher heme degradation. The results showed no effect of quercetin exposure on the expression of *Ho1* (1.4-fold, P=0.43, n=4 for both diets).

Finally, EPO, the principal regulator of erythropoiesis which stimulates erythrocyte production in response to a decrease in oxygen delivery (13), was not significantly modulated in the amniotic fluid of fetuses exposed to quercetin as compared with control fetuses (3.1 ± 0.5 U/ mL for quercetin exposed fetuses *versus* 2.6 ± 0.8 U/ mL for control fetuses; P=0.19; amniotic fluid was pooled per litter, n=3 in case of both diets).

Table 2. Characteristics of the control and quercetin exposed litters during fetal period and after birth

	Control	Quercetin	P-value
Litter size during gestation	4.6 ± 1.6	7.3 ± 1.2	0.28
Fetal weight (mg)	192.4 ± 22.8	212.6 ± 32.8	0.62
Placental weight (mg)	91.9 ± 11.0	84.5 ± 4.6	0.64
Litter size at birth	6.4 ± 0.7	5.1 ± 0.8	0.27
Pup weight (g) at day 5	3.2 ± 0.3	3.1 ± 0.1	0.87

General characteristics of the litters prenatally exposed to quercetin-supplemented or normal diet. Results represent the mean ± SE. At day 14.5 of gestation: n=5 for control litters and n=3 for quercetin exposed litters. N=8 for litters that were born in case of both diets. No significant differences were found by nested ANOVA accounting for litter effects.

Prenatal exposure to quercetin and erythropoiesis in adulthood

Since there were no statistically significant differences in litter characteristics at day 14.5 of gestation, we did not expect any differences in litter characteristics at birth (average litter size and pup weight (g) at day 5 after birth), as was confirmed and shown in Table 2. Additionally, we investigated whether prenatal exposure to quercetin resulted in changes in globin gene expression at adult age by determining the globin mRNA expression levels in the bone marrow of 12 week old mice using quantitative real-time PCR (Figure 1A-B). At adult age there were also no differences in globin gene expression noticeable.

Next, RBC maturation in the bone marrow of three 12 week old mice from 2 different litters per diet group was analyzed using May-Grünwald staining. Bone marrow smears showed that prenatal quercetin exposure had no effect on maturation of RBCs as there were also no observed differences in the number of RBC progenitors (proerythroblast, basophilic erythroblast, ortochromic eryhtroblast, reticulocyte) and pyrenocytes (measure for enucleation of immature RBCs, Figure 1C).

Hemoglobin degradation was examined by determining *Ho1* mRNA expression level (Figure 1D), which catalyzes the rate-limiting step in heme degradation during RBC degradation (12). *Ho1* gene expression was determined by quantitative real-time PCR in the spleen which is the main site for RBC degradation and in the bone marrow, where RBC production takes place. Results did not indicate an increase in heme degradation and therefore in RBC turnover in the bone marrow (n=4 for both diets) in 12 week old mice prenatally exposed to quercetin. In spleen, however, a significant increase in *Ho1* expression (P=0.04, n=4 from different litters for both diets) was detected, suggesting that there is an increase in heme degradation in the spleen of 12 week old mice prenatally exposed to quercetin.
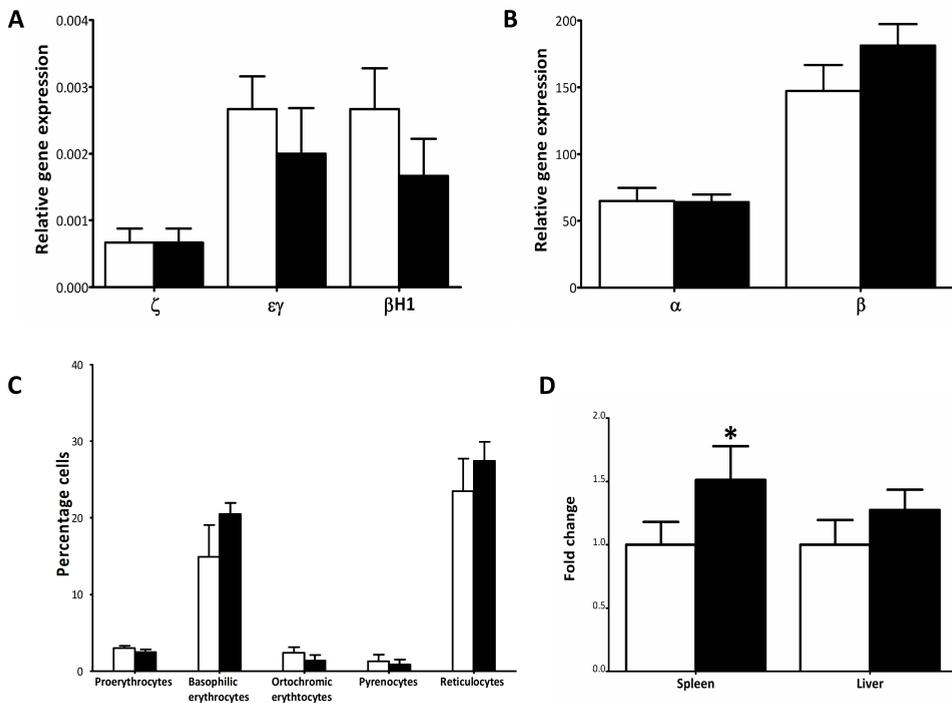
Figure 1. Prenatal exposure to quercetin has no effect on erythropoiesis at adulthood.
mRNA expression level of embryonic globins (ζ, $\beta H1$, $\epsilon\gamma$; A) and adult globins (α, β; B) were measured in the bone marrow of 12 week old control (n=6) or quercetin exposed (n=6) mice. Bars represent the average fold change (calculated as $2^{-\Delta CT}$) of each globin gene quantified by quantitative real-time PCR. C. Bone marrow smears of 12 week old mice from the control (n=3) and quercetin exposed (n=3) group were analyzed to determine the different RBC progenitors cells in the bone marrow. Bars represent the percentage of cells of each cell type. D. mRNA expression level of *Ho1* measured in the spleen and bone marrow of 12 week old mice from the control group (n=4) and the quercetin exposed group (n=4). Bars represent the normalized average fold change (calculated as $2^{-\Delta\Delta CT}$) of *Ho1* gene quantified by quantitative real-time PCR. White bars represent the control group, black bars the quercetin exposed group. Error bars represent the standard error. *: P≤0.05

Finally, gene expression analysis of the bone marrow cells of 12 week old mice (n=3 for both groups) were performed using microarrays and showed that of the 88 genes involved in erythropoiesis or iron homeostasis according to their Gene-Ontology terms, there was only one gene differentially expressed, namely *Steap4* (-1.7-fold, P=0.009, Supplementary data, Table 3[†]), indicating that these pathways were not affected by prenatal maternal intake of quercetin during gestation.

Prenatal quercetin exposure and liver iron storage

Previously, it has been shown that quercetin chelates iron *in vitro* (2, 3) and attenuates iron induced liver injury *in vivo* (14). We assessed the amount of total iron at 12 weeks of age in the liver and spleen, which are respectively the major and minor iron storage organs. In the liver, iron levels were indeed significantly increased in case of *in utero* quercetin exposure (94.74 ± 5.3 ng/ mg *versus* 62.9 ± 5.0 ng/ mg for control animals, P=0.002, n=5 for both diets, Figure 2A). Iron levels measured in the spleen of mice prenatally exposed to quercetin were also higher, although not statistically significant (929.5 ± 265.1 ng/ mg *versus* 724.5 ± 124.3 ng/ mg for control animals, P=0.41, n=5 for both diets, Figure 2B).
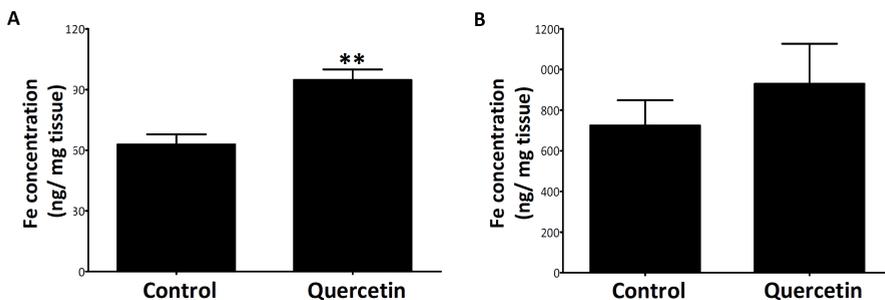
Figure 2. Prenatal quercetin exposure results in increased iron levels in the liver of 12 week old mice. Iron levels were measured in liver (A) and spleen (B) of control (n=5) and quercetin exposed (n=5) mice at 12 weeks of age. Bars represent the amount of total iron expressed as ng/ mg tissue. Error bars represent the standard error. [**]: P≤0.01

The increased iron storage in the liver of 12 week old mice prenatally exposed to quercetin could be the result of a long-lasting change in iron homeostasis. Therefore, we examined the expression of iron homeostasis related genes using quantitative real-time PCR. *Hepcidin* showed a slightly, though not significant increase in expression in mice prenatally exposed to quercetin (n=10) compared with the control group (n=5, Figure 3A). The same was found for *IL-6* that normally regulates hepcidin production (15), which showed a slight, although not significant increased expression in quercetin exposed mice (n=10) compared to control mice (n=5, Figure 3B). However, when comparing the overall median of *hepcidin* and *IL-6* expression with the individual data in the quercetin exposed and control group, we found that in case of the control group only 1 in 5 mice had an expression of *hepcidin* or *IL-6* above the median, while in the quercetin exposed group this was 6 out of 10 mice. Other cytokines, namely *IL-1β*, a component of IL-1, which induces ferritin expression resulting in an increased storage of iron (15), and *IL-10*, which stimulates the uptake of iron *via* the transferrin-receptor (15), showed a statistically significant increase in expression in quercetin exposed mice (n=10, *IL-1β*: P=0.05; *IL-*

10: P=0.02, Figure 3C, E) compared to controls (n=5). Although the gene expression of *hepcidin* and ILs involved in iron homeostasis were generally increased in adult mice prenatally exposed to quercetin, they were not all statistically significant increased. Interestingly, gene expression of *IL-1ß* was not altered in bone marrow of 12-week old mice prenatally exposed to quercetin (*IL-1ß*: -1.2-fold change, P=0.5; no microarray data were available for *IL-6*, *IL-10* and *hepcidin* as the expression signal could not be detected).

As iron overload could result in oxidative stress, 8-oxo-dG levels were measured in the livers of mice prenatally exposed to quercetin (n=5) compared to controls (n=5). Interestingly, mice prenatally exposed to quercetin had lower 8-oxo-dG levels (14.6 ± 1.5 x 10^{-6} 8-oxo-dG/ dG *versus* 26.8 ± 2.0 x 10^{-6} 8-oxo-dG/ dG for control, P=0.001).
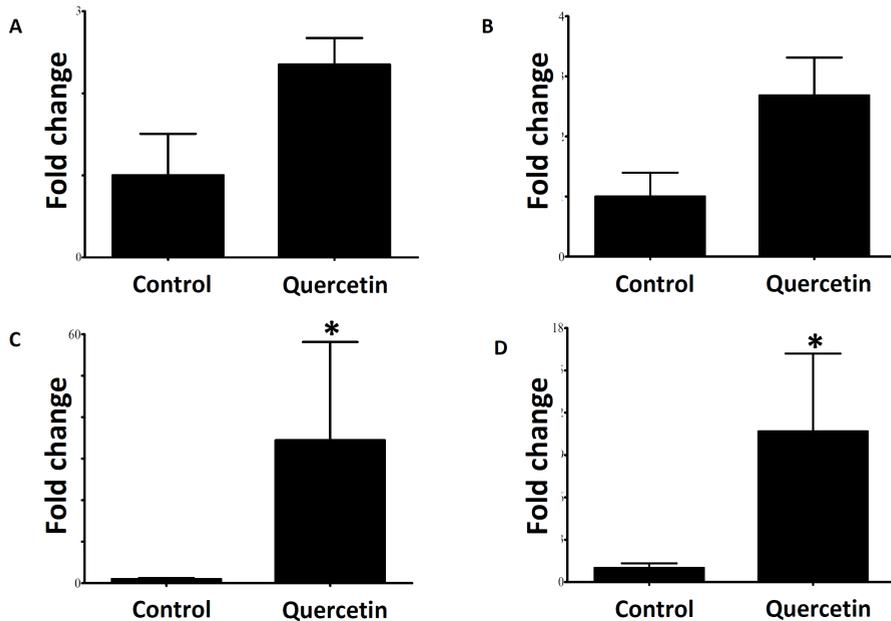
Figure 3. Prenatal quercetin exposure induces *hepcidin* and interleukins involved in iron uptake in the liver of 12 week old mice.

mRNA expression levels of *hepcidin* (A), *IL-6* (B), *IL-1ß* (C), *IL-10* (D) were measured in the liver of 12 week old mice from the control group (n=5) and the quercetin exposed group (n=7). Bars represent the normalized average fold change (calculated as $2^{-\Delta\Delta CT}$) of *hepcidin* and interleukin genes quantified by quantitative real-time PCR. Error bars represent the standard error. *: P≤0.05.

Prenatal quercetin leads to long-term epigenetic changes

As quercetin exposure early in development exerted long-term changes in the expression of certain genes, we assumed that these effects might be mediated through epigenetic modifications. Therefore, we employed an assay in which the methylation-sensitive endonuclease McrBC cleaves methylated DNA, but leaves unmethylated CpGs intact. By subsequent PCR amplification, one could quantify the unmethylated repetitive elements. Using this assay, we determined the methylation status of Major and Minor satellites, intracisternal-A-particle (IAP-GAG), long interspersed nucleotide elements (LINE1) and short interspersed nucleotide elements (SINEB1 and SINEB2), which constitute almost 37.5 % of the mouse genome (1), in bone marrow cells.

Overall, bone marrow cells of adult mice prenatally exposed to quercetin (n=3) showed moderate hypermethylation of the repetitive elements observed as lower level of PCR products after McrBC digestion compared with the control group (n=3, Figure 4A). Especially, the SINEB1 elements were hypermethylated (P=0.01).

In the fetuses, quercetin exposure exerted no effect on the gene expression. Still, to investigate whether global epigenetic modifications had already occurred *in utero*, we studied the methylation status of the repetitive DNA elements in the liver of control (n=4) and quercetin exposed fetuses (n=4) at E14.5. Indeed, we did not observe any difference in the methylation status of repetitive elements between the diet groups at this particular developmental time point (Figure 4B).
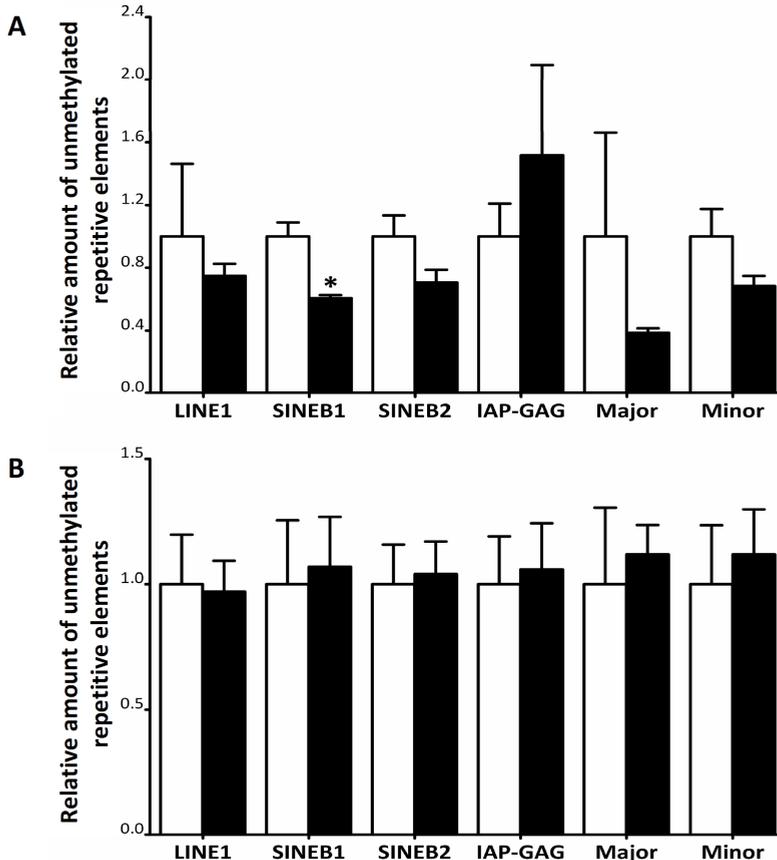
Figure 4. The effect of quercetin on DNA methylation of repetitive elements.
A. Long-term effect of prenatal exposure to quercetin on DNA methylation of repetitive elements in bone marrow cells of quercetin exposed mice (n=3) compared to control mice (n=3). B. Short-term effect of prenatal exposure to quercetin on DNA methylation of repetitive elements in fetal liver of quercetin exposed fetuses (n=4) compared to control fetuses (n=4) at day 14.5 of gestation. The degree of methylation of repetitive elements: long interspersed nucleotide elements (LINE1), short interspersed nucleotide elements (SINEB1 and SINEB2), intracisternal-A-particle (IAP-GAG), and Major and Minor satellites were determined by methylation-sensitive McrBC-real-time PCR assay. Bars represent the normalized average expression level of the unmethylated repetitive elements between the quercetin-exposed (black bars) and the control group (white bars). Error bars represent the standard error. *: P≤0.05.

Discussion

In this study, pregnant mice were exposed to a high dose of quercetin (302 mg/ kg feed), which is higher than the average daily dietary intake of quercetin in humans (4–68 mg per day) (17). However, we do not expect that this dose has been toxic to the fetuses as Prater *et al.* (18) showed that a high dose of quercetin (333 mg/ kg feed) administered to mice throughout their pregnancy, did not have teratogenic effects on the fetuses. Moreover, the quercetin dose administered to mice in our study is comparable with quercetin supplement intake: the daily dose of quercetin supplements lies between 200-1800 mg quercetin (manufacturers' recommendations) (19, 20). These high dose supplements are freely available in pharmacies and drugstores. Besides, it has also been shown that daily quercetin supplementation results in increased plasma quercetin levels (19), suggesting that the fetus could also be exposed to increased concentrations of quercetin and its metabolites.

In this study, we show that prenatal exposure to quercetin results in an adapted iron homeostasis as seen in the significantly increased iron storage in the liver of adult mice. We assumed that the effects seen in adult mice prenatally exposed to quercetin were most probably the result of a disturbed iron homeostasis *in utero* with persisting consequences for iron homeostasis and erythropoiesis later in life.

At gestational day 14.5 no clear effects of quercetin on erythropoiesis or iron levels could be observed. Iron levels measured in the liver of mothers and gene expression levels of liver *hepcidin* did not indicate any differences between control and quercetin exposed mothers, suggesting that mothers fed a diet containing iron chelators did not suffer from severe iron depletion during gestation. In addition, maternal iron deficiency normally results in a reduced fetal weight (21). However, E14.5 fetuses exposed to quercetin had an average weight comparable to control. High placental weight is usually also associated with iron deficient pregnancies (22), but no difference in placental weight were noticeable between quercetin exposed and control fetuses. Finally, the average weight at day 5 after birth did not differ between control and quercetin exposed mice. Taken together, this would suggest that the situation created by quercetin exposure did not result in severe iron deficiency and did not cause early termination of gestation, as the litter size both at day 14.5 of gestation and at birth did not differ between both diet groups.

However, mother mice exposed to quercetin did have increased expression of *Ho1*, which could suggest that quercetin did decrease the amount of available iron and therefore the mother had to make more iron available for the fetuses by degradation of heme. Indeed, during a normal gestation, when the mother needs to cope with the increasing iron requirement of the fetus, she first addresses her own iron stores. Only when the fetal demands are too high or there is maternal iron deficiency, hematocrit levels will decrease (23), because iron is made available by increased degradation of heme. Degradation of heme by Ho1 results in the formation of bilirubin, (12), and bilirubin levels were increased in the amniotic fluid of fetuses exposed to quercetin. Please note that this increase was not statistically significant, but fetal bilirubin is transfered *via* the placenta to the mother (24), suggesting that the levels of bilirubin actually formed in the fetus could have been even higher. However, as increased levels of bilirubin are normally found in anemic fetuses (24), and because we did not see any differences in globin expression or RBC composition in fetuses exposed to quercetin, we

assume that quercetin exposure at this time point in gestation had a very mild effect that cannot be distinguished from a non-anemic situation. In addition, it should also be emphasized that the iron requirement of the fetus increases throughout the pregnancy (25). Therefore, pronounced effects of quercetin may not be present at this time point in gestation. Moreover, as flavonoids have shown to accumulate in fetal tissue (7), the direct effects of quercetin exposure may be visible at a later time point of gestation. Nonetheless, we had chosen day 14.5 of gestation to sacrifice the pregnant mice, since at that time point the switch from primitive to adult erythroid lineage and concomitantly the switch from fetal to adult hemoglobin occurs (26-28). However, exposure to quercetin did not have any effects on these events.

Once born, pups were no longer exposed to high levels of quercetin. We assumed that this resulted in the increased availability of dietary iron, which could be experienced as an iron overload as we suspect that these animals were 'developmentally programmed' to cope with lower iron levels *in utero*. The mismatch between the *in utero* environment and postnatal life with regard to iron availability resulted in a differently regulated iron homeostasis in mice prenatally exposed to quercetin. However erythropoiesis was not affected, since we did not observe any differences in globin status and RBC maturation in the bone marrow. Interestingly, *Ho1* gene expression in the spleen was significantly up-regulated, suggesting increased RBC turnover. However, as there were no observed differences in bone marrow maturation or the amount of red blood cells (7.2 ± 0.1 x 10^{12}/ L *versus* 7.0 ± 0.1 x 10^{12}/ L for control group, P=0.53, n=39 for quercetin group and n=41 for control group), we assumed that the increase in *Ho1* gene expression is the result of an increase in hemoglobin per RBC. Indeed, there were 1) a slight elevation in hemoglobin level of reticulocytes (1.03 ± 0.01 fmol *versus* 0.96 ± 0.01 fmol in control group, P=0.07, n=39 for quercetin group and n=41 for control group) and 2) an increase in the mean corpuscular volume of red blood cells (mean ± SE, 46.33 ± 0.50 fL *versus* 44.96 ± 0.45 fL for control group, n=39 for quercetin group and n=41 for control group) compared with control mice, which both indicate that more hemoglobin is formed by RBCs of mice prenatally exposed to quercetin.

In addition, gene expression profile analysis of bone marrow cells did not show any alterations in gene expression of genes involved in erythropoiesis or iron homeostasis between prenatally exposed and control mice. Only the expression of *Steap4* was significantly decreased in mice prenatally exposed to quercetin. Steap4 has been shown to function as a ferrireductase, capable of increasing the uptake of iron by cells *in vitro*. It has also been postulated that Steap4 could play a role in the pathologic deposition of iron in many tissues (29). Decreased expression of *Steap4* by prenatal exposure to quercetin thus limits the iron uptake by the bone marrow, probably to protect the bone marrow from excessive iron uptake, which could induce oxidative stress.

The increase in iron availability after birth could activate pathways to overcome the iron 'overload' as it is toxic to cells (4, 30). Indeed, expression of *hepcidin* and *IL-1β*, *IL-6* and *IL-10*, all involved in the storage of iron, were up-regulated resulting in increased storage of iron in the liver. It is remarkable to see that this pathway is activated, as it is normally triggered during chronic inflammation induced anemia (4, 15). An overview of the mechanism by which we assume that iron accumulation occurs in the liver of mice prenatally exposed to high levels of quercetin is given in Figure 5.
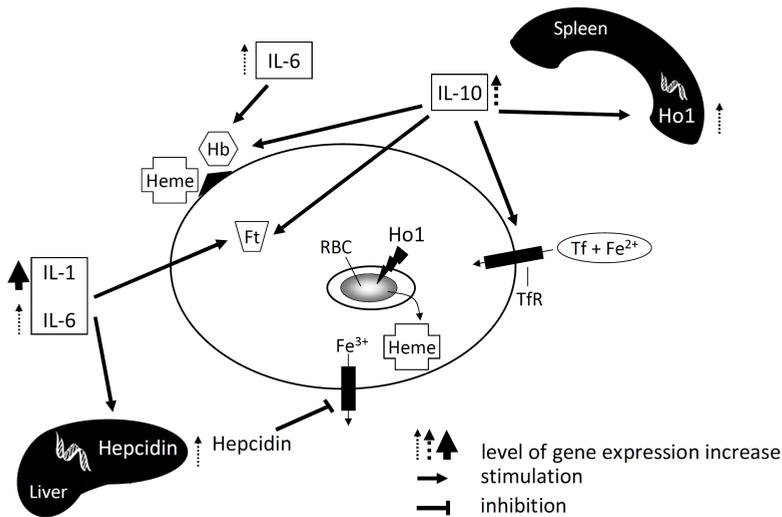
Figure 5. Overview of the hypothesis.
Prenatal quercetin exposure results in a relative iron excess after birth because iron chelation by quercetin ceases, which will activate pathways to overcome the iron 'overload' as it is toxic to cells. Since these pathways are permanently activated in adult life, iron will accumulate in the liver. Iron accumulation in cells occurs through the up-regulation of several interleukins (IL1β, IL6 and IL10) involved in the storage of iron. The increase in IL-1 and IL-6 will up-regulate the expression of hepcidin by the liver, resulting in iron retention by limiting the iron efflux. In addition, IL-1 and IL-6, but also IL-10, is known to increase the production of ferritin (Ft), increasing the iron storage capacity of cells. Moreover, IL-6 and IL-10 increase the uptake of heme and hemoglobin (Hb). IL-10 also induces the production of hemeoxygenase-1 (Ho1) by the spleen, resulting in the release of iron from heme. In addition, it increases the uptake of transferrin (Tf) bound iron. The increase in gene expression is indicated by 3 different arrows which represent a fold change of approximately 5, 20 and 60, respectively.

As quercetin exposure early in development was found to exert long-term changes in the expression of certain genes, we assume that these effects might be mediated through epigenetic modifications. Our results show that prenatal exposure to quercetin was associated with a moderate hypermethylation of repetitive elements (SINEB1, SINEB2, LINE1, Major and Minor satellites) in bone marrow cells at 12 weeks of age. However the methylation status of these repeats was not affected directly by quercetin exposure during fetal development, therefore we assume that this occurred at a later time point. However, one should keep in mind that for some experiments performed in this study numbers of animals were on the low side.

The expression of inflammation related cytokines was enhanced, although there was no apparent inflammation present. Therefore, future studies should further elucidate the critical window of exposure, as this study suggests that quercetin exerts an effect after day 14.5 of gestation in mice. Furthermore, it also needs to be investigated whether these mice would react differently if inflammation would indeed be triggered at adult age.

Besides quercetin, other iron chelators which are administered to overcome iron overload resulting from for instance chronic blood transfusion (31, 32), should be used with caution during pregnancy.

Acknowledgments

†Supplementary data

The supplementary data can be viewed at:
http://www.sciencedirect.com/science/article/pii/S0300483X11004562.

Supplementary Table 1. Gene expression profile by Q-PCR of globins in the liver of quercetin exposed fetuses at embryonic day 14.5.

Supplementary Table 2. Red blood cell composition in blood of quercetin exposed fetuses at embryonic day 14.5.

Supplementary Table 3. Go term analysis of the mRNA expression of bone marrow cells of 12 week old mice prenatally exposed to quercetin.

Supplementary data. Identification of relevant genes by Gene Ontology terms. Statistical analysis of microarray data.

References

1. Ross, J. A., and Kasum, C. M. (2002) Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annu Rev Nutr* 22, 19-34
2. Ferrali, M., Signorini, C., Caciotti, B., Sugherini, L., Ciccoli, L., Giachetti, D., and Comporti, M. (1997) Protection against oxidative damage of erythrocyte membrane by the flavonoid quercetin and its relation to iron chelating activity. *FEBS Lett* 416, 123-129
3. Kitagawa, S., Sakamoto, H., and Tano, H. (2004) Inhibitory effects of flavonoids on free radical-induced hemolysis and their oxidative effects on hemoglobin. *Chem Pharm Bull (Tokyo)* 52, 999-1001
4. Munoz, M., Villar, I., and Garcia-Erce, J. A. (2009) An update on iron physiology. *World J Gastroenterol* 15, 4617-4626
5. Cao, G., Sofic, E., and Prior, R. L. (1997) Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free Radic Biol Med* 22, 749-760
6. Laughton, M. J., Halliwell, B., Evans, P. J., and Hoult, J. R. (1989) Antioxidant and pro-oxidant actions of the plant phenolics quercetin, gossypol and myricetin. Effects on lipid peroxidation, hydroxyl radical generation and bleomycin-dependent damage to DNA. *Biochem Pharmacol* 38, 2859-2865
7. Todaka, E., Sakurai, K., Fukata, H., Miyagawa, H., Uzuki, M., Omori, M., Osada, H., Ikezuki, Y., Tsutsumi, O., Iguchi, T., and Mori, C. (2005) Fetal exposure to phytoestrogens--the difference in phytoestrogen status between mother and fetus. *Environ Res* 99, 195-203
8. Vanhees, K., de Bock, L., Godschalk, R. W., van Schooten, F. J., and van Waalwijk van Doorn-Khosrovani, S. B. (2011) Prenatal exposure to flavonoids: implication for cancer risk. *Toxicol Sci* 120, 59-67
9. Godfrey, K. M., and Barker, D. J. (2000) Fetal nutrition and adult disease. *Am J Clin Nutr* 71, 1344S-1352S
10. Vanhees, K., Coort, S., Ruijters, E. J., Godschalk, R. W., van Schooten, F. J., and van Doorn-Khosrovani, S. B. (2011) Epigenetics: prenatal exposure to genistein leaves a permanent signature on the hematopoietic lineage. *FASEB J* 25, 797-807
11. Langie, S. A., Kowalczyk, P., Tudek, B., Zabielski, R., Dziaman, T., Olinski, R., van Schooten, F. J., and Godschalk, R. W. (2010) The effect of oxidative stress on nucleotide-excision repair in colon tissue of newborn piglets. *Mutat Res* 695, 75-80
12. Maines, M. D. (1988) Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. *FASEB J* 2, 2557-2568
13. Koury, M. J., and Ponka, P. (2004) New insights into erythropoiesis: the roles of folate, vitamin B12, and iron. *Annu Rev Nutr* 24, 105-131
14. Zhang, Y., Li, H., Zhao, Y., and Gao, Z. (2006) Dietary supplementation of baicalin and quercetin attenuates iron overload induced mouse liver injury. *Eur J Pharmacol* 535, 263-269
15. Weiss, G. (2009) Iron metabolism in the anemia of chronic disease. *Biochim Biophys Acta* 1790, 682-693
16. Waterston, R. H., Lindblad-Toh, K., Birney, E., Rogers, J., Abril, J. F., Agarwal, P., Agarwala, R., Ainscough, R., Alexandersson, M., An, P., Antonarakis, S. E., Attwood, J., Baertsch, R., Bailey, J., Barlow, K., Beck, S., Berry, E., Birren, B., Bloom, T., Bork, P., Botcherby, M., Bray, N., Brent, M. R., Brown, D. G., Brown, S. D., Bult, C., Burton, J., Butler, J., Campbell, R. D., Carninci, P., Cawley, S., Chiaromonte, F., Chinwalla, A. T., Church, D. M., Clamp, M., Clee, C., Collins, F. S., Cook, L. L., Copley, R. R., Coulson, A., Couronne, O., Cuff, J., Curwen, V., Cutts, T., Daly, M., David, R., Davies, J., Delehaunty, K. D., Deri, J., Dermitzakis, E. T., Dewey, C., Dickens, N. J., Diekhans, M., Dodge, S., Dubchak, I., Dunn, D. M., Eddy, S. R., Elnitski, L., Emes, R. D., Eswara, P., Eyras, E., Felsenfeld, A., Fewell, G. A., Flicek, P., Foley, K., Frankel, W. N., Fulton, L. A., Fulton, R. S., Furey, T. S., Gage, D., Gibbs, R. A., Glusman, G., Gnerre, S., Goldman, N., Goodstadt, L., Grafham, D., Graves, T. A., Green, E. D., Gregory, S., Guigo, R., Guyer, M., Hardison, R. C., Haussler, D., Hayashizaki, Y., Hillier, L. W., Hinrichs, A., Hlavina, W., Holzer, T., Hsu, F., Hua, A., Hubbard, T., Hunt, A., Jackson, I., Jaffe, D. B., Johnson, L. S., Jones, M., Jones, T. A., Joy, A., Kamal, M., Karlsson, E. K., Karolchik, D., Kasprzyk, A., Kawai, J., Keibler, E., Kells, C., Kent, W. J., Kirby, A., Kolbe, D. L., Korf, I., Kucherlapati, R. S., Kulbokas, E. J., Kulp, D., Landers, T., Leger, J. P., Leonard, S., Letunic, I., Levine, R., Li, J., Li, M., Lloyd, C., Lucas, S., Ma, B., Maglott, D. R., Mardis, E. R., Matthews, L., Mauceli, E., Mayer, J. H., McCarthy, M., McCombie, W. R., McLaren, S., McLay, K., McPherson, J. D., Meldrim, J., Meredith, B., Mesirov, J. P., Miller, W., Miner, T. L., Mongin, E., Montgomery, K. T., Morgan, M., Mott, R., Mullikin, J. C., Muzny, D. M., Nash, W. E., Nelson, J. O., Nhan, M. N., Nicol, R., Ning, Z., Nusbaum, C., O'Connor, M. J., Okazaki, Y., Oliver, K., Overton-Larty, E., Pachter, L., Parra, G., Pepin, K. H., Peterson, J., Pevzner, P., Plumb, R., Pohl, C. S., Poliakov, A., Ponce, T. C., Ponting, C. P., Potter, S., Quail, M., Reymond, A., Roe, B. A., Roskin, K. M., Rubin, E. M., Rust, A. G., Santos, R., Sapojnikov, V., Schultz, B., Schultz, J., Schwartz, M. S., Schwartz, S., Scott, C., Seaman, S., Searle, S., Sharpe, T., Sheridan, A., Shownkeen, R., Sims, S., Singer, J. B., Slater, G., Smit, A., Smith, D. R., Spencer, B., Stabenau, A., Stange-Thomann, N., Sugnet, C., Suyama, M., Tesler, G., Thompson, J., Torrents, D., Trevaskis, E., Tromp,

J., Ucla, C., Ureta-Vidal, A., Vinson, J. P., Von Niederhausern, A. C., Wade, C. M., Wall, M., Weber, R. J., Weiss, R. B., Wendl, M. C., West, A. P., Wetterstrand, K., Wheeler, R., Whelan, S., Wierzbowski, J., Willey, D., Williams, S., Wilson, R. K., Winter, E., Worley, K. C., Wyman, D., Yang, S., Yang, S. P., Zdobnov, E. M., Zody, M. C., and Lander, E. S. (2002) Initial sequencing and comparative analysis of the mouse genome. *Nature* 420, 520-562

17. Skibola, C. F., and Smith, M. T. (2000) Potential health impacts of excessive flavonoid intake. *Free Radic Biol Med* 29, 375-383

18. Prater, M. R., Laudermilch, C. L., Liang, C., and Holladay, S. D. (2008) Placental oxidative stress alters expression of murine osteogenic genes and impairs fetal skeletal formation. *Placenta* 29, 802-808

19. Egert, S., Wolffram, S., Bosy-Westphal, A., Boesch-Saadatmandi, C., Wagner, A. E., Frank, J., Rimbach, G., and Mueller, M. J. (2008) Daily quercetin supplementation dose-dependently increases plasma quercetin concentrations in healthy humans. *J Nutr* 138, 1615-1621

20. Mennen, L. I., Walker, R., Bennetau-Pelissero, C., and Scalbert, A. (2005) Risks and safety of polyphenol consumption. *Am J Clin Nutr* 81, 326S-329S

21. Gambling, L., Charania, Z., Hannah, L., Antipatis, C., Lea, R. G., and McArdle, H. J. (2002) Effect of iron deficiency on placental cytokine expression and fetal growth in the pregnant rat. *Biol Reprod* 66, 516-523

22. Hindmarsh, P. C., Geary, M. P., Rodeck, C. H., Jackson, M. R., and Kingdom, J. C. (2000) Effect of early maternal iron stores on placental weight and structure. *Lancet* 356, 719-723

23. Gambling, L., Czopek, A., Andersen, H. S., Holtrop, G., Srai, S. K., Krejpcio, Z., and McArdle, H. J. (2009) Fetal iron status regulates maternal iron metabolism during pregnancy in the rat. *Am J Physiol Regul Integr Comp Physiol* 296, R1063-1070

24. Sikkel, E., Pasman, S. A., Oepkes, D., Kanhai, H. H., and Vandenbussche, F. P. (2004) On the origin of amniotic fluid bilirubin. *Placenta* 25, 463-468

25. Bothwell, T. H. (2000) Iron requirements in pregnancy and strategies to meet them. *Am J Clin Nutr* 72, 257S-264S

26. Palis, J. (2008) Ontogeny of erythropoiesis. *Curr Opin Hematol* 15, 155-161

27. Keller, G., Lacaud, G., and Robertson, S. (1999) Development of the hematopoietic system in the mouse. *Exp Hematol* 27, 777-787

28. Kingsley, P. D., Malik, J., Emerson, R. L., Bushnell, T. P., McGrath, K. E., Bloedorn, L. A., Bulger, M., and Palis, J. (2006) "Maturational" globin switching in primary primitive erythroid cells. *Blood* 107, 1665-1672

29. Ohgami, R. S., Campagna, D. R., McDonald, A., and Fleming, M. D. (2006) The Steap proteins are metalloreductases. *Blood* 108, 1388-1394

30. Ganz, T. (2006) Hepcidin and its role in regulating systemic iron metabolism. *Hematology Am Soc Hematol Educ Program*, 29-35, 507

31. Olivieri, N. F., and Brittenham, G. M. (1997) Iron-chelating therapy and the treatment of thalassemia. *Blood* 89, 739-761

32. Taher, A., Sheikh-Taha, M., Sharara, A., Inati, A., Koussa, S., Ellis, G., Dhillon, A. P., and Hoffbrand, A. V. (2005) Safety and effectiveness of 100 mg/kg/day deferiprone in patients with thalassemia major: a two-year study. *Acta Haematol* 114, 146-149

Chapter 6

Maternal intake of quercetin during gestation alters *ex vivo* benzo[a]pyrene metabolism and DNA-adduct formation in adult offspring

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Abstract

Variation in xenobiotic metabolism can not entirely be explained by genetic diversity in metabolic enzymes. We suggest that maternal diet during gestation can contribute to variation in metabolism by creating an *in utero* environment that shapes the offspring's defence against chemical carcinogens. Therefore, pregnant mice were supplemented with the natural aryl hydrocarbon receptor (AhR) agonist quercetin (1 mmol quercetin/ kg feed) until delivery. Next, it was investigated whether the adult offspring at the age of 12 weeks had altered biotransformation of the environmental pollutant benzo[a]pyrene (B[a]P). *In utero* quercetin exposure resulted in significantly enhanced gene expression of *Cyp1a1*, *Cyp1b1*, *Nqo1* and *Ugt1a6* in liver of fetuses at day 14.5 of gestation. Despite cessation of supplementation after delivery, altered gene expression persisted into adulthood, but in a tissue and gender dependent manner. Expression of phase I enzymes (*Cyp1a1* and *Cyp1b1*) was up-regulated in liver of adult female mice *in utero* exposed to quercetin, whereas expression of phase II enzymes (*Gstp1*, *Nqo1* and *Ugt1a6*) was predominantly enhanced in lung tissue of female mice. Epigenetic mechanisms may contribute to this adapted gene expression, as the repetitive elements (SINEB1) were hypomethylated in liver of female mice prenatally exposed to quercetin. Studies on *ex vivo* metabolism of B[a]P by lung and liver microsomes showed that the amount of B[a]P-9,10-dehydrodiol, B[a]P-7,8-dihydrodiol and 3-hydroxy-B[a]P did not change, but the amount of unmetabolized B[a]P was significantly lower after incubation with lung microsomes from offspring that received quercetin during gestation. Moreover, *ex vivo* B[a]P induced DNA-adduct formation was significantly lower for liver microsomes of offspring that were exposed to quercetin during gestation. These results suggest that prenatal diet leads to persistent alterations in phase I and II enzymes of adult mice and may affect cancer risk.

Introduction

A vast amount of research has been performed to determine the effects of genetic polymorphisms in enzymes involved in xenobiotic metabolism on inter-individual variation in cancer susceptibility (1-4). However, analysis of genetic polymorphisms did not explain a large part of the variation and other factors, especially the diet, have been postulated as additional contributors to individual reactions after exposure to xenobiotics (5). For instance, intake of dietary flavonoids is common in the human diet as they are found in fruit, vegetables and beverages such as wine, tea and coffee. They are mostly known for their anticancer properties, as they are potent antioxidants (6). Quercetin is the most widely consumed flavonoid in the diet. This polyphenolic compound is not only a potent free radical scavenger and iron chelator (7, 8), it can also modulate the expression and activity of cytochrome P450 (CYP450) and phase II detoxifying enzymes (9-11). The expression of phase I and phase II enzymes is important for the metabolism of many xenobiotics, including benzo[a]pyrene (B[a]P). B[a]P is produced during the incomplete combustion of organic compounds like tobacco, fuels, wood and meat (12-14). On its own, B[a]P has no mutagenic or carcinogenic properties (10, 15). However, it can diffuse into the cell due to its lipophilic character, bind to the aryl hydrocarbon receptor (AhR) and translocate into the nucleus. Here, AhR heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT) and this complex binds to xenobiotic responsive elements, leading to the transcription of genes of phase I and II enzymes (10, 16). The metabolic activation of B[a]P is thought to be principally performed by cytochrome P450 (CYP450) enzymes (CYP1A1, CYP1B1), resulting in the formation of the reactive B[a]P-7,8-dihydrodiol-9,10-epoxide (BPDE), leading to DNA-adduct formation and subsequent mutations, thus increasing the risk of cancer. Although it is known that quercetin prevents B[a]P induced DNA damage, there is a disagreement about the underlying mechanism (10, 17-20). Similar to B[a]P, quercetin is a potent ligand for AhR (9) and could thus alter the expression of enzymes involved in both activation as well as detoxification of B[a]P.

A diet rich in fruit and vegetables and therefore containing high amounts of flavonoids is thought to protect against cancer development (6). However, the time point of exposure to these flavonoids can also contribute to the inter-individual differences (21). Without any doubt, maternal diets can affect fetal programming by inducing epigenetic alterations that can lead to long-lasting changes in gene expression (21, 22). Due to altered genetic imprinting, individuals may acquire distinct responses to carcinogenic exposures and may therefore have different risks for developing DNA damage and cancer. The fetal origin of adult cancer needs more attention.

Therefore, in this study we aim to investigate whether gene expression of B[a]P metabolizing enzymes can be modulated by prenatal exposure to quercetin (1 mmol (302 mg) quercetin/ kg feed) *via* the maternal diet, starting 3 days before conception until delivery. At the adult age of 12 weeks male and female offspring mice were sacrificed and phase I and II gene expression was determined. Direct effect of quercetin was investigated by determining gene expression levels of phase I and II enzymes in the liver of fetuses at day 14.5 of gestation. B[a]P metabolism and DNA-adduct formation was determined *ex vivo* using S9 mix of liver and lung tissue of 12 week old male and female offspring.

Material and methods

Mice and sample collection

Mice (129/SvJ:C57BL/6J background) approximately 8 weeks of age, received either normal chow (n=8, low phytoestrogen content complete feed for mice breeding, ssniff®, Soest, Germany) or the same chow (ssniff®) supplemented with quercetin (n=8, 1 mmol (302 mg)/ kg feed) (Sigma, Zwijndrecht, the Netherlands) from 3 days before conception until the end of gestation. Male mice were placed in the cage only for the duration of copulation. After delivery all mothers and pups received normal chow. Offspring mice were anesthetized and sacrificed by cardiac puncture at 12 weeks of age, and liver and lung were removed and frozen at -80 °C until analysis.

To study the direct effects of the maternal diet on fetal phase I and II gene expression, a different group of mice were mated overnight. The presence of a vaginal plug the next morning was defined as 0.5 day post conception. On day 14.5 of gestation, mice were sacrificed to isolate the fetuses and their livers, as *AhR* mRNA and protein expression peaks between gestational day 14 and 16 (23).

Quantitative real-time polymerase chain reaction

The liver of fetuses and one half of the lung and liver of 12 week old mice were homogenized, using the Ultra-Turrax homogenizer (IKA, Staufen, Germany). Next, RNA was isolated using TRIzol Reagent (Invitrogen, Breda, the Netherlands) according to the manufacturer's instructions.

Using iScript reverse transcriptase (Bio-rad, Hercules, USA), 1 µg RNA was transcribed into cDNA. An aliquot of one 10th of the resulting cDNA was applied for quantitative PCR amplification together with 12.5 µl SYBR Green (Qiagen, Venlo, the Netherlands) and 7.5 pmol of each primer (see Table 1, Eurogentec, Maastricht, the Netherlands) in a volume of 25 µl. The reactions were carried out using a MyiQ Single Color RT-PCR detection system (Bio-rad), under the following conditions: 1 cycle at 95 °C for 3 minutes and then 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute followed by 1 cycle at 95 °C for 1 minute and 1 cycle at 65 °C. Data were analyzed by MyiQ Software system (Bio-rad), Ct values were normalized for an endogenous reference gene (*β-actin*), compared with the calibrator (i.e. average Ct value of control samples) and expressed as fold change ($2^{-\Delta\Delta Ct}$).

Table 1: Forward and reverse primers used for quantitative real-time PCR

	Forward primer (5'-3')	Reverse primer (5'-3')
Cyp1a1	TGCCCTTCATTGGTCACATG	CGTCCCCATACTGCTGACTCA
Cyp1b1	AACGCAGCCGGTGATTGT	TGTACCGACAGCCGAAGCA
Comt	AGAGAAGGAGTGGGCCATGA	CCGAATCACTGCATCCATGA
Gstp1	AGAACCAGGGAGGCAAAGCT	CAGGTCCAGCAAGTTGTAATCG
Nqo1	CAGTTCCCATTGCAGTGGTTT	CCTGCTACGAGCACTCTCTCAA
Ugt1a6	GCTACCCCAAAATGATCTGCTT	AATACCATGGGAGCCAGAGTGT
AhR	TGTGCAGAATCCCACATCCG	AATCAAGCGTGCATTGGACTG
β-actin	ACGGCCAGGTCATCACTATTG	CAAGAAGGAAGGCTGGAAAAGA
SINEB1	GTGGCGCACGCCTTTAATC	GACAGGGTTTCTCTGTGTAG
SINEB2	GAGATGGCTCAGTGGTTAAG	CTGTCTTCAGACACTCCAG
LINE1	TTTGGGACACAATGAAAGCA	CTGCCGTCTACTCCTCTTGG

Methylation-sensitive McrBC-real-time polymerase chain reaction (PCR) assay of fetuses and adult mice

Analysis of methylation patterns of the short- and long interspersed nucleotide elements (SINEB1, SINEB2 and LINE1) was performed using the methylation-sensitive McrBC-real-time PCR assay (24). 1 µg of genomic DNA was digested overnight at 37 °C using 10 U of McrBC (New England Biolabs, Beverly, MA, USA), an endonuclease which cleaves DNA containing 5-methylcytosine, but will not cleave unmethylated DNA. The DNA strand-breaks prevent amplification of methylated DNA in the quantitative real-time PCR assay. Two-step quantitative real-time PCR was performed using IQ SYBR Green Supermix (Bio-Rad Laboratories, Veenendaal, the Netherlands) with 4 ng of McrBC-digested DNA and 25 pmol of each primer (Table 1, Eurogentec, Maastricht, the Netherlands) in a reaction volume of 25 µl. The cycling conditions consisted of an initial denaturation at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 45 seconds, 58 °C for 90 seconds, using the iCycler (Bio-Rad). Data were analyzed by MyiQ Software system (Bio-rad), Ct values were normalized for Ct values of undigested DNA and subsequently compared to a calibrator (i.e. average Ct value of 5 control samples for the adult mice and of 4 control samples for the fetuses). Results were expressed as relative expression ($2^{-\Delta\Delta Ct}$). An increase in PCR amplification is indicative of hypomethylation, whereas a decrease in PCR amplification products indicates hypermethylation. Alterations in DNA methylation were assessed in fetal liver and adult liver and lung.

High-performance liquid chromatography (HPLC) and fluorescence detection of B[a]P metabolites

To mimic lung and liver biotransformation, an aliquot of S9 mix (4 mg of proteins), obtained from homogenizing the other half of the lung and liver of 12 week old mice in lysis buffer (10 mM Tris, 150 mM KCl, pH 7.4) using the Ultra-Turrax homogenizer (IKA), was incubated with a mixture consisting of 1 µM B[a]P in DMSO and a NADPH-generating system (10 mM NADPH, 100 mM glucose-6-phosphate, 40 mM $MgCl_2$, 400 mM Tris (pH 8), 17.8 µg glucose-6-phoshate dehydrogenase and milli-Q in a final volume of 500 µl), without addition of cofactors for phase II metabolizing enzymes. The incubation was carried out at 37 °C for 1 hour. Afterwards, metabolites were extracted using ethyl-acetate, evaporated and dissolved in methanol. Next, samples were applied to a HPLC system equipped with a fluorescence detector (excitation 257 nm, emission 350 nm) and an analytical reversed-phase column (ODS Hypersil, length 2.5 cm, I.D. 3 mm, particle size 5 µm; Supelco, Belafonte, USA). Separation was performed with solvent A (100 % methanol) and solvent B (40 % methanol, mobile phase), using following elution gradient: 30 % A and 70 % B (0-29 minutes), 90 % A and 10 % B (30-35 minutes), 30 % A and 70 % B (36-40 minutes) while the flow rate was maintained at 0.5 ml/ minutes.

[32]P-postlabeling analysis

To determine the BPDE induced DNA-adduct formation 10 µg calf thymus DNA (Sigma) was incubated with the S9 mix of lung and liver tissue of 12 week old mice and 1 µM of B[a]P. The DNA was isolated using a phenol-chloroform extraction method and BPDE DNA-adduct levels were determined by the nuclease P1 enrichment technique as described by Reddy and Randerath (25) with the modifications described by Godschalk *et al.* (26). In all

experiments, three BPDE DNA standards with known BPDE DNA-adduct levels (one adduct per 10^6, 10^7 and 10^8 normal nucleotides) were analyzed in parallel for quantification purposes. Adduct spots on the chromatograms were quantified using Phosphor-Imaging technology (Fujifilm FLA-3000, Rotterdam, the Netherlands) using the Aida Imager Analyser (Raytest, Milan, Italy), with a detection limit of approximately 1 adduct per 10^9 nucleotides per individual DNA adduct spot.

Statistical analysis

Statistical analysis was performed with Statistical Package for Social Sciences (SPSS version 17 for Windows, SPSS Inc., Chicago, IL, USA). Nested ANOVA was used to compare the mRNA expression level of phase I and II enzymes in the liver of fetuses. The non-parametric Mann-Whitney test was used to determine the mRNA expression level of phase I and II enzymes in the liver and lung of adults, unconjugated B[a]P metabolites, unmetabolized B[a]P and BPDE DNA-adducts formed after incubation with liver or lung S9 mix of adult mice. To avoid litter effects no more than two adult mice per litter were used for analysis. One-way ANOVA was used to test differences in methylation pattern of SINEB1, SINEB2 and LINE1 between control and quercetin exposed fetuses and adult mice.

Results

Quercetin exposure increased gene expression of phase I and II enzymes in fetuses

To investigate the effects of quercetin exposure on the fetuses' gene expression of phase I and II enzymes, mice fetuses were exposed to quercetin (1 mmol/ kg feed) *via* the maternal diet. Pregnant mice were sacrificed at day 14.5 of gestation. Phase I and II gene expression levels were examined in the liver of the fetuses (n=5 per diet group). In line with the AhR agonistic action of quercetin maternal exposure resulted in a significant increase in expression of both phase I (*Cyp1a1*: $F_{1,3}$: 22.5, P=0.004; *Cyp1b1*: $F_{1,3}$: 76.1, P<0.001; Table 2) and phase II (*Gstp1*: $F_{1,3}$: 5.2, P=0.07; *Nqo1*: $F_{1,3}$: 20.5, P=0.005 and *Ugt1a6*: $F_{1,3}$: 1.8, P=0.004) enzymes, without a statistically significant litter effect. AhR gene expression did not change by quercetin exposure.

Table 2. Relative gene expression profile of phase I and II enzymes in the liver of quercetin exposed fetuses at day 14.5 of gestation

	Control (n=5)	Quercetin (n=5)		
	Mean ± SE	Mean ± SE	Diet	Litter effect
Cyp1a1	1.0 ± 0.4	16.5 ± 3.1	$F_{1,3}$: 22.5; P=0.004	$F_{5,3}$: 0.9; P=0.6
Cyp1b1	1.0 ± 0.4	7.9 ± 1.3	$F_{1,3}$: 76.1; P<0.001	$F_{5,3}$: 0.1; P=1.0
Comt	1.0 ± 0.4	2.5 ± 0.2	$F_{1,3}$: 3.8; P=0.1	$F_{5,3}$: 3.5; P=0.2
Gstp1	1.0 ± 0.4	2.5 ± 0.4	$F_{1,3}$: 5.2; P=0.07	$F_{5,3}$: 3.7; P=0.2
Nqo1	1.0 ± 0.2	2.5 ± 0.3	$F_{1,3}$: 20.5; P=0.005	$F_{5,3}$: 0.5; P=0.7
Ugt1a6	1.0 ± 0.5	3.9 ± 1.0	$F_{1,3}$: 1.8; P=0.004	$F_{5,3}$: 4.3; P=0.6
AhR	1.0 ± 0.4	1.2 ± 0.1	$F_{1,3}$: 0.1; P=0.7	$F_{5,3}$: 9.0 P=0.05

F-value with corresponding degrees of freedom for respectively diet groups and number of litters

Prenatal exposure to quercetin affects gene expression profile of B[a]P metabolizing enzymes in adult liver and lung

As *in utero* exposure to quercetin up-regulated gene expression of phase I and II enzymes, we investigated whether these changes persisted into adulthood. Therefore mice were *in utero* exposed to quercetin (1 mmol/ kg feed) and they were sacrificed at the age of 12 weeks. Basal gene expression of phase I and II enzymes was determined in liver and lung tissue of 5 male and 5 female offspring mice. As shown in Table 3, prenatal exposure to quercetin enhanced gene expression in the liver of female offspring mice of phase I enzymes, *Cyp1b1* (P=0.01) and *Cyp1a1* (P=0.05), involved in B[a]P metabolism in the liver of female offspring mice. However, the gene expression of *AhR* was significantly induced in the liver of males prenatally exposed to quercetin (P=0.009). On the contrary, the expression of phase II enzymes was not altered in the liver.

In lungs of these mice, no significant changes in phase I or *AhR* gene expression levels were observed (Table 3). On the other hand, female mice prenatally exposed to quercetin showed an increase in expression of the following phase II enzymes in their lung: *Gstp1* (P=0.009), *Nqo1* (P=0.009) and *Ugt1a6* (P<0.05) compared to control females.

Also interesting to see were the significant tissue specific differences in gene expression between male and female mice (Table 4). In case of the liver, *Cyp1b1* (P=0.05), *Gstp1* (P=0.009), *Nqo1* (P=0.009) and *Ugt1a6* (P=0.02) were differentially regulated in control males and females. In the lung, only the expression of *Gstp1* (P<0.05) and *Nqo1* (P=0.009) differed between male and female control mice. Moreover, prenatal exposure to quercetin seemed to counteract these gender differences for almost all genes, with an exception for *Gstp1* in liver (P=0.009) and *Nqo1* (P=0.01) in lung.

Table 3. Relative gene expression of phase I and II enzymes in the liver and lung of 12 week old male and female offspring mice prenatally exposed to quercetin

		Males[1] (n=5)		Females[1] (n=5)	
		Control	Quercetin	Control	Quercetin
Liver	Cyp1a1	1.0 ± 0.3	1.0 ± 0.1	1.0 ± 0.1	1.5 ± 0.2[*]
	Cyp1b1	1.0 ± 0.2	1.9 ± 0.6	1.0 ± 0.3	2.9 ± 0.8[**]
	Comt	1.0 ± 0.1	0.9 ± 0.2	1.0 ± 0.2	1.2 ± 0.3
	Gstp1	1.0 ± 0.4	0.9 ± 0.2	1.0 ± 0.2	1.4 ± 0.4
	Nqo1	1.0 ± 0.1	4.5 ± 2.9	1.0 ± 0.5	2.4 ± 1.0
	Ugt1a6	1.0 ± 0.2	1.0 ± 0.3	1.0 ± 0.2	1.0 ± 0.4
	AhR	1.0 ± 0.1	2.6 ± 0.4[**]	1.0 ± 0.3	1.5 ± 0.4
Lung	Cyp1a1	1.0 ± 0.5	0.9 ± 0.3	1.0 ± 0.2	0.9 ± 0.1
	Cyp1b1	1.0 ± 0.2	1.2 ± 0.5	1.0 ± 0.4	1.4 ± 0.2
	Comt	1.0 ± 0.3	1.5 ± 0.2	1.0 ± 0.1	1.2 ± 0.2
	Gstp1	1.0 ± 0.1	1.4 ± 0.2	1.0 ± 0.1	2.2 ± 0.3[**]
	Nqo1	1.0 ± 0.7	1.6 ± 0.6	1.0 ± 0.2	11.3 ± 3.8[**]
	Ugt1a6	1.0 ± 0.3	1.6 ± 0.6	1.0 ± 0.1	2.3 ± 0.4[*]
	AhR	1.0 ± 0.4	1.9 ± 0.6	1.0 ± 0.6	1.0 ± 0.2

[1] Control male and female mice were used as reference sample to calculate ΔΔCT for respectively males and females. Results represent mean ± SE. Significant differences between diets: *: P<0.05, **: P≤0.01

Prenatal exposure to quercetin results in long-lasting epigenetic modifications of repetitive elements in liver of female mice prenatally exposed to quercetin

As gene expression of phase I and phase II enzymes were altered by prenatal exposure to quercetin and were maintained till adulthood, we also investigated whether exposure to quercetin could affect the methylation status of the repetitive elements SINEB1, SINEB2 and

LINE1. SINE and LINE repetitive elements are normally expressed during early embryogenesis, after which their expression rapidly decreases with development. Later in life, expression of SINEB1, SINEB2 and LINE1 is associated with cell stress, for instance by DNA-damaging agents (27, 28). At gestational day 14.5, no changes in methylation of repetitive elements were detected in fetuses exposed to quercetin (Figure 1A), suggesting that quercetin exposure did not influence methylation status at that point in gestation.

Although no differences in global methylation status could be detected in the liver at gestational day 14.5, SINEB1 in the liver of adult female mice prenatally exposed to quercetin was hypomethylated compared to control female mice (P=0.01, Figure 1B). DNA methylation of SINEB2 and LINE1 repetitive elements was also decreased in female mice prenatally exposed to quercetin, though not significantly when analysed seperately. Combined analysis of all three repetitive sequences also indicated that these were hypomethylated in liver of female offspring that received quercetin *in utero*, with borderline significance (P=0.06). No alterations in methylation were observed for male liver or lung of both genders (Figure 1B and 1C). These data suggests that *in utero* exposure to quercetin results in the hypomethylation of the repetitive elements SINEB1, SINEB2 and LINE1 in the liver of adult female mice.

Prenatal exposure to quercetin diminished the B[a]P-induced DNA-adduct formation ex vivo using liver microsomes of adult mice

Prenatal exposure to quercetin resulted in long-lasting alterations in the gene expression profile of enzymes involved in B[a]P metabolism in liver and lung of mainly female offspring. However, as mRNA expression levels do not necessarily reflect enzyme activity, S9 mix from liver or lung tissue of 5 male and 5 female mice prenatally exposed to quercetin were incubated *ex vivo* with B[a]P. S9 mix of liver of mice prenatally exposed to quercetin did not show an altered B[a]P metabolism (Table 5). On the other hand, lung S9 mix of mice prenatally exposed to quercetin resulted in decreased amounts of B[a]P-9,10-dihydrodiol (9,10-diOH-B[a]P, P=0.007) and B[a]P-7,8-dihydrodiol (7,8–diOH-B[a]P, P=0.05). The amount of 9,10-diOH-B[a]P was especially decreased in case of S9 mix derived from male mice prenatally exposed to quercetin compared to control males (P=0.03). In addition, the total amount of unmetabolized B[a]P was significantly lower (P=0.01) when using lung S9 mix of mice prenatally exposed to quercetin compared to the control group, especially in males (P<0.05).

Despite the clear differences in gene expression between male and female mice, no gender differences were observed for B[a]P metabolism, except maybe for the liver S9 mix where 9,10-diOH-B[a]P levels were higher for control males compared to their female littermates, but this did not reach statistical significance (P=0.09). The overall profile of metabolites strongly differed between lung and liver S9 mix; using S9 mix obtained from the lung, the major metabolite was 3-hydroxy-B[a]P (3-OH-B[a]P), which is considered to be a detoxification product of B[a]P. No B[a]P DNA-adducts could be detected in calf thymus DNA that was added to the S9 mix obtained from lung tissue, regardless of the prenatal diet (Table 5). When using liver derived S9 mix, prenatal quercetin exposure resulted in a significant decrease in DNA-adduct formation (P=0.04), with no differences between genders.
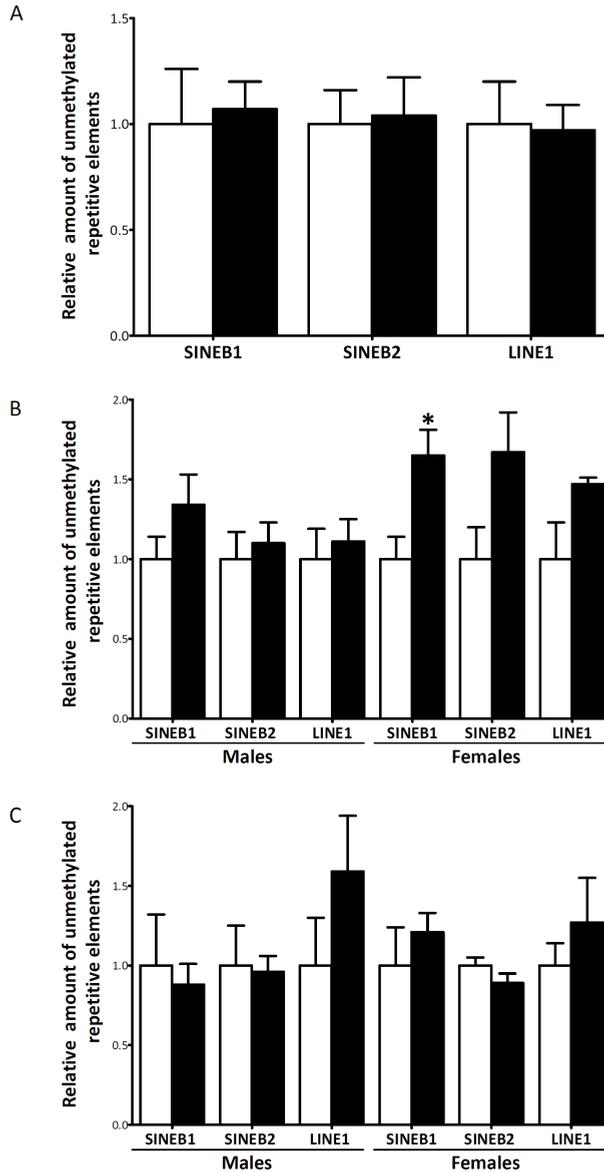
Figure 1. Methylation status of SINEB1, SINEB2 and LINE1 repetitive elements in fetal and adult liver and lung after (prenatal) exposure to quercetin

Short-term effects of prenatal exposure to quercetin on DNA methylation of SINEB1, SINEB2 and LINE1 repetitive elements in fetal liver of quercetin exposed fetuses (n=4, black bars) compared to control fetuses (n=4, white bars) at day 14.5 of gestation (A). Long-term effects of prenatal exposure to quercetin on DNA methylation of SINEB1, SINEB2 and LINE1 repetitive elements in liver (B) and lung (C) of male and female mice prenatally exposed to quercetin (n=5 for each gender, black bars) compared with control mice (n=5 for each gender, white bars). The degree of methylation of SINEB1, SINEB2 and LINE1 repetitive elements were determined by methylation-sensitive McrBC-real-time PCR assay. Bars represent the normalized average expression level of the unmethylated repetitive elements. Error bars represent standard error. Statistical significance was determined by One-way ANOVA: *: $P<0.05$.

Table 4. Gender effect on phase I and II gene expression in the liver and lung of 12 week old mice

		Control		Quercetin			Control		Quercetin	
		Males[1] (n=5)	Females[1] (n=5)	Males[1] (n=5)	Females[1] (n=5)		Males[1] (n=5)	Females[1] (n=5)	Males[1] (n=5)	Females[1] (n=5)
Cyp1a1	liver	1.0 ± 0.3	0.9 ± 0.1	1.0 ± 0.1	1.4 ± 0.2	Lung	1.0 ± 0.5	1.1 ± 0.2	0.9 ± 0.3	0.9 ± 0.1
Cyp1b1		1.0 ± 0.2	0.5 ± 0.1*	1.9 ± 0.6	1.2 ± 0.3		1.0 ± 0.2	0.6 ± 0.2	1.2 ± 0.5	0.7 ± 0.1
Comt		1.0 ± 0.1	0.8 ± 0.1	0.9 ± 0.2	0.8 ± 0.2		1.0 ± 0.3	0.9 ± 0.1	1.5 ± 0.2	1.0 ± 0.3
Gstp1		1.0 ± 0.4	0.1 ± 0.02**	0.9 ± 0.2	0.1 ± 0.04**		1.0 ± 0.1	0.8 ± 0.1*	1.4 ± 0.2	1.8 ± 0.3
Nqo1		1.0 ± 0.1	3.3 ± 1.4**	4.5 ± 2.9	6.2 ± 2.6		1.0 ± 0.7	0.3 ± 0.1**	1.6 ± 0.6	7.3 ± 1.8**
Ugt1a6		1.0 ± 0.2	2.1 ± 0.3*	1.0 ± 0.3	2.1 ± 0.7		1.0 ± 0.3	0.8 ± 0.1	1.6 ± 0.7	2.0 ± 0.2
AhR		1.0 ± 0.01	1.2 ± 0.3	2.6 ± 0.4	1.7 ± 0.5		1.0 ± 0.4	3.4 ± 1.7	1.9 ± 0.6	2.2 ± 1.0

[1] Control male mice were used as reference sample to calculate ΔΔCT for males and females. Results represent mean ± SE. Significant differences between gender: *: P<0.05, **: P≤0.01.

Table 5. B[a]P metabolism by liver and lung S9 mix of 12 week old male and female offspring mice prenatally exposed to quercetin and resulting BPDE-adducts

	Mean ± SE	Overall (n=10)		Males (n=5)		Females (n=5)	
		Control	Quercetin	Control	Quercetin	Control	Quercetin
Liver	9,10-diOH-B[a]P (ng/ mL)	12.4 ± 3.0	8.6 ± 1.4	17.3 ± 3.5[a]	10.6 ± 2.2	6.2 ± 3.2[a]	6.7 ± 1.6
	7,8-diOH-B[a]P (ng/ mL)	1.0 ± 0.2	0.8 ± 0.1	1.2 ± 0.2	0.8 ± 0.1	0.8 ± 0.4	0.8 ± 0.2
	3-OH-B[a]P (ng/ mL)	2.7 ± 0.4	2.6 ± 0.3	2.3 ± 0.7	2.4 ± 0.2	3.1 ± 0.6	2.7 ± 0.7
	Unmetabolized B[a]P (AUC)	359.7 ± 35.0	375.3 ± 15.6	339.3 ± 64.1	389.5 ± 8.1	380.1 ± 34.5	361.2 ± 30.4
	BPDE DNA-adducts/ 10^8 nucleotides	33.4 ± 7.4	17.0 ± 3.3*	28.6 ± 3.7	13.1 ± 3.7	36.2 ± 11.9	20.1 ± 5.1
Lung	9,10-diOH-B[a]P (ng/ mL)	0.5 ± 0.04	0.3 ± 0.03**	0.5 ± 0.03	0.3 ± 0.06*	0.5 ± 0.07	0.3 ± 0.04
	7,8-diOH-B[a]P (ng/ mL)	0.2 ± 0.04	0.1 ± 0.02*	0.3 ± 0.06	0.1 ± 0.03	0.2 ± 0.04	0.1 ± 0.02
	3-OH-B[a]P (ng/ mL)	3.1 ± 0.4	3.1 ± 0.8	3.0 ± 1.5	3.3 ± 1.5	3.4 ± 0.7	2.9 ± 0.9
	Unmetabolized B[a]P (AUC)	148.9 ± 14	81.9 ± 14.8**	154.3 ± 26.3	65.9 ± 17.8*	143.4 ± 13.1	98.0 ± 23.3
	BPDE DNA-adducts/ 10^8 nucleotides	nd	nd	nd	nd	nd	nd

Results represent mean ± SE. Significant differences between diets: *: P≤0.05, **: P≤0.01. Significant differences between gender within diet: [a]: P=0.09. AUC= area under the curver. Nd= not detected.

Discussion

As genetic polymorphisms (1-4) do not entirely explain the inter-individual differences in xenobiotic metabolism, we investigated whether prenatal exposure to the natural flavonoid quercetin, which is a potent AhR ligand, could permanently alter the expression of enzymes involved in both activation as well as detoxification of B[a]P.

We found that *in utero* exposure to quercetin affected the gene expression of phase I and II enzymes of the fetal liver at gestational day 14.5. Gene expression of *Cyp1a1* and *Cyp1b1* was significantly increased, indicating that quercetin or its metabolites functioned as an AhR ligand in the fetus. However, these alterations in gene expression could also have other reasons. For instance, Santini *et al.* (29) showed that exposure of swine granulose cells *in vitro* to a low dose of quercetin resulted in increased levels of 17β-estradiol. As 17β-estradiol is a substrate for CYP1B1 and 1A1 (30) and because fetuses, both males and females, are continuously exposed to 17β-estradiol throughout gestation *via* the mother (31) this route of exposure could additionally increase the gene expression of phase I enzymes. Moreover, as CYP's are hemeproteins that contain iron (32), the iron scavenging properties of quercetin (7) could result in less CYP activity, therefore enhanced gene expression is needed as compensation. Gene expression of the phase II enzymes *Ugt1a6* and *Nqo1* was also significantly enhanced in the liver of quercetin exposed fetuses. This increase could theoretically be the result of an increase in CYP activity, detoxifying the reactive compounds formed by the phase I reactions. Regardless of the underlying mechanism, from our experiments it is clear that supplementation with quercetin altered gene expression *in utero*.

According to the hypothesis of 'fetal programming' impaired maternal nutrition during pregnancy results in fetal adaptations that are meant to improve survival. However, when these adaptations are permanent, they could also alter the risk for diseases later in life. In this study, we investigated whether the changes in phase I and II gene expression due to the *in utero* environment are maintained throughout life. Indeed, 12 week old female mice prenatally exposed to quercetin had increased phase I (*Cyp1a1* and *Cyp1b1*) gene expression levels in their liver, while the phase II enzymes (*Gstp1*, Nqo1 and *Ugt1a6*) were predominantly enhanced in lung tissue of these female mice. Interestingly, *AhR* gene expression was only increased in the liver of male mice prenatally exposed to quercetin.

The assumed AhR-induced up-regulation of Cyp450s seen in the liver due to prenatal exposure to quercetin did not occur in the lung. Since the lung develops mainly in the final stages of pregnancy and even continues to develop after birth (33), while the liver is largely differentiated before birth (34), the different responses of both tissues to quercetin exposure could be due to the difference in maturation state of the organ during exposure. The enhanced expression of phase II enzymes in mice prenatally exposed to quercetin could be the result of the pro-oxidant capacity of quercetin (35, 36), resulting in the up-regulation of phase II enzymes through the Nrf2-pathway (9), which could then be maintained throughout life.

In the control group significant differences were observed between male and female offspring regarding the gene expression of certain phase I and II enzymes both in liver as well as lung. This has already been reported for several species (37-40). However, prenatal exposure to quercetin seemed to reduce this gender difference. Only for *Gstp1* (liver) and *Nqo1* (lung) the gender effect was persistent. Makaji *et al.* (11) also reported that prenatal

intake of flavonoids only resulted in long-term alterations of CYP activity in female rat offspring, by making the CYP profile more masculine, as was also observed in our study. They suggested that quercetin could influence phase I and II gene expression by regulating constitutive androstene receptor or Ah receptor. They also suggested that the gender differences in mRNA expression of phase I enzymes seen in adult animals is imprinted by the presence (or absence) of increased androgen levels at puberty.

We suspected that epigenetic modulations underlie the persistent changes in phase I and II gene expression. Therefore the potential of quercetin to alter the epigenome was assessed and showed that prenatal quercetin exposure resulted in the hypomethylation of repetitive elements in liver of adult female mice. Interestingly, most pronounced changes in gene expression were observed in liver and lung of female mice prenatally exposed to quercetin. However, changes in DNA methylation of repetitive elements were only seen in liver. As we did not measure gene specific DNA methylation levels, we can only assume that *in utero* exposure to quercetin resulted in gene specific hypomethylation for the genes or their regulators that were up-regulated in liver and lung tissue of male and female mice prenatally exposed to quercetin. Since these epigenetic modifications could not be detected in fetuses at day 14.5 of gestation, we suggest that they occurred at a later time point in pregnancy or even after birth. This is plausible as during the late gestational and early postnatal period, tissue maturation results in most epigenetic modifications. Moreover, it has been suggested that transcriptional inactivity attracts *de novo* methylation while transcriptional activity can eventually override DNA methylation (41).

To study the effects of maternal quercetin intake during gestation on B[a]P metabolism by adult liver and lung, we used S9 mix of lungs and livers of animals that did not receive B[a]P at adult age. In that case, analysis of *ex vivo* B[a]P metabolism using S9 mix is more straightforward, because there is no bias by for instance B[a]P uptake and kinetics, DNA repair and altered cell turnover. The identified metabolites formed using liver and lung S9 mix did not differ between control and quercetin exposed animals. However, due to the limited number of metabolites detected, false representation of the B[a]P metabolite profile can not be excluded. Although there were no differences in the amount of unmetabolized B[a]P for liver S9 mix after the one hour incubation, the BPDE DNA-adduct levels were lower for mice prenatally exposed to quercetin. This suggests that prenatal exposure to quercetin did not result in an accelerated metabolism of B[a]P (as seen in lung), but in B[a]P detoxification, e.g. less formation of DNA reactive metabolites including BPDE.

No BPDE DNA-adducts could be detected for the lung S9 mix of both control and quercetin exposed mice, indicating that the B[a]P-adduct forming ability of the lung is lower compared to the liver. Moreover, for the lung less unmetabolized B[a]P was detected after incubation, indicating that the conversion of B[a]P to water soluble derivatives was significantly increased in offspring that received quercetin during gestation. This suggested that more detoxification of B[a]P can take place in the lung of mice prenatally exposed to quercetin as a result of the adapted gene expression of phase II enzymes, which is an important finding as the lung is the primary target organ for airborne B[a]P (42). This finding therefore indicates that prenatal exposure to quercetin could decrease the risk on B[a]P induced DNA damage and therefore the susceptibility to lung cancer.

These results show that prenatal exposure to the natural AhR agonist quercetin leads to tissue and gender specific long-lasting alterations in the gene expression of phase I and II enzymes of adult mice, probably by epigenetic modifications. This seems to result in

increased protection against B[a]P-induced DNA-adduct formation. However, as gene expression levels of phase I and phase II enzymes do not reflect actual enzyme activities, and because B[a]P metabolism and BPDE DNA-adduct formation experiments occurred *ex vivo*, it is now warranted to continue with similar studies with *in vivo* exposure to B[a]P of adult mice prenatally exposed to quercetin to confirm our theory.

Acknowledgements

References

1. Cabral, R. E., Caldeira-de-Araujo, A., Cabral-Neto, J. B., and Costa Carvalho Mda, G. (2010) Analysis of GSTM1 and GSTT1 polymorphisms in circulating plasma DNA of lung cancer patients. *Mol Cell Biochem* 338, 263-269

2. Rojas, M., Cascorbi, I., Alexandrov, K., Kriek, E., Auburtin, G., Mayer, L., Kopp-Schneider, A., Roots, I., and Bartsch, H. (2000) Modulation of benzo[a]pyrene diolepoxide-DNA adduct levels in human white blood cells by CYP1A1, GSTM1 and GSTT1 polymorphism. *Carcinogenesis* 21, 35-41

3. Wang, S., Chanock, S., Tang, D., Li, Z., Jedrychowski, W., and Perera, F. P. (2008) Assessment of interactions between PAH exposure and genetic polymorphisms on PAH-DNA adducts in African American, Dominican, and Caucasian mothers and newborns. *Cancer Epidemiol Biomarkers Prev* 17, 405-413

4. Pande, M., Amos, C. I., Eng, C., and Frazier, M. L. (2010) Interactions between cigarette smoking and selected polymorphisms in xenobiotic metabolizing enzymes in risk for colorectal cancer: A case-only analysis. *Mol Carcinog* 49, 974-980

5. Doll, R., and Peto, R. (1981) The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J Natl Cancer Inst* 66, 1191-1308

6. Ross, J. A., and Kasum, C. M. (2002) Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annu Rev Nutr* 22, 19-34

7. Ferrali, M., Signorini, C., Caciotti, B., Sugherini, L., Ciccoli, L., Giachetti, D., and Comporti, M. (1997) Protection against oxidative damage of erythrocyte membrane by the flavonoid quercetin and its relation to iron chelating activity. *FEBS Lett* 416, 123-129

8. Kitagawa, S., Sakamoto, H., and Tano, H. (2004) Inhibitory effects of flavonoids on free radical-induced hemolysis and their oxidative effects on hemoglobin. *Chem Pharm Bull (Tokyo)* 52, 999-1001

9. Moon, Y. J., Wang, X., and Morris, M. E. (2006) Dietary flavonoids: effects on xenobiotic and carcinogen metabolism. *Toxicol In Vitro* 20, 187-210

10. Ciolino, H. P., Daschner, P. J., and Yeh, G. C. (1999) Dietary flavonols quercetin and kaempferol are ligands of the aryl hydrocarbon receptor that affect CYP1A1 transcription differentially. *Biochem J* 340 (Pt 3), 715-722

11. Makaji, E., Ho, S. H., Holloway, A. C., and Crankshaw, D. J. (2011) Effects in rats of maternal exposure to raspberry leaf and its constituents on the activity of cytochrome p450 enzymes in the offspring. *Int J Toxicol* 30, 216-224

12. Gelboin, H. V. (1980) Benzo[alpha]pyrene metabolism, activation and carcinogenesis: role and regulation of mixed-function oxidases and related enzymes. *Physiol Rev* 60, 1107-1166

13. Kim, J. H., Stansbury, K. H., Walker, N. J., Trush, M. A., Strickland, P. T., and Sutter, T. R. (1998) Metabolism of benzo[a]pyrene and benzo[a]pyrene-7,8-diol by human cytochrome P450 1B1. *Carcinogenesis* 19, 1847-1853

14. Long, D. J., 2nd, and Jaiswal, A. K. (2000) NRH:quinone oxidoreductase2 (NQO2). *Chem Biol Interact* 129, 99-112

15. Uno, S., Dalton, T. P., Derkenne, S., Curran, C. P., Miller, M. L., Shertzer, H. G., and Nebert, D. W. (2004) Oral exposure to benzo[a]pyrene in the mouse: detoxication by inducible cytochrome P450 is more important than metabolic activation. *Mol Pharmacol* 65, 1225-1237

16. Singhal, R., Badger, T. M., and Ronis, M. J. (2008) Rats fed soy protein isolate (SPI) have impaired hepatic CYP1A1 induction by polycyclic aromatic hydrocarbons as a result of interference with aryl hydrocarbon receptor signaling. *Toxicol Appl Pharmacol* 227, 275-283

17. Kang, Z. C., Tsai, S. J., and Lee, H. (1999) Quercetin inhibits benzo[a]pyrene-induced DNA adducts in human Hep G2 cells by altering cytochrome P-450 1A1 gene expression. *Nutr Cancer* 35, 175-179

18. Sergent, T., Dupont, I., Van der Heiden, E., Scippo, M. L., Pussemier, L., Larondelle, Y., and Schneider, Y. J. (2009) CYP1A1 and CYP3A4 modulation by dietary flavonoids in human intestinal Caco-2 cells. *Toxicol Lett* 191, 216-222

19. Delgado, M. E., Haza, A. I., Arranz, N., Garcia, A., and Morales, P. (2008) Dietary polyphenols protect against N-nitrosamines and benzo(a)pyrene-induced DNA damage (strand breaks and oxidized purines/pyrimidines) in HepG2 human hepatoma cells. *Eur J Nutr* 47, 479-490

20. Walle, T., and Walle, U. K. (2007) Novel methoxylated flavone inhibitors of cytochrome P450 1B1 in SCC-9 human oral cancer cells. *J Pharm Pharmacol* 59, 857-862

21. Vanhees, K., Coort, S., Ruijters, E. J., Godschalk, R. W., van Schooten, F. J., and Barjesteh van Waalwijk van Doorn-Khosrovani, S. (2011) Epigenetics: prenatal exposure to genistein leaves a permanent signature on the hematopoietic lineage. *FASEB J* 25, 797-807

22. Dolinoy, D. C., Weidman, J. R., Waterland, R. A., and Jirtle, R. L. (2006) Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. *Environ Health Perspect* 114, 567-572

23. Abbott, B. D., Birnbaum, L. S., and Perdew, G. H. (1995) Developmental expression of two members of a new class of transcription factors: I. Expression of aryl hydrocarbon receptor in the C57BL/6N mouse embryo. *Dev Dyn* 204, 133-143

24. Pogribny, I. P., Ross, S. A., Tryndyak, V. P., Pogribna, M., Poirier, L. A., and Karpinets, T. V. (2006) Histone H3 lysine 9 and H4 lysine 20 trimethylation and the expression of Suv4-20h2 and Suv-39h1 histone methyltransferases in hepatocarcinogenesis induced by methyl deficiency in rats. *Carcinogenesis* 27, 1180-1186

25. Reddy, M. V., and Randerath, K. (1986) Nuclease P1-mediated enhancement of sensitivity of 32P-postlabeling test for structurally diverse DNA adducts. *Carcinogenesis* 7, 1543-1551

26. Godschalk, R. W., Maas, L. M., Van Zandwijk, N., van 't Veer, L. J., Breedijk, A., Borm, P. J., Verhaert, J., Kleinjans, J. C., and van Schooten, F. J. (1998) Differences in aromatic-DNA adduct levels between alveolar macrophages and subpopulations of white blood cells from smokers. *Carcinogenesis* 19, 819-825

27. Kramerov, D. A., and Vassetzky, N. S. (2005) Short retroposons in eukaryotic genomes. *Int Rev Cytol* 247, 165-221

28. Stribinskis, V., and Ramos, K. S. (2006) Activation of human long interspersed nuclear element 1 retrotransposition by benzo(a)pyrene, an ubiquitous environmental carcinogen. *Cancer Res* 66, 2616-2620

29. Santini, S. E., Basini, G., Bussolati, S., and Grasselli, F. (2009) The phytoestrogen quercetin impairs steroidogenesis and angiogenesis in swine granulosa cells in vitro. *J Biomed Biotechnol* 2009, 419891

30. Hanna, I. H., Dawling, S., Roodi, N., Guengerich, F. P., and Parl, F. F. (2000) Cytochrome P450 1B1 (CYP1B1) pharmacogenetics: association of polymorphisms with functional differences in estrogen hydroxylation activity. *Cancer Res* 60, 3440-3444

31. Delbes, G., Levacher, C., and Habert, R. (2006) Estrogen effects on fetal and neonatal testicular development. *Reproduction* 132, 527-538

32. Nelson, D. R., Koymans, L., Kamataki, T., Stegeman, J. J., Feyereisen, R., Waxman, D. J., Waterman, M. R., Gotoh, O., Coon, M. J., Estabrook, R. W., Gunsalus, I. C., and Nebert, D. W. (1996) P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* 6, 1-42

33. Ten Have-Opbroek, A. A. (1991) Lung development in the mouse embryo. *Exp Lung Res* 17, 111-130

34. Zaret, K. S. (2002) Regulatory phases of early liver development: paradigms of organogenesis. *Nat Rev Genet* 3, 499-512

35. Boots, A. W., Li, H., Schins, R. P., Duffin, R., Heemskerk, J. W., Bast, A., and Haenen, G. R. (2007) The quercetin paradox. *Toxicol Appl Pharmacol* 222, 89-96

36. Choi, E. J., Chee, K. M., and Lee, B. H. (2003) Anti- and prooxidant effects of chronic quercetin administration in rats. *Eur J Pharmacol* 482, 281-285

37. Delaunois, A., Florquin, S., Segura, P., Montano, L. M., Vargas, M. H., and Gustin, P. (1999) Interactions between cytochrome P-450 activities and ozone-induced modulatory effects on endothelial permeability in rabbit lungs: influence of gender. *Inhal Toxicol* 11, 999-1014

38. Scandlyn, M. J., Stuart, E. C., and Rosengren, R. J. (2008) Sex-specific differences in CYP450 isoforms in humans. *Expert Opin Drug Metab Toxicol* 4, 413-424

39. Timofeeva, M. N., Kropp, S., Sauter, W., Beckmann, L., Rosenberger, A., Illig, T., Jager, B., Mittelstrass, K., Dienemann, H., Bartsch, H., Bickeboller, H., Chang-Claude, J. C., Risch, A., and Wichmann, H. E. (2009) CYP450 polymorphisms as risk factors for early-onset lung cancer: gender-specific differences. *Carcinogenesis* 30, 1161-1169

40. Lofgren, S., Hagbjork, A. L., Ekman, S., Fransson-Steen, R., and Terelius, Y. (2004) Metabolism of human cytochrome P450 marker substrates in mouse: a strain and gender comparison. *Xenobiotica* 34, 811-834

41. Waterland, R. A., Kellermayer, R., Rached, M. T., Tatevian, N., Gomes, M. V., Zhang, J., Zhang, L., Chakravarty, A., Zhu, W., Laritsky, E., Zhang, W., Wang, X., and Shen, L. (2009) Epigenomic profiling indicates a role for DNA methylation in early postnatal liver development. *Hum Mol Genet* 18, 3026-3038

42. Uppstad, H., Ovrebo, S., Haugen, A., and Mollerup, S. (2010) Importance of CYP1A1 and CYP1B1 in bioactivation of benzo[a]pyrene in human lung cell lines. *Toxicol Lett* 192, 221-228

Chapter 7

Summary & General discussion

Adult chronic illnesses, such as cancer, cardiovascular and respiratory diseases are a major burden on human health worldwide (1). Exposure to genotoxins and mutagens are thought to play an important role in the etiology of these diseases (2-6). On the other hand, a healthy diet is though to protect against genotoxins and mutagens and would therefore also protect against these chronic diseases to maintain health. One major dietary group receiving a lot of attention regarding the treatment and prevention of adult chronic diseases are the flavonoids, because of their potent antioxidant capacity (7-10). They comprise a large group of polyphenolic compounds widely distributed in the human diet (9), resulting in an average intake from 65 to 250 mg of mixed flavonoids a day (11). The most predominant flavonoid in the human diet is quercetin, which is present in for instance onions, apples, tea and red wine (9, 12-14). Besides being a potent free radical scavenger, it also chelates iron (15, 16). Genistein is another important flavonoid, with antioxidant properties and is mainly found in soybeans. However, it also has estrogen-like properties due to its structural similarities to 17β-estradiol. Whether it works as an estrogen agonist or antagonist depends on the situation (17, 18).

Due to the health benefits they are presumed to provide, flavonoids are freely available as high dose supplements, with a recommended daily dose ranging between 1-2 gram (19, 20). Additionally, disturbingly is the increase in supplement consumption of woman during pregnancy. In the US 78 % of the pregnant woman take dietary supplements (21), which includes the intake of folic acid. However, folic acid supplementation in adults has been shown to increase the risk on cancer (22). Yet little is known about the biological actions of flavonoids in pregnancy and the short-term and/ or long-term effects they exert on the offspring's health. It is known that flavonoids can cross the placenta and may accumulate in the fetus (23, 24). Therefore research is needed to assess the safety of intake of flavonoid supplements during pregnancy.

Flavonoids during pregnancy: Infant leukemia

Excessive intake of flavonoids could play a role in the onset of infant leukemia as they are efficient topoisomerase II inhibitors (25, 26). Flavonoids could therefore induce DNA double-strand breaks (DSB) and rearrangements in the mixed-lineage leukemia (*MLL*) gene, which is frequently observed in childhood leukemia (26, 27). In Chapter 2 we mated *Atm*-ΔSRI heterozygote mice to obtain pups with a different genetic capacity for the DNA repair of DSBs, namely *Atm* wild-type, *Atm*-ΔSRI heterozygote and *Atm*-ΔSRI mutant offspring mice and found that prenatal exposure to flavonoids indeed induced *Mll* translocations in the bone marrow of both wild-type and *Atm*-ΔSRI mutant mice. Moreover, *Atm*-ΔSRI mutant mice did show a higher susceptibility to develop these chromosomal aberrations. We also showed that mice with an impaired DNA repair had an increased risk on developing other malignancies when prenatally exposed to genistein besides an increased risk on infant leukemia, which indicates that the DSB DNA repair pathway is relevant for maintaining genomic integrity in the developing fetus.

The *Atm*-ΔSRI mutation corresponds to one of the most common *Atm* mutations found to date in humans and these mice have a phenotype which is distinct from *Atm* knock-out mice. It is known that *Atm*-ΔSRI mutant mice have a 50 % chance of developing leukemia and lymphoma without additional exposures (28). The age of tumor onset was

decreased dramatically by prenatal exposure to genistein and quercetin from an average of 18.6 months (28, 29) to only 3 months in our study.

Although the number of animals in which leukemia could actually be identified was low in our study, analysis of *Mll* translocations established in offspring mice prenatally exposed to both flavonoids, suggested that the risk of developing cancer later in life for these animals was elevated.

Flavonoids during pregnancy: Fetal programming

Besides directly increasing cancer susceptibility, flavonoids are also thought to be involved in so-called 'fetal programming'. This concept defines an attempt of the fetus to adapt to adverse conditions encountered *in utero*, which results in adaptations that will be detrimental when these conditions will not prevail later in life (30). A clear example of fetal programming by genistein was given by Dolinoy *et al.* (31), as they showed that prenatal exposure of agouti mice to genistein resulted in a change in coat color, but it also protected the mouse from obesity later in life, which is distinctive for these mice. These effects were caused by epigenetic changes, namely hypermethylation of a repetitive element upstream of the transcription start site of the *Agouti* gene and the *Agouti* gene was silenced as a result of this hypermethylation. Formation of altered DNA methylation patterns in cells during fetal development by flavonoid intake may thus have long-lasting effects, because patterns can be transmitted to daughter cells after cell division

Persistent changes in gene expression

In Chapter 3, the effect of prenatal exposure to genistein on gene expression and global methylation of bone marrow cells of 12 week old male mice was investigated. Prenatal exposure to genistein resulted in a pronounced down-regulation of estrogen responsive genes and of genes involved in inhibiting hematopoiesis, consequently up-regulating erythropoiesis and granulopoiesis in these mice. The effect of genistein on estrogen responsive genes was to some extent expected as genistein has structural similarities with 17β-estradiol, exerting its effect through the estrogen receptor (18). In addition, the effect of genistein on hematopoiesis was also found by Zhou *et al.* (32) who pretreated mice with genistein for 7 days prior to whole-body irradiation, protecting them from death due to enhanced regeneration of hematopoietic stem cells. We hypothesize that the changes in gene expression were a result of epigenetic mechanisms, because hypermethylation of repetitive elements could be distinguished in the bone marrow cells of these animals.

Oxidative stress related processes

Since flavonoids are well known for their antioxidant properties (9) and they can cross the placenta to reach the developing fetus, we investigated whether prenatal exposure to genistein and quercetin could also have long-lasting effects on systems that contribute to the resistance to oxidative stress, which is involved in the onset of cancer (33). To this end,

the antioxidant capacity of liver and lung tissue of adult male mice prenatally exposed to genistein or quercetin was assessed in Chapter 4. Quercetin exposure during pregnancy resulted in the up-regulation of genes involved in the enzymatic antioxidant defense system of the liver of fetuses at day 14.5 of gestation. Especially *Nrf2*, which functions as an important transcription factor responding to oxidative stress, showed increased gene expression levels during quercetin exposure. This indicated that despite quercetin's antioxidant property, it probably exerted pro-oxidant effects during *in utero* exposure. Pro-oxidant effects have previously also been ascribed to quercetin (34, 35). Genistein exposure on the contrary, had no effect on the enzymatic antioxidant defense system at this time point in gestation. This could be the result of a less pro-oxidant or even antioxidant effect of genistein. However, it has also been reported for genistein that in case of Nrf2 genistein impedes its nuclear export and degradation (36), making the up-regulation in gene expression unnecessary.

However, at adult age, mice prenatally exposed to either genistein or quercetin still had a differential expression of genes involved in the enzymatic antioxidant defense in their liver. This suggests that the adaptations made by the fetus in response to the *in utero* conditions by the increased supply of genistein or quercetin to the mother are maintained throughout life. We also investigated the effect of prenatal exposure to genistein or quercetin on the non-enzymatic antioxidant defense system and could only find an effect of prenatal exposure to genistein in the lung compared to control animals. Oxidative stress induced DNA damage, namely direct oxidizing of the DNA (8-oxo-dG levels) or indirect DNA damage induced by the lipid peroxidation product MDA (M_1dG), was also assessed in the liver and lung of adult male mice prenatally exposed to quercetin or genistein. Prenatal exposure to genistein but especially to quercetin resulted in lower levels of oxidative stress induced DNA damage, however, only in the liver of these animals. As we mainly found changes in the gene expression of enzymatic antioxidants in the liver, it seems that the pre-emptive trigger of the enzymatic antioxidant defense system appeared to be beneficial and protective against oxidative DNA damage in the liver of adult mice prenatally exposed to genistein but especially to quercetin.

This effect was tissue specific as in the lung only modest effects of maternal flavonoid supplementation on gene expression of enzymatic antioxidants were observed and the non-enzymatic antioxidant defense system was only increased due to prenatal genistein exposure. Therefore the minor statistically non significant decrease in 8-oxo-dG levels and M_1dG in the lung of male mice prenatally exposed to either genistein or quercetin was as expected.

This study showed that although genistein and quercetin are mainly known for their antioxidant properties, they probably functioned as pro-oxidants during gestation, as has been reported previously (35, 37-40), probably due to their accumulation in the fetus (23, 24), because antioxidant properties mainly occur at lower concentrations. This study also showed that genistein and quercetin did not exerted the same effects, with regard to long-lasting changes in the enzymatic antioxidant defense system; quercetin was more effective than genistein. Though it had already been shown that exposure to genistein and quercetin during adult life protects against oxidative stress (41, 42), we have now shown that *in utero* exposure also results in protection at adulthood, but it remains to be established whether this beneficial effect outweighs the oxidative stress that is induced *in utero*.

Iron chelating properties of quercetin as programming trigger

Quercetin is known to be a very potent iron chelator (15, 16). Therefore, the effect of prenatal quercetin exposure on iron homeostasis was assessed during fetal development and at adult age in Chapter 5. First, we investigated whether quercetin exposure *in utero* indeed resulted in iron chelation. However, at day 14.5 of gestation, no differences in the total amount of iron measured in amniotic fluid could be detected. Next, since the largest amount of iron is used for the production of hemoglobin (43, 44), gene expression of globin genes was assessed. At day 14.5 of gestation, fetuses are undergoing a switch from the embryonic hemoglobin, solely produced by primitive erythrocytes, to the adult hemoglobin, produced by primitive and definitive erythrocytes (45, 46). The main difference between both forms of hemoglobin is their affinity for oxygen. During early development, oxygen levels are low due to limited vascularisation to ensure proper organogenesis, because at this time point the fetus is the most vulnerable for oxidative stress, which could lead to damage or disruption of the fetus (47, 48). Nevertheless, the fetus needs to subtract oxygen from the maternal blood. Therefore, in the early phase of pregnancy the fetus produces embryonic hemoglobin as it has a higher affinity for oxygen compared to adult hemoglobin present in the maternal blood (49, 50). However, once the utero-placental circulation is well established (between day 9.5 and 11 of gestation) (47) a switch to the adult hemoglobin occurs due to the increase in oxygen concentration (45, 46, 48). In addition, embryonic hemoglobin is less stable compared to adult hemoglobin (48) making it more prone to oxidation, resulting in the formation of methemoglobin, which is incapable of binding oxygen. Hence, embryonic hemoglobin also releases iron more easily from heme (51). As it has been shown that quercetin exposure results in the formation of methemoglobin by entering red blood cells (RBCs) and oxidizing the iron ion (15, 16), we expected to see an accelerated switch from the fetal to the adult hemoglobin in fetuses exposed to quercetin. Though the results were not significant, we did observe a trend in globin gene expression, namely the decrease in gene expression of embryonic globins in fetuses exposed to quercetin. However, the gene expression of adult globins remained unaltered while the switch from the primitive erythroid lineage towards the definitive erythroid lineage was accelerated due to increased quercetin intake by the mothers. This could indicate that, thought iron levels were not significantly lower quercetin did chelate the iron delivered by the mother, making the amount of iron available for hemoglobin production limited. Moreover, these data also suggested that quercetin chelated the iron from embryonic hemoglobin, which is less stable, accelerating the switch from embryonic to adult hemoglobin. However, the level of adult hemoglobin was not elevated as the globin production is dependent on iron availability (52), which we suspected to be low. However, the accelerated switch in hemoglobin production seems reasonable as the switch in erythroid lineage was also accelerated in these mice, and RBCs from the definitive lineage mainly produce adult hemoglobin.

The amount of iron stored in the liver of adult offspring was much higher in mice prenatally exposed to quercetin compared to control animals. We assume that exposure to quercetin resulted in lower levels of available iron *in utero*, to which the fetus adapted. Once born, fetuses were exposed to normal levels of iron, because the chelating activity of quercetin ceased. However, due to this fetal programming towards a life with lower levels of iron, these levels exceeded the levels they perceived as normal. Therefore, pathways

were activated to cope with this "iron overload". Hence, we found an increased gene expression of several interleukins and hepcidin, all involved in iron storage normally seen with anemia of chronic disease also known as 'anemia of inflammation'. Interestingly, anemia is seen more frequently at advanced age and in people with cancer, where 'anemia of inflammation' accounts for 77 % of the underlying causes of anemia (53). In addition, a decreased gene expression of Steap4, involved in the iron uptake by cells (54), was found in bone marrow cells of mice prenatally exposed to quercetin. We assumed that this measure was taken to protect the bone marrow from excess iron and subsequent DNA damage.

These data suggested that although mice were adapted to an *in utero* situation, with scarcity of iron due to quercetin chelation, they did cope with the increased iron availability later in life. Moreover, they even decreased the risk on iron induced ROS formation, as a decrease in 8-oxo-dG levels was found for the liver of these mice. However, as gene expression of inflammation related pathways were induced in the absence of an inflammatory trigger, one could expect that these mice could react differently if inflammation would be triggered. As inflammation is involved in many chronic diseases, including cancer (33), one can hypothesize that although these animals seemed to be protected against oxidative stress induced diseases, an inflammatory stressor could alter this protective mechanism towards a harmful one, but this needs confirmation.

Quercetin as AhR agonist modifies xenobiotic metabolism

AhR-signaling in the liver is of major importance in determining the capacity to metabolize certain well known carcinogens. It therefore determines an individual's risk to develop cancer after exposure to genotoxic compounds. We further elucidated the potential of quercetin exposure on carcinogen metabolism, more precisely on the metabolism of B[a]P (Chapter 6). B[a]P is know to be a potent mutagen and carcinogen when metabolically activated. This activation is performed by CYP1A1 and CYP1B1 (55, 56), which both have been shown to be modulated by quercetin. Moreover, quercetin can also modulate phase II enzymes (57-59). However, no straightforward effect of quercetin on phase I and II expression has been reported (56, 57, 60-62). Here, we showed that exposure to quercetin induced Cyp1a1 and Cyp1b1 gene expression in mice fetuses at day 14.5 of gestation. This increase could be due to the fact that CYPs are hemoproteins, containing heme-iron (63), which could be chelated by quercetin resulting in less CYP activity that needs to be compensated by an increase in gene expression. Another explanation is the fact that quercetin can increase the 17β-estradiol level, which is a known substrate for Cyp1b1 and Cyp1a1 (64). Moreover, AhR gene expression was not induced in these fetuses. However, as in the liver of developing mice the AhR mRNA and protein expression peaks between gestational day 14 and 16 (65), it may be that the effect of quercetin on AhR gene expression may not be visible at that time point. Furthermore, gene expression of Nqo1, Ugt1a6 and Gstp1 was also induced in fetuses exposed to quercetin. We suspect that this is the result of the activation of the Nrf2-pathway as all three genes are target genes of the transcription factor Nrf2 (66, 67). Indeed, Nrf2 gene expression was up-regulated in these fetuses (Chapter 4).

At 12 weeks of age, mice prenatally exposed to quercetin still had an up-regulation in the expression of Cyp1b1 in their liver, while the up-regulation of the other genes was not maintained throughout life. Interesting to see was that in adult mice, AhR gene

expression was increased, which could at least partly explain the increase in *Cyp1b1* expression. We compared male and female littermates and found that female mice prenatally exposed to quercetin developed a more masculine profile regarding the phase I and II enzymes compared to control female mice. In rats it has also been shown that prenatal exposure to quercetin resulted in long-term alterations of CYP activity, predominantly in the female offspring. Here, the CYP profile of the female offspring also showed to be more masculine compared to control females. It was suggested that quercetin exerted these effects by regulating constitutive androstene receptor or Ah receptor (58). Indeed, *AhR* gene expression was slightly increased in female mice prenatally exposed to quercetin.

Gene expression of phase I and II enzymes demonstrated a different profile in the lung of 12 week old mice prenatally exposed to quercetin. Here phase II enzymes (*Gstp1*, *Nqo1* and *Ugt1a6*) were mainly induced. The difference in tissue response to prenatal quercetin exposure could be due to the fact that the lung develops mainly in the final stages of pregnancy and even continues to develop after birth (68), while the liver is largely differentiated before birth (69). Hence, less gender effects were noticeable in the lung, which is in line with the fact that lung is not a main organ involved in estrogen metabolism. However, in fetuses at gestational day 14.5, increased gene expression of *Nqo1* and *Ugt1a6* was detected and was suggested to be the result of the activation of the Nrf2-pathway, as quercetin can also function as pro-oxidant (Chapter 4). As the lung is directly exposed to oxygen concentrations higher than in most tissues (33), a pre-emptive oxidative stress trigger *in utero* could result in the up-regulation of the Nrf2-pathway and therefore of phase II enzymes. However, as mentioned above, Nrf2-pathway was probably only modestly induced in the lung of these mice (Chapter 4).

Although prenatal exposure to quercetin resulted in an increased expression of phase I and II enzymes in liver and lung, differences in *ex vivo* B[a]P metabolism were only found in the lung of mice prenatally exposed to quercetin as they showed a decrease in the amount of B[a]P-9,10-dihydrodiol and B[a]P-7,8-dihydrodiol. Moreover, mice prenatally exposed to quercetin showed an increase in B[a]P metabolism. However, due to the high detoxification of B[a]P towards 3-hydroxy-B[a]P in all diet groups, no BPDE-induced DNA-adducts could be detected. However, as the lung is the primary target organ for airborne B[a]P the increased B[a]P metabolism and phase II gene expression suggests that prenatal exposure to quercetin could decrease the susceptibility to develop lung cancer *in vivo*.

Surprisingly, in case of the liver the *ex vivo* induction of BPDE DNA-adducts was decreased for mice prenatally exposed to quercetin, although they did not show a change in the rate of B[a]P metabolism or a changed profile of the 3 major B[a]P metabolites, including the 7,8-dihydrodiol of B[a]P which is thought to be the precursor of the ultimate genotoxic metabolite, BPDE. This suggests that prenatal exposure to quercetin results in the protection from B[a]P induced DNA damage in the liver, we suspect due to up-regulation of phase I enzymes. Although CYP1A1 and CYP1B1 are mainly thought to be important for metabolic activation of B[a]P, we suspect that they also contributed to the detoxification of B[a]P, as previously also has been suggested by Uno *et al.* (70).

Fetal programming is regulated *via* epigenetics

The changes in gene expression discussed in Chapter 3-6, as a result of *in utero* exposure to genistein or quercetin, are maintained throughout life. We therefore expected epigenetic mechanisms to be involved. Therefore, the induction of changes in methylation status of repetitive elements due to prenatal exposure to genistein or quercetin was investigated, as the mouse genome constitutes almost for 37.5 % out of these repetitive elements (71). Results showed that that both flavonoids did not induce changes in global methylation (namely, methylation of SINEB1, SINEB2, LINE1, IAP, Major and Minor satellites) in the liver of fetuses at gestational day 14.5 (Chapter 3, 5, 6). However, when investigating methylation patterns in the bone marrow of 12 week old mice, an overall increase in methylation could be distinguished and this especially for SINEB1 and SINEB2 (Chapter 3, 5). Moreover, in liver and lung tissue of 12 week old mice prenatally exposed to genistein or quercetin mild hypomethylation of the repetitive elements SINEB1, SINEB2, and LINE1 occurred (Chapter 6). The fact that no changes in methylation could be distinguished in fetuses at day 14.5 of gestation could suggest that *de novo* methylation induced by flavonoids took place at a later time point in gestation or even after birth. This is plausible as during the late gestational and early postnatal period, tissue maturation can result in epigenetic modifications. Moreover, it has been suggested that transcriptional inactivity attracts *de novo* methylation while transcriptional activity can override DNA methylation, inducing developmental hypomethylation (72). Besides, the level of exposure to genistein and quercetin only increases throughout gestation, enhancing their effects.

Prenatal exposure to genistein has previously been shown to alter gene expression by affecting methylation patterns, namely prenatal exposure of yellow agouti mice altered coat color and body weight of agouti mice by inducing hypermethylation of transposable repetitive elements upstream of the transcription start site of the *Agouti* gene (31). Hence, both genistein and quercetin have been shown to inhibit DNA methyltransferases, inducing hypomethylation, namely of hypermethylated tumor suppressor genes, to which their anticancer effects are ascribed (73, 74). However, prenatal exposure to flavonoids resulted in a tissue dependent effect on methylation, namely hypermethylation in case of the bone marrow and hypomethylation in case of liver and lung tissue, especially of SINE transcripts. During early embryogenesis SINE and LINE repetitive elements are normally expressed, after which their expression rapidly decreases with development. Therefore, SINEB1, SINEB2 and LINE1 expression later in life is associated with cell stress, for instance induced by DNA-damaging agents (75, 76), which we have shown in Chapter 2 quercetin and genistein are capable of due to their topoII inhibiting properties. As SINE and LINE expression are also associated with cancer (75, 76), this could suggest that an *in utero* flavonoid trigger can result in an increased silencing of SINEB1, SINEB2 and LINE1 to protect the genome from any future genomic instability.

Implications and future research

As this is only the beginning of understanding how maternal intake of genistein and quercetin can affect offspring's risk on developing cancer later in life, further elaborate studies need to be performed:

- Concerning the epigenetic mechanisms behind the genistein and quercetin induced fetal programming. Moreover, the critical window of exposure should be assessed.
- Although genistein and quercetin both are flavonoids, they exert different effects. Therefore their specific actions should be further elucidated.
- At 12 weeks of age it seemed that prenatal exposure to genistein and quercetin overall had beneficial effects regarding cancer susceptibility. However, as these mice are relatively young, the true contribution of the adaptations made by the fetus regarding genistein and quercetin exposure *in utero* will not be visible at this age and additional studies should thus include aging mice. Moreover, the effects of fetal programming should also be investigated at a later time point in life, when these mice normally are subjected to adult chronic diseases. These chronic diseases could also be induced by for instance pro-inflammatory triggers.
- It would also be interesting to investigate what the effects of life-long exposure to both flavonoids would be regarding the cancer susceptibility.
- It would also be valuable to study the effects of prenatal exposure to genistein or quercetin on following generations. However, to examine the true transgenerational inheritance, the third generation, the first unexposed generation, needs to be examined, as the first generation is directly exposed to genistein or quercetin and the second generation results from gametes that are exposed to genistein or quercetin (Figure 1).

Mother: parental generation
Intake of macro- and micronutrients

Gametes: **2nd** generation
Exposed to macro- and micronutrients during gametogenesis

Fetus: **1st** generation
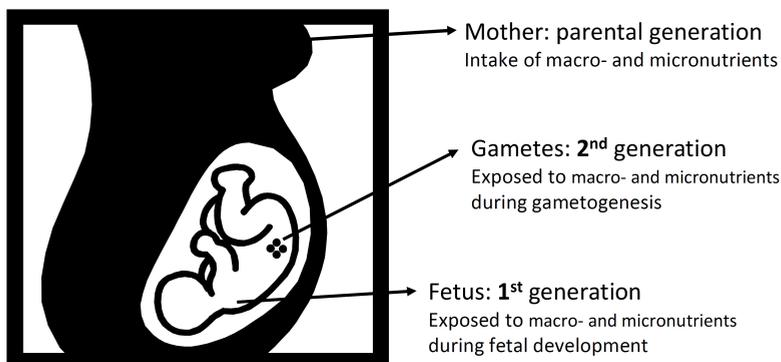Exposed to macro- and micronutrients during fetal development

Figure 1. Overview of transgenerational inheritance of effects induced by genistein or quercetin exposure.

When summarizing the results as described in this thesis it can be concluded that prenatal exposure to quercetin and genistein resulted in altered fetal programming as compared to a non-supplemented diet during pregnancy. During gestation, when fetuses are directly exposed to both flavonoids, changes in expression of genes involved in antioxidant defense and carcinogen metabolism occurred, suggesting that these fetuses adapted to

the *in utero* environment. However, once born genistein and quercetin exposure ceased, but some changes in gene expression were maintained until adult life, while others were gained throughout life, showing that the offspring was trying to cope with its new 'unexpected' environment.

At the age of 12 weeks, prenatal exposure to genistein and quercetin seemed to be beneficial as these mice showed to have less oxidative stress induced DNA damage as a result of increased enzymatic antioxidant defense system and more efficient iron storage. It also seemed that they could better cope with B[a]P induced DNA-damage due to the up-regulation of phase I and II enzymes. Moreover, the increased methylation in certain repetitive elements suggested that these animals were more protected from genomic instability, overall suggesting they were less susceptible to develop cancer. On the other hand, prenatal exposure to genistein and quercetin induced *MII* translocations, increasing the risk on leukemia especially in mice with an impaired DNA repair. Additionally, some inflammatory cytokines were increased in their basal expression, which could affect the inflammatory response.

A general overview of the effects of prenatal genistein and quercetin exposure on the offspring's risk to develop cancer is depicted in Figure 2.
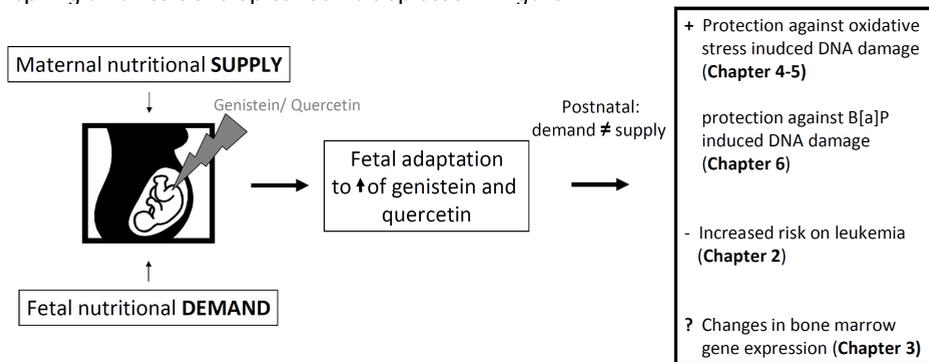

Figure 2. General overview of the effects of prenatal exposure to genistein or quercetin on the offspring's cancer risk discussed in this thesis

References

1. Darnton-Hill, I., Nishida, C., and James, W. P. (2004) A life course approach to diet, nutrition and the prevention of chronic diseases. *Public Health Nutr* 7, 101-121

2. Khan, T. H., Jahangir, T., Prasad, L., and Sultana, S. (2006) Inhibitory effect of apigenin on benzo(a)pyrene-mediated genotoxicity in Swiss albino mice. *J Pharm Pharmacol* 58, 1655-1660

3. Muhammad, S., Bierhaus, A., and Schwaninger, M. (2009) Reactive oxygen species in diabetes-induced vascular damage, stroke, and Alzheimer's disease. *J Alzheimers Dis* 16, 775-785

4. Tabner, B. J., Turnbull, S., El-Agnaf, O., and Allsop, D. (2001) Production of reactive oxygen species from aggregating proteins implicated in Alzheimer's disease, Parkinson's disease and other neurodegenerative diseases. *Curr Top Med Chem* 1, 507-517

5. Touyz, R. M. (2004) Reactive oxygen species and angiotensin II signaling in vascular cells -- implications in cardiovascular disease. *Braz J Med Biol Res* 37, 1263-1273

6. Weinberg, F., and Chandel, N. S. (2009) Reactive oxygen species-dependent signaling regulates cancer. *Cell Mol Life Sci* 66, 3663-3673

7. Arts, I. C., and Hollman, P. C. (2005) Polyphenols and disease risk in epidemiologic studies. *Am J Clin Nutr* 81, 317S-325S

8. Hollman, P. C., and Katan, M. B. (1999) Dietary flavonoids: intake, health effects and bioavailability. *Food Chem Toxicol* 37, 937-942

9. Ross, J. A., and Kasum, C. M. (2002) Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annu Rev Nutr* 22, 19-34

10. Yao, L. H., Jiang, Y. M., Shi, J., Tomas-Barberan, F. A., Datta, N., Singanusong, R., and Chen, S. S. (2004) Flavonoids in food and their health benefits. *Plant Foods Hum Nutr* 59, 113-122

11. Erdman, J. W., Jr., Balentine, D., Arab, L., Beecher, G., Dwyer, J. T., Folts, J., Harnly, J., Hollman, P., Keen, C. L., Mazza, G., Messina, M., Scalbert, A., Vita, J., Williamson, G., and Burrowes, J. (2007) Flavonoids and heart health: proceedings of the ILSI North America Flavonoids Workshop, May 31-June 1, 2005, Washington, DC. *J Nutr* 137, 718S-737S

12. Aherne, S. A., and O'Brien, N. M. (2002) Dietary flavonols: chemistry, food content, and metabolism. *Nutrition* 18, 75-81

13. Diplock, A. T., Charleux, J. L., Crozier-Willi, G., Kok, F. J., Rice-Evans, C., Roberfroid, M., Stahl, W., and Vina-Ribes, J. (1998) Functional food science and defence against reactive oxidative species. *Br J Nutr* 80 Suppl 1, S77-112

14. Skibola, C. F., and Smith, M. T. (2000) Potential health impacts of excessive flavonoid intake. *Free Radic Biol Med* 29, 375-383

15. Ferrali, M., Signorini, C., Caciotti, B., Sugherini, L., Ciccoli, L., Giachetti, D., and Comporti, M. (1997) Protection against oxidative damage of erythrocyte membrane by the flavonoid quercetin and its relation to iron chelating activity. *FEBS Lett* 416, 123-129

16. Kitagawa, S., Sakamoto, H., and Tano, H. (2004) Inhibitory effects of flavonoids on free radical-induced hemolysis and their oxidative effects on hemoglobin. *Chem Pharm Bull (Tokyo)* 52, 999-1001

17. Marik, R., Allu, M., Anchoori, R., Stearns, V., Umbricht, C. B., and Khan, S. (2011) Potent genistein derivatives as inhibitors of estrogen receptor alpha-positive breast cancer. *Cancer Biol Ther* 11

18. Miodini, P., Fioravanti, L., Di Fronzo, G., and Cappelletti, V. (1999) The two phyto-oestrogens genistein and quercetin exert different effects on oestrogen receptor function. *Br J Cancer* 80, 1150-1155

19. Espin, J. C., Garcia-Conesa, M. T., and Tomas-Barberan, F. A. (2007) Nutraceuticals: facts and fiction. *Phytochemistry* 68, 2986-3008

20. Harwood, M., Danielewska-Nikiel, B., Borzelleca, J. F., Flamm, G. W., Williams, G. M., and Lines, T. C. (2007) A critical review of the data related to the safety of quercetin and lack of evidence of in vivo toxicity, including lack of genotoxic/carcinogenic properties. *Food Chem Toxicol* 45, 2179-2205

21. Sullivan, K. M., Ford, E. S., Azrak, M. F., and Mokdad, A. H. (2009) Multivitamin use in pregnant and nonpregnant women: results from the Behavioral Risk Factor Surveillance System. *Public Health Rep* 124, 384-390

22. Baggott, J. E., Oster, R. A., and Tamura, T. (2011) Meta-analysis of cancer risk in folic acid supplementation trials. *Cancer Epidemiol*

23. Bonacasa, B., Siow, R. C., and Mann, G. E. (2011) Impact of dietary soy isoflavones in pregnancy on fetal programming of endothelial function in offspring. *Microcirculation* 18, 270-285

24. Schroder-van der Elst, J. P., van der Heide, D., Rokos, H., Morreale de Escobar, G., and Kohrle, J. (1998) Synthetic flavonoids cross the placenta in the rat and are found in fetal brain. *Am J Physiol* 274, E253-256

25. Ross, J. A., Potter, J. D., Reaman, G. H., Pendergrass, T. W., and Robison, L. L. (1996) Maternal exposure to potential inhibitors of DNA topoisomerase II and infant leukemia (United States): a report from the Children's Cancer Group. *Cancer Causes Control* 7, 581-590

26. Spector, L. G., Xie, Y., Robison, L. L., Heerema, N. A., Hilden, J. M., Lange, B., Felix, C. A., Davies, S. M., Slavin, J., Potter, J. D., Blair, C. K., Reaman, G. H., and Ross, J. A. (2005) Maternal diet and infant leukemia: the DNA topoisomerase II inhibitor hypothesis: a report from the children's oncology group. *Cancer Epidemiol Biomarkers Prev* 14, 651-655

27. Barjesteh van Waalwijk van Doorn-Khosrovani, S., Janssen, J., Maas, L. M., Godschalk, R. W., Nijhuis, J. G., and van Schooten, F. J. (2007) Dietary flavonoids induce MLL translocations in primary human CD34+ cells. *Carcinogenesis* 28, 1703-1709

28. Spring, K., Cross, S., Li, C., Watters, D., Ben-Senior, L., Waring, P., Ahangari, F., Lu, S. L., Chen, P., Misko, I., Paterson, C., Kay, G., Smorodinsky, N. I., Shiloh, Y., and Lavin, M. F. (2001) Atm knock-in mice harboring an in-frame deletion corresponding to the human ATM 7636del9 common mutation exhibit a variant phenotype. *Cancer Res* 61, 4561-4568

29. Spring, K., Ahangari, F., Scott, S. P., Waring, P., Purdie, D. M., Chen, P. C., Hourigan, K., Ramsay, J., McKinnon, P. J., Swift, M., and Lavin, M. F. (2002) Mice heterozygous for mutation in Atm, the gene involved in ataxia-telangiectasia, have heightened susceptibility to cancer. *Nat Genet* 32, 185-190

30. Barker, D. J., and Clark, P. M. (1997) Fetal undernutrition and disease in later life. *Rev Reprod* 2, 105-112

31. Dolinoy, D. C., Weidman, J. R., Waterland, R. A., and Jirtle, R. L. (2006) Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. *Environ Health Perspect* 114, 567-572

32. Zhou, Y., and Mi, M. T. (2005) Genistein stimulates hematopoiesis and increases survival in irradiated mice. *J Radiat Res (Tokyo)* 46, 425-433

33. Reuter, S., Gupta, S. C., Chaturvedi, M. M., and Aggarwal, B. B. (2010) Oxidative stress, inflammation, and cancer: how are they linked? *Free Radic Biol Med* 49, 1603-1616

34. Boots, A. W., Li, H., Schins, R. P., Duffin, R., Heemskerk, J. W., Bast, A., and Haenen, G. R. (2007) The quercetin paradox. *Toxicol Appl Pharmacol* 222, 89-96

35. Choi, E. J., Chee, K. M., and Lee, B. H. (2003) Anti- and prooxidant effects of chronic quercetin administration in rats. *Eur J Pharmacol* 482, 281-285

36. Kaspar, J. W., and Jaiswal, A. K. (2011) Tyrosine phosphorylation controls nuclear export of Fyn, allowing Nrf2 activation of cytoprotective gene expression. *FASEB J* 25, 1076-1087

37. Cao, G., Sofic, E., and Prior, R. L. (1997) Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free Radic Biol Med* 22, 749-760

38. Galati, G., Sabzevari, O., Wilson, J. X., and O'Brien, P. J. (2002) Prooxidant activity and cellular effects of the phenoxyl radicals of dietary flavonoids and other polyphenolics. *Toxicology* 177, 91-104

39. Ullah, M. F., Shamim, U., Hanif, S., Azmi, A. S., and Hadi, S. M. (2009) Cellular DNA breakage by soy isoflavone genistein and its methylated structural analogue biochanin A. *Mol Nutr Food Res* 53, 1376-1385

40. Vargas, A. J., and Burd, R. (2010) Hormesis and synergy: pathways and mechanisms of quercetin in cancer prevention and management. *Nutr Rev* 68, 418-428

41. Boots, A. W., Drent, M., de Boer, V. C., Bast, A., and Haenen, G. R. (2011) Quercetin reduces markers of oxidative stress and inflammation in sarcoidosis. *Clin Nutr* 30, 506-512

42. Siow, R. C., and Mann, G. E. (2010) Dietary isoflavones and vascular protection: activation of cellular antioxidant defenses by SERMs or hormesis? *Mol Aspects Med* 31, 468-477

43. Munoz, M., Villar, I., and Garcia-Erce, J. A. (2009) An update on iron physiology. *World J Gastroenterol* 15, 4617-4626

44. Zhang, A. S., and Enns, C. A. (2009) Molecular mechanisms of normal iron homeostasis. *Hematology Am Soc Hematol Educ Program*, 207-214

45. Kingsley, P. D., Malik, J., Emerson, R. L., Bushnell, T. P., McGrath, K. E., Bloedorn, L. A., Bulger, M., and Palis, J. (2006) "Maturational" globin switching in primary primitive erythroid cells. *Blood* 107, 1665-1672

46. Palis, J. (2008) Ontogeny of erythropoiesis. *Curr Opin Hematol* 15, 155-161

47. Dennery, P. A. (2007) Effects of oxidative stress on embryonic development. *Birth Defects Res C Embryo Today* 81, 155-162

48. Jauniaux, E., Gulbis, B., and Burton, G. J. (2003) The human first trimester gestational sac limits rather than facilitates oxygen transfer to the foetus--a review. *Placenta* 24 Suppl A, S86-93

49. Amoyal, I., and Fibach, E. (2007) Hemoglobin switch in the newborn: a flow cytometry analysis. *Neonatology* 91, 61-68

50. Purdie, A., Wells, R. M., and Brittain, T. (1983) Molecular aspects of embryonic mouse haemoglobin ontogeny. *Biochem J* 215, 377-383

51. Comporti, M., Signorini, C., Buonocore, G., and Ciccoli, L. (2002) Iron release, oxidative stress and erythrocyte ageing. *Free Radic Biol Med* 32, 568-576

52. Koury, M. J., and Ponka, P. (2004) New insights into erythropoiesis: the roles of folate, vitamin B12, and iron. *Annu Rev Nutr* 24, 105-131

53. Weiss, G. (2009) Iron metabolism in the anemia of chronic disease. *Biochim Biophys Acta* 1790, 682-693

54. Ohgami, R. S., Campagna, D. R., McDonald, A., and Fleming, M. D. (2006) The Steap proteins are metalloreductases. *Blood* 108, 1388-1394

55. Alexandrov, K., Rojas, M., and Satarug, S. (2010) The critical DNA damage by benzo(a)pyrene in lung tissues of smokers and approaches to preventing its formation. *Toxicol Lett* 198, 63-68

56. Delgado, M. E., Haza, A. I., Arranz, N., Garcia, A., and Morales, P. (2008) Dietary polyphenols protect against N-nitrosamines and benzo(a)pyrene-induced DNA damage (strand breaks and oxidized purines/pyrimidines) in HepG2 human hepatoma cells. *Eur J Nutr* 47, 479-490

57. Ciolino, H. P., Daschner, P. J., and Yeh, G. C. (1999) Dietary flavonols quercetin and kaempferol are ligands of the aryl hydrocarbon receptor that affect CYP1A1 transcription differentially. *Biochem J* 340 (Pt 3), 715-722

58. Makaji, E., Ho, S. H., Holloway, A. C., and Crankshaw, D. J. (2011) Effects in rats of maternal exposure to raspberry leaf and its constituents on the activity of cytochrome p450 enzymes in the offspring. *Int J Toxicol* 30, 216-224

59. Moon, Y. J., Wang, X., and Morris, M. E. (2006) Dietary flavonoids: effects on xenobiotic and carcinogen metabolism. *Toxicol In Vitro* 20, 187-210

60. Kang, Z. C., Tsai, S. J., and Lee, H. (1999) Quercetin inhibits benzo[a]pyrene-induced DNA adducts in human Hep G2 cells by altering cytochrome P-450 1A1 gene expression. *Nutr Cancer* 35, 175-179

61. Sergent, T., Dupont, I., Van der Heiden, E., Scippo, M. L., Pussemier, L., Larondelle, Y., and Schneider, Y. J. (2009) CYP1A1 and CYP3A4 modulation by dietary flavonoids in human intestinal Caco-2 cells. *Toxicol Lett* 191, 216-222

62. Walle, T., and Walle, U. K. (2007) Novel methoxylated flavone inhibitors of cytochrome P450 1B1 in SCC-9 human oral cancer cells. *J Pharm Pharmacol* 59, 857-862

63. Nelson, D. R., Koymans, L., Kamataki, T., Stegeman, J. J., Feyereisen, R., Waxman, D. J., Waterman, M. R., Gotoh, O., Coon, M. J., Estabrook, R. W., Gunsalus, I. C., and Nebert, D. W. (1996) P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* 6, 1-42

64. Santini, S. E., Basini, G., Bussolati, S., and Grasselli, F. (2009) The phytoestrogen quercetin impairs steroidogenesis and angiogenesis in swine granulosa cells in vitro. *J Biomed Biotechnol* 2009, 419891

65. Abbott, B. D., Birnbaum, L. S., and Perdew, G. H. (1995) Developmental expression of two members of a new class of transcription factors: I. Expression of aryl hydrocarbon receptor in the C57BL/6N mouse embryo. *Dev Dyn* 204, 133-143

66. Kohle, C., and Bock, K. W. (2007) Coordinate regulation of Phase I and II xenobiotic metabolisms by the Ah receptor and Nrf2. *Biochem Pharmacol* 73, 1853-1862

67. Niestroy, J., Barbara, A., Herbst, K., Rode, S., van Liempt, M., and Roos, P. H. (2011) Single and concerted effects of benzo[a]pyrene and flavonoids on the AhR and Nrf2-pathway in the human colon carcinoma cell line Caco-2. *Toxicol In Vitro* 25, 671-683

68. Ten Have-Opbroek, A. A. (1991) Lung development in the mouse embryo. *Exp Lung Res* 17, 111-130

69. Zaret, K. S. (2002) Regulatory phases of early liver development: paradigms of organogenesis. *Nat Rev Genet* 3, 499-512

70. Uno, S., Dalton, T. P., Dragin, N., Curran, C. P., Derkenne, S., Miller, M. L., Shertzer, H. G., Gonzalez, F. J., and Nebert, D. W. (2006) Oral benzo[a]pyrene in Cyp1 knockout mouse lines: CYP1A1 important in detoxication, CYP1B1 metabolism required for immune damage independent of total-body burden and clearance rate. *Mol Pharmacol* 69, 1103-1114

71. Waterston, R. H., Lindblad-Toh, K., Birney, E., Rogers, J., Abril, J. F., Agarwal, P., Agarwala, R., Ainscough, R., Alexandersson, M., An, P., Antonarakis, S. E., Attwood, J., Baertsch, R., Bailey, J., Barlow, K., Beck, S., Berry, E., Birren, B., Bloom, T., Bork, P., Botcherby, M., Bray, N., Brent, M. R., Brown, D. G., Brown, S. D., Bult, C., Burton, J., Butler, J., Campbell, R. D., Carninci, P., Cawley, S., Chiaromonte, F., Chinwalla, A. T., Church, D. M., Clamp, M., Clee, C., Collins, F. S., Cook, L. L., Copley, R. R., Coulson, A., Couronne, O., Cuff, J., Curwen, V., Cutts, T., Daly, M., David, R., Davies, J., Delehaunty, K. D., Deri, J., Dermitzakis, E. T., Dewey, C., Dickens, N. J., Diekhans, M., Dodge, S., Dubchak, I., Dunn, D. M., Eddy, S. R., Elnitski, L., Emes, R. D., Eswara, P., Eyras, E., Felsenfeld, A., Fewell, G. A., Flicek, P., Foley, K., Frankel, W. N., Fulton, L. A., Fulton, R. S., Furey, T. S., Gage, D., Gibbs, R. A., Glusman, G., Gnerre, S., Goldman, N., Goodstadt, L., Grafham, D., Graves, T. A., Green, E. D., Gregory, S., Guigo, R., Guyer, M., Hardison, R. C., Haussler, D., Hayashizaki, Y., Hillier, L. W., Hinrichs, A., Hlavina, W., Holzer, T., Hsu, F., Hua, A., Hubbard, T., Hunt, A., Jackson, I., Jaffe, D. B., Johnson,

L. S., Jones, M., Jones, T. A., Joy, A., Kamal, M., Karlsson, E. K., Karolchik, D., Kasprzyk, A., Kawai, J., Keibler, E., Kells, C., Kent, W. J., Kirby, A., Kolbe, D. L., Korf, I., Kucherlapati, R. S., Kulbokas, E. J., Kulp, D., Landers, T., Leger, J. P., Leonard, S., Letunic, I., Levine, R., Li, J., Li, M., Lloyd, C., Lucas, S., Ma, B., Maglott, D. R., Mardis, E. R., Matthews, L., Mauceli, E., Mayer, J. H., McCarthy, M., McCombie, W. R., McLaren, S., McLay, K., McPherson, J. D., Meldrim, J., Meredith, B., Mesirov, J. P., Miller, W., Miner, T. L., Mongin, E., Montgomery, K. T., Morgan, M., Mott, R., Mullikin, J. C., Muzny, D. M., Nash, W. E., Nelson, J. O., Nhan, M. N., Nicol, R., Ning, Z., Nusbaum, C., O'Connor, M. J., Okazaki, Y., Oliver, K., Overton-Larty, E., Pachter, L., Parra, G., Pepin, K. H., Peterson, J., Pevzner, P., Plumb, R., Pohl, C. S., Poliakov, A., Ponce, T. C., Ponting, C. P., Potter, S., Quail, M., Reymond, A., Roe, B. A., Roskin, K. M., Rubin, E. M., Rust, A. G., Santos, R., Sapojnikov, V., Schultz, B., Schultz, J., Schwartz, M. S., Schwartz, S., Scott, C., Seaman, S., Searle, S., Sharpe, T., Sheridan, A., Shownkeen, R., Sims, S., Singer, J. B., Slater, G., Smit, A., Smith, D. R., Spencer, B., Stabenau, A., Stange-Thomann, N., Sugnet, C., Suyama, M., Tesler, G., Thompson, J., Torrents, D., Trevaskis, E., Tromp, J., Ucla, C., Ureta-Vidal, A., Vinson, J. P., Von Niederhausern, A. C., Wade, C. M., Wall, M., Weber, R. J., Weiss, R. B., Wendl, M. C., West, A. P., Wetterstrand, K., Wheeler, R., Whelan, S., Wierzbowski, J., Willey, D., Williams, S., Wilson, R. K., Winter, E., Worley, K. C., Wyman, D., Yang, S., Yang, S. P., Zdobnov, E. M., Zody, M. C., and Lander, E. S. (2002) Initial sequencing and comparative analysis of the mouse genome. *Nature* 420, 520-562

72. Waterland, R. A., Kellermayer, R., Rached, M. T., Tatevian, N., Gomes, M. V., Zhang, J., Zhang, L., Chakravarty, A., Zhu, W., Laritsky, E., Zhang, W., Wang, X., and Shen, L. (2009) Epigenomic profiling indicates a role for DNA methylation in early postnatal liver development. *Hum Mol Genet* 18, 3026-3038

73. Duthie, S. J. (2011) Epigenetic modifications and human pathologies: cancer and CVD. *Proc Nutr Soc* 70, 47-56

74. Li, Y., and Tollefsbol, T. O. (2010) Impact on DNA methylation in cancer prevention and therapy by bioactive dietary components. *Curr Med Chem* 17, 2141-2151

75. Kramerov, D. A., and Vassetzky, N. S. (2005) Short retroposons in eukaryotic genomes. *Int Rev Cytol* 247, 165-221

76. Stribinskis, V., and Ramos, K. S. (2006) Activation of human long interspersed nuclear element 1 retrotransposition by benzo(a)pyrene, an ubiquitous environmental carcinogen. *Cancer Res* 66, 2616-2620

Nederlandse samenvatting

Chronische ziektes die op volwassen leeftijd optreden, zoals kanker, cardiovasculaire en respiratoire aandoeningen zijn een grote last voor de gezondheid van de mens wereldwijd (1). Blootstelling aan genotoxische en mutagene stoffen lijkt een belangrijke rol te spelen in de ontwikkeling van dit soort ziektes (2-6). Van gezonde voeding wordt vermoed dat ze beschermen tegen deze genotoxische en mutagene stoffen en zo ook bescherming bieden tegen chronische ziektes en de gezondheid te bevorderen. Een voedingscomponent dat veel aandacht krijgt in verband met de behandeling en preventie van chronische ziektes zijn de flavonoïden (7-10). Flavonoïden bestaan uit een grote groep polyfenolen die wijdverspreid voorkomen in onze voeding (9), waardoor de gemiddelde inname varieert tussen 65 en 250 mg flavonoïden per dag (11). Quercetine is de meest voorkomende flavonoïd in ons dieet en wordt teruggevonden in uien, appels, thee en rode wijn (9, 12-14). Het is een zeer krachtige radicaalvanger en kan ook ijzer cheleren (15, 16). Genisteïne is ook een belangrijk flavonoïd dat voornamelijk wordt teruggevonden in sojabonen. Naast een antioxidant werking heeft het ook oestrogeen agonistische/ antagonistische werkingen, afhankelijk van de situatie, als gevolg van structurele overeenkomsten met 17β-oestradiol (17, 18).

Aangezien flavonoïden momenteel geacht worden voordeling te zijn voor de gezondheid, zijn ze vrij verkrijgbaar als voedingssupplementen en dit in hoge dosissen, met een aangeraden dagelijkse dosis tussen 1-2 gram (19, 20). Zorgwekkend is de toename in supplement inname door zwangere vrouwen. In de VS nemen 78 % van de zwangere vrouwen voedingssupplementen (21), waaronder foliumzuur. Nochtans verhoogt de inname van foliumzuur het risico op kanker bij volwassenen (22). Er is echter weinig bekend over de biologische werking van flavonoïden tijdens de zwangerschap en over de kort en/ of lange termijn effecten die ze kunnen hebben op de gezondheid van het kind. Aangezien flavonoïden de placenta kunnen doorkruisen om zo te accumuleren in de foetus (23, 24), is het nodig onderzoek te verrichten naar de veiligheid van verhoogde flavonoïd inname tijdens de zwangerschap.

Flavonoïden tijdens de zwangerschap: Kinderleukemie

Een verhoogde flavonoïd inname wordt ook in verband gebracht met het ontstaan van kinderleukemie, omdat ze topoïsomerase II kunnen remmen (25, 26) en zo dubbelstrengsbreuken in DNA en herschikkingen van het *mixed-lineage leukemia* (*MLL*) gen kunnen induceren, wat veel voorkomt bij kinderleukemie (26, 27). In Hoofdstuk 2 werden *Atm*-ΔSRI heterozygote muizen gepaard om nakomelingen te verkrijgen met een verschillende genetische mogelijkheid om DNA schade te herstellen, namelijk *Atm* wild-type, *Atm*-ΔSRI heterozygote en *Atm*-ΔSRI mutante nakomelingen. Wij konden vaststellen dat prenatale blootstelling aan flavonoïden inderdaad *Mll* translocaties in het beenmerg van zowel wild-type als *Atm*-ΔSRI mutante muizen veroorzaakte. Bovendien waren *Atm*-ΔSRI mutante muizen gevoeliger voor het ontwikkelen van deze chromosomale afwijkingen. Ook werd aangetoond dat muizen met een slecht werkend DNA herstelmechanisme een verhoogd risico hadden op het ontwikkelen van andere aandoeningen buiten kinderleukemie, wanneer ze prenataal blootgesteld waren aan genisteïne. Dit geeft aan dat het DNA dubbelstrengsbreuk herstelmechanisme van belang is voor het behoud van de integriteit van het genoom in de ontwikkelende foetus.

De *Atm*-ΔSRI mutatie komt overeen met een van de meest voorkomende *Atm* mutaties teruggevonden in de mens. *Atm*-ΔSRI muizen hebben ook een fenotype dat verschilt van *Atm* knock-out muizen. *Atm*-ΔSRI mutant muizen hebben 50 % kans op het ontwikkelen van leukemie en lymfoma, zonder bijkomende blootstellingen (28). In onze studie lag de leeftijd waarop deze muizen tumoren ontwikkelden dramatisch lager wanneer ze prenataal blootgesteld waren aan genisteïne en quercetine, namelijk van een gemiddelde leeftijd van 18,6 maanden (28, 29) naar slechts 3 maanden.

Ondanks dat het aantal dieren waarin leukemie vastgesteld kon worden tamelijk laag was in onze studie, toonden de *Mll* translocatie analyse wel aan dat muizen die prenataal blootgesteld waren aan een van beide flavonoïden een verhoogd risico liepen op het krijgen van kanker op latere leeftijd.

Flavonoïden tijdens de zwangerschap: Foetale programmering

Naast het direct verhogen van de gevoeligheid voor het ontwikkelen van kanker wordt van flavonoïden ook vermoed dat ze betrokken zijn bij 'foetale programmering'. Dit concept houdt in dat de foetus zich tracht aan te passen aan ongunstige omstandigheden dat het tegenkomt *in utero*, wat kan resulteren in aanpassingen die schadelijk zijn wanneer deze omstandigheden niet overheersen tijdens het verdere leven (30). Dolinoy *et al.* (31) geeft een duidelijk voorbeeld van foetale programmering door aan te tonen dat agouti muizen die prenataal blootgesteld werden aan genisteïne een andere vachtkleur hadden, terwijl ze ook beschermd waren tegen obesitas op latere leeftijd, wat kenmerkend is voor agouti muizen. Deze effecten werden veroorzaakt door epigenetische veranderingen, namelijk door hypermethylatie van een repetitief element stroomopwaarts van de transcriptie start site van het *Agouti* gen dat resulteerde in de verminderde expressie van dit gen. De verandering in het DNA methylatie patroon tijdens de foetale ontwikkeling door blootstelling aan flavonoïden kan dus langdurige effecten hebben, omdat dit patroon doorgegeven wordt aan dochtercellen tijdens de celdeling.

Aanhoudende veranderingen in gen expressie

In Hoofdstuk 3 werd het effect van prenatale blootstelling aan genisteïne op gen expressie en globale methylatie van beenmerg cellen van 12 weken oude, mannelijke muizen onderzocht. Prenatale blootstelling aan genisteïne resulteerde overduidelijk in een verminderde expressie van oestrogeengevoelige genen en genen betrokken bij het remmen van de hematopoëse. Dit had als gevolg dat de erythropoëse en granulopoëse toenam in deze muizen. Het effect dat genisteïne uitoefende op de oestrogeengevoelige genen was min of meer verwacht, aangezien genisteïne structurele gelijkenissen heeft met 17β-oestradiol, waardoor het via de oestrogeenreceptor zijn effect kan uitoefenen (18). Daarnaast werd het effect van genisteïne op de hematopoëse ook teruggevonden door Zhou *et al.* (32), waar muizen voorbehandeld werden met genisteïne alvorens ze 7 dagen later aan een gehele lichaamsbestraling blootgesteld werden. De voorbehandeling met genisteïne had een beschermend effect op deze muizen doordat het de regeneratie van hematopoëtische stamcellen verbeterde. We vermoeden dat de

verandering in gen expressie in onze studie het resultaat is van epigenetische mechanismen, aangezien de repetitieve elementen een toename in methylatie vertoonden in het beenmerg van deze dieren.

Oxidatieve stress gerelateerde processen

Flavonoïden zijn voornamelijk gekend omwille van hun werking als antioxidant (9) en ze kunnen de placenta doorkruisen om zo de ontwikkelende foetus te bereiken. Daarom hebben wij onderzocht of prenatale blootstelling aan genisteïne of quercetine ook lange termijn effecten kon hebben op systemen die bijdragen aan de bescherming tegen oxidatieve stress, aangezien dit betrokken is bij het ontstaan van kanker (33). Hiervoor hebben we de antioxidant capaciteit van lever en long weefsel van volwassen, mannelijke muizen die prenataal blootgesteld werden aan genisteïne en quercetine, onderzocht in Hoofdstuk 4. Quercetine blootstelling tijdens de dracht resulteerde in een verhoogde expressie van genen die betrokken zijn bij het enzymatische antioxidant verdedigingssysteem van de lever van foetussen op dag 14,5 van de dracht. Vooral *Nrf2*, een belangrijke transcriptie factor die reageert op oxidatieve stress, toonde een verhoogde expressie bij blootstelling aan quercetine. Dit toont aan dat, ondanks quercetine's eigenschappen als antioxidant, het waarschijnlijk een pro-oxidante werking uitoefende tijdens de *in utero* blootstelling. Pro-oxidante effecten zijn reeds eerder aangetoond voor quercetine (34, 35). Blootstelling aan genisteïne daarentegen had op dit moment van de dracht geen effect op het enzymatische antioxidant e verdedigingssysteem. Dit zou kunnen zijn doordat genisteïne een minder sterke pro-oxidant is of zelfs eerder een antioxidant werking had. Het is echter wel reeds aangetoond dat genisteïne het nucleaire transport en de degradatie van Nrf2 verhinderd (36), waardoor de verhoogde gen expressie overbodig is.

Op volwassen leeftijd vertoonde de muizen die prenataal blootgesteld werden aan genisteïne of quercetine nog altijd een verandering in de expressie van genen die betrokken zijn bij de enzymatische antioxidante verdediging van de lever. Dit suggereert dat de foetus zich aanpast aan de *in utero* omgeving, gecreëerd door de verhoogde aanlevering van genisteïne en quercetine door de moeder, en dat deze aanpassingen behouden blijven tijdens het leven. We onderzochten ook het effect van prenatale blootstelling aan genisteïne en quercetine op de non-enzymatische antioxidant e verdediging. Enkel prenatale blootstelling aan genisteïne had een effect op de non-enzymatische antioxidante capaciteit van de long. Oxidatieve stress-geïnduceerde DNA schade, namelijk directe oxidatie van het DNA (8-oxo-dG levels) of indirecte DNA schade geïnduceerd door het lipidenperoxidatie product MDA (M_1dG) werd ook gemeten in de lever en longen van volwassen muizen die prenataal blootgesteld werden aan quercetine of genisteïne. Zowel prenatale blootstelling aan genisteïne, maar vooral aan quercetine, resulteerde in minder oxidatieve stress geïnduceerd DNA schade, maar dit enkel in de lever van de dieren. Aangezien we in de lever voornamelijk veranderingen in gen expressie van de enzymatische antioxidanten vonden, lijkt het erop dat het preventief stimuleren van het enzymatische antioxidant verdedigingssysteem voordelig en beschermend werkt tegen oxidatieve DNA schade in de lever van volwassen muizen die prenataal blootgesteld waren aan genisteïne, maar vooral aan quercetine.

Dit effect was weefselspecifiek, aangezien in de long enkel bescheiden effecten in gen expressie van de enzymatische antioxidanten terug te vinden waren ten gevolge van toevoeging van flavonoïden aan het dieet van de moeder en de non-enzymatische antioxidanten verdedigingssysteem enkel toegenomen was ten gevolge van blootstelling aan genisteïne, in vergelijking met de lever. Daarom dat de beperkte, niet statistisch significante, daling in 8-oxo-dG en M_1dG in de long van volwassen muizen die prenataal blootgesteld werden aan genisteïne of quercetine te verwachten was.

Deze studie toonde aan dat, ondanks het feit dat genisteïne en quercetine voornamelijk gekend zijn omwille van hun antioxidante eigenschappen, ze waarschijnlijk als pro-oxidanten fungeerden tijdens de dracht, zoals ook al eerder gemeld werd (35, 37-40). Dit is aannemelijk aangezien ze accumuleren in de foetus (23, 24) terwijl een antioxidante werking optreed bij lagere concentraties. Deze studie toonde ook aan dat genisteïne en quercetine niet hetzelfde effect uitoefenden als we kijken naar de lange termijn veranderingen in het enzymatische antioxidante verdedigingssysteem. Het effect van quercetine was meer uitgesproken dan dat van genisteïne. Hoewel het al eerder was aangetoond dat blootstelling aan genisteïne en quercetine op volwassen leeftijd beschermt tegen oxidatieve stress (41, 42), hebben wij nu aangetoond dat *in utero* blootstelling ook voor bescherming zorgt op volwassen leeftijd. Het moet echter nog vastgesteld worden of dit voordelige effect kan opwegen tegen de oxidatieve stress veroorzaakt *in utero*.

De ijzer chelerende eigenschap van quercetine als programmeringsstimulus

Quercetine is een gekende, zeer krachtige ijzer chelator (15, 16). Dit is de rede dat het effect van prenatale blootstelling aan quercetine op de ijzerhuishouding onderzocht werd tijdens de foetale ontwikkeling en op volwassen leeftijd in Hoofdstuk 5. Eerst werd onderzocht of *in utero* blootstelling aan quercetine wel degelijk resulteerde in ijzer chelatie, maar op dag 14,5 van de dracht was er echter geen verschil in de totale hoeveelheid ijzer meetbaar in het vruchtwater. Aangezien het merendeel van het ijzer gebruikt wordt voor hemoglobine vorming (43, 44) werd de gen expressie van globine genen bepaald. Op dag 14,5 van de dracht ondergaan foetusen een omschakeling van de embryonische vorm van hemoglobine, dat enkel gevormd wordt door primitieve rode bloedcellen (RBCs), naar de volwassen vorm van hemoglobine, dat gevormd wordt door zowel de primitieve als de definitieve RBCs (45, 46). Het belangrijkste verschil tussen beide vormen van hemoglobine is hun affiniteit voor zuurstof. Tijdens de vroege ontwikkeling zijn de zuurstof niveaus laag door de beperkte doorbloeding, om een optimale orgaanontwikkeling te garanderen. Dit is de periode waarin de foetus het meest kwetsbaar is voor oxidatieve stress, wat kan leiden tot beschadiging van de foetus en vroegtijdig beëindigen van de zwangerschap (47, 48). Niettemin moet de foetus ook zuurstof krijgen via het bloed van de moeder. Daarom vormt de foetus in de eerste fase van de zwangerschap embryonische hemoglobine, welke een hogere affiniteit voor zuurstof heeft in vergelijking met de adulte hemoglobine, wat gevormd wordt door de moeder (49, 50). Eens de utero-placentale circulatie gevormd is (tussen dag 9,5 en 11 van de dracht) (47), komt er een overschakeling naar de adulte hemoglobine, als gevolg van de toename in zuurstof concentratie (45, 46, 48). Maar de embryonische hemoglobine is ook minder stabiel in vergelijking met de adulte hemoglobine (48), waardoor het gevoeliger is voor oxidatie, wat resulteert in de

vorming van methemoglobine dat niet in staat is zuurstof te binden. Daarbij laat embryonische hemoglobine ook makkelijker ijzer vrij van zijn heem groep (51). Aangezien het aangetoond is dat blootstelling aan quercetine leidt tot de vorming van methemoglobine, doordat quercetine de RBCs ingaat en het ijzer ion oxideert (15, 16), verwachtten wij ook een versnelde overgang van embryonische naar adulte hemoglobine waar te nemen bij blootstelling aan quercetine. Ondanks dat de resultaten niet significant waren, zagen we toch een trend in de expressie van de globine genen, namelijk een vermindering in gen expressie van de embryonische globines in foetussen die blootgesteld waren aan quercetine. De gen expressie van de adulte globines was echter onveranderd, terwijl de overgang van de primitieve RBC lijn naar de definitieve RBC lijn versneld was ten gevolg van de verhoogde inname van quercetine door de moeders. Dit kan betekenen dat ondanks dat de ijzer niveaus niet significant afgenomen waren, quercetine toch het ijzer dat geleverd werd door de moeder cheleerde, waardoor er minder ijzer beschikbaar was voor de vorming van hemoglobine. Daarbij doet deze data ook vermoeden dat quercetine het ijzer van de embryonische hemoglobine cheleerde, omdat deze minder stabiel is, waardoor de overgang van embryonische naar adult hemogelobine versneld werd. Er was echter geen verandering in de hoeveelheid adulte hemoglobine detecteerbaar. Dit kan verklaard worden doordat de globine productie afhankelijk is van de hoeveelheid beschikbaar ijzer (52), waarvan wij vermoeden dat er minder beschikbaar was. Desondanks lijkt de versnelde overgang van embryonisch naar adult hemoglobine aannemelijk aangezien de overgang in RBC lijn ook versneld was in deze muizen en RBCs van de definitieve lijn voornamelijk adult hemoglobine produceren.

De hoeveelheid ijzer dat opgeslagen was in de lever van volwassen nakomelingen lag veel hoger in muizen die prenataal blootgesteld waren aan quercetine in vergelijking met controle dieren. Wij vermoeden dat de foetus zich aangepast heeft aan de lagere hoeveelheden bruikbaar ijzer waaraan het *in utero* blootgesteld was door de chelatie van quercetine. Eens geboren worden de foetussen blootgesteld aan een normaal ijzer niveau, aangezien de chelerende werking van quercetine wegvalt. Doordat deze foetussen echter geprogrammeerd werden voor een leven met een laag ijzer niveau voelden deze niveaus aan alsof ze de normale waarden overschreden. Hierdoor werden pathways geactiveerd om deze 'ijzer overlast' te verwerken. We vonden ook een verhoogde gen expressie terug voor verschillende interleukines en *hepcidin* die allemaal betrokken zijn bij de opslag van ijzer wanneer 'anemie van chronische ziekten', ook wel 'inflammatoire anemie' genoemd, optreed. Interessant om te weten is dat anemie vaker voorkomt op oudere leeftijd en bij personen die lijden aan kanker, waarvan 'inflammatoire anemie' meetelt voor 77 % van de onderliggende oorzaken van de anemie (53). In beenmerg cellen van muizen die prenataal blootgesteld werden aan quercetine werd ook een daling teruggevonden in de gen expressie van *Steap4*, wat normaal betrokken is bij de opname van ijzer door cellen (54). Wij vermoeden dat deze maatregel genomen was om het beenmerg te beschermen tegen een overmaat aan ijzer en de DNA schade die hierdoor veroorzaakt kan worden.

Deze data doet vermoeden dat de nakomelingen konden omgaan met de toename in beschikbaar ijzer tijdens hun leven, ondanks dat ze zich hadden aangepast aan de *in utero* omstandigheden, waar ijzer schaars was door de chelatie door quercetine. Sterker nog, er werden minder reactieve zuurstof soorten (ROS) gevormd door ijzer aangezien

lagere 8-oxo-dG levels teruggevonden werden in de lever van deze muizen. Maar omdat de expressie van genen betrokken bij inflammatie ook verhoogd was in afwezigheid van een inflammatoire stimulus, zou het kunnen dat deze muizen anders zullen reageren als inflammatie zou plaats vinden. Aangezien inflammatie betrokken is bij vele chronische ziektes, waaronder kanker (33), lijkt het erop dat, ondanks dat deze dieren beschermd zijn tegen oxidatieve stress gerelateerde ziektes, een inflammatoire stimulus dit beschermend mechanisme misschien kan veranderen in een schadelijk mechanisme. Dit moet echter nog bevestigd worden.

Quercetine als AhR agonist wijzigt het xenobiotisch metabolisme

AhR-signalering in de lever speelt een belangrijke rol in het metabolisme van bepaalde, gekende carcinogenen. Zo bepaalt het het risico van een individu op het ontwikkelen van kanker na blootstelling aan genotoxische stoffen. Daarom hebben wij uitgezocht of blootstelling aan quercetine het carcinogeen metabolisme kon beïnvloeden, meer bepaald het metabolisme van B[a]P (Hoofdstuk 6). B[a]P is een gekend, krachtig mutageen en carcinogeen dat metabolisch geactiveerd moet worden. Deze activatie wordt uitgevoerd door CYP1A1 en CYP1B1 (55, 56), die beiden gereguleerd kunnen worden door quercetine. Quercetine kan zelfs fase II enzymen reguleren (57-59). Desondanks is er geen eenduidig effect van quercetine op fase I en II enzyme expressie gemeld (56, 57, 60-62). In deze studie toonden we aan dat blootstelling aan quercetine *Cyp1a1* en *Cyp1b1* gen expressie verhoogde in foetussen op dag 14,5 van de dracht. Aangezien CYPs hemoproteïnes zijn, die ijzer bevatten in hun heemgroep (63), kan het zijn dat de toename in gen expressie het resultaat is van de chelerende werking van quercetine. Dit kan namelijk resulteren in een mindere CYP activiteit wat gecompenseerd wordt door een toename in gen expressie. Een andere verklaring kan zijn dat quercetine het 17β-oestradiol niveau verhoogt, wat ook een substraat is voor Cyp1b1 en Cyp1a1 (64). *AhR* gen expressie was bovendien niet verhoogd in foetussen. Dit kan zijn omdat in de lever van ontwikkelende muizen foetussen de *AhR* mRNA en proteïne expressie een piek kent tussen dag 14 en 16 van de dracht (65), waardoor het effect van quercetine op de *AhR* gen expressie misschien nog niet zichtbaar is op dit tijdstip. Daarbuiten was de gen expressie van *Nqo1*, *Ugt1a6* and *Gstp1* wel verhoogd in foetussen die blootgesteld waren aan quercetine. Wij vermoeden dat dit het resultaat is van de activatie van het Nrf2-pathway, aangezien deze 3 genen doelgenen zijn van de transcriptie factor Nrf2 (66, 67). *Nrf2* gen expressie was inderdaad verhoogd in foetussen blootgesteld aan quercetine (Hoofdstuk 4).

Op 12 weken vertoonde de muizen die prenataal blootgesteld waren aan quercetine nog altijd een verhoogde expressie van *Cyp1b1* in hun lever, terwijl de verhoogde expressie van de overige genen niet behouden bleef over tijd. Interessant om te zien was dat in volwassen dieren de gen expressie van *AhR* verhoogd was, wat de toename in *Cyp1b1* gen expressie kan verklaren. Wanneer mannelijke en vrouwelijke muizen van eenzelfde nest met elkaar vergeleken werden, vonden we dat vrouwelijke muizen die prenataal blootgesteld waren aan quercetine een mannelijker fase I en II enzyme profiel ontwikkelden in vergelijking met vrouwelijke controle muizen. In ratten werd ook aangetoond dat prenatale blootstelling aan quercetine lange termijn veranderingen in CYP activiteit veroorzaakte, voornamelijk in de vrouwelijke nakomelingen. Het CYP

profiel van de vrouwelijke nakomelingen toonde ook in dit geval een mannelijker profiel in vergelijking met controle vrouwen. Er werd vermoed dat quercetine zijn effecten uitoefende via de regulatie van de androstene of Ah receptor (58). Inderdaad, *AhR* gen expressie was licht verhoogd in vrouwelijke muizen die prenataal blootgesteld waren aan quercetine.

Gen expressie van fase I en II enzymen toonde een ander profiel in de longen van 12 weken oude muizen die prenataal blootgesteld waren aan quercetine. In dit geval was de gen expressie van de fase II enzymen (Gs*tp1*, *Nqo1* and *Ugt1a6*) verhoogd. Het feit dat beide weefsels anders reageren op prenatale blootstelling aan quercetine kan verklaard worden doordat de longen zich voornamelijk ontwikkelen tijdens de laatste fase van de zwangerschap en zelfs nog verder ontwikkelen na de geboorte (68), terwijl de lever grotendeels gevormd is voor de geboorte (69). De minder aanwezige geslachtseffecten in de long kunnen ook het gevolg zijn van de minder belangrijke rol die de long speelt in het metabolisme van oestrogeen. Desondanks vertoonden de foetussen op dag 14,5 van de dracht wel een toename in *Nqo1* en *Ugt1a6* gen expressie, waarvan wij vermoeden dat dit het resultaat was van de activering van het Nrf2-pathway, aangezien quercetine ook als pro-oxidant kan werken (Hoofdstuk 4). Aangezien de long blootgesteld wordt aan hogere zuurstof concentraties in vergelijking met andere weefsels (33) kan een preventieve oxidatieve stress stimulus *in utero* resulteren in de verhoogde expressie van de Nrf2-pathway en zo ook van de fase II enzymen. Maar, zoals hierboven aangegeven, was het Nrf2-pathway slechts matig geïnduceerd in de longen van muizen die prenataal blootgesteld waren aan quercetine (Hoofdstuk 4).

Hoewel prenatale blootstelling aan quercetine resulteerde in een verhoogde expressie van fase I en II enzymen in lever en longen, werden er slechts enkele veranderingen teruggevonden in het *ex vivo* B[a]P metabolisme van de longen van muizen die prenataal blootgesteld werden aan quercetine. Dit was zichtbaar als een afname in B[a]P-9,10-dihydrodiol en B[a]P-7,8-dihydrodiol. Bovendien hadden muizen die prenataal blootgesteld waren aan quercetine een verhoogd B[a]P metabolisme. Omwille van de hoge detoxificatie van B[a]P tot 3-hydroxy-B[a]P konden er echter geen BPDE-geïnduceerde DNA-adducten gedetecteerd worden in geval van alle 3 de dieetgroepen. Aangezien de long het doelorgaan is voor vluchtig B[a]P zou de toename in B[a]P metabolisme en fase II gen expressie erop kunnen wijzen dat prenatale blootstelling aan quercetine de gevoeligheid voor het ontwikkelen van longkanker *in vivo* kan verminderen.

In de lever was verrassend genoeg te zien dat *ex vivo* inductie van BPDE DNA-adducten verminderd was wanneer muizen prenataal blootgesteld waren aan quercetine. Desondanks was er geen verandering in de snelheid waarmee B[a]P gemetaboliseerd werd of in het profiel van de 3 belangrijkste B[a]P metabolieten, waaronder de 7,8-dihydrodiol van B[a]P wat een voorloper is van de uiteindelijke genotoxische metaboliet BPDE. Dit wijst erop dat prenatale blootstelling aan quercetine resulteert in de bescherming tegen B[a]P geïnduceerde DNA schade in de lever, waarschijnlijk door een verhoogde expressie van fase I enzymen. Ondanks dat CYP1A1 en CYP1B1 belangrijk zijn voor de activering van B[a]P, vermoeden wij dat ze ook bijdragen aan B[a]P detoxificatie, wat voorheen ook voorgesteld werd door Uno *et al.* (70).
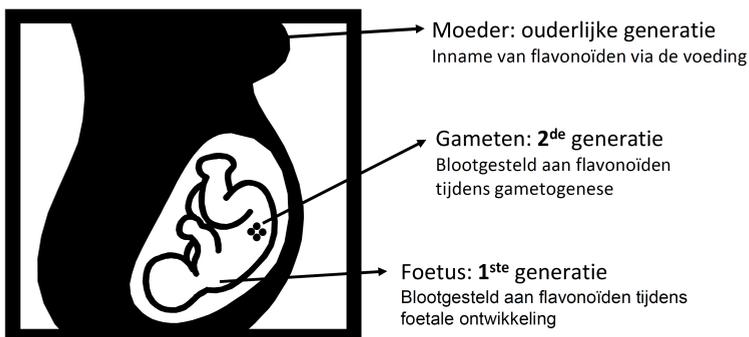
Foetale programmering wordt gereguleerd via epigenetica

De verandering in gen expressie ten gevolge van *in utero* blootstelling aan genisteïne of quercetine, besproken in Hoofdstuk 3-6, werd behouden doorheen het leven van de muis. Wij vermoeden dus dat epigenetische mechanismen betrokken zijn. Daarom werden in dit onderzoek de veranderingen in methylatie status van repetitieve elementen ten gevolge van prenatale blootstelling aan genisteïne en quercetine nagegaan, aangezien het muizen genoom voor 37,5 % uit repetitieve elementen bestaat (71). De resultaten toonden aan dat beide flavonoïden geen veranderingen in globale methylatie (namelijk methylatie van SINEB1, SINEB2, LINE1, IAP, major en minor satellieten) veroorzaakten in de lever van foetussen op dag 14,5 van de dracht (Hoofdstuk 3, 5, 6). Wanneer echter het methylatie patroon van het beenmerg van 12 weken oude muizen onderzocht werd, was een algemene toename in methylatie zichtbaar, vooral in geval van SINEB1 en SINEB2, wanneer muizen prenataal blootgesteld waren aan genisteïne of quercetine (Hoofdstuk 3, 5). In lever en long weefsel van 12 weken oude muizen die prenataal blootgesteld waren aan genisteïne of quercetine bleken de repetitieve elementen SINEB1, SINEB2 en LINE1 licht gehypomethyleerd te zijn (Hoofdstuk 6). Het feit dat er nog geen verandering in methylatie vastgesteld kon worden in foetussen op dag 14,5 van de dracht kan betekenen dat *de novo* methylatie veroorzaakt door flavonoïden op een later tijdstip in de dracht of zelfs na de geboorte pas plaatsvindt. Dit is aannemelijk aangezien tijdens de laatste fase van de dracht en tijdens de vroege postnatale periode weefsel maturatie plaatsvindt, waardoor epigenetische modificaties plaatsvinden. Bovendien wordt beweerd dat transcriptionele inactivatie *de novo* methylatie aantrekt, terwijl transcriptionele activitie *de novo* methylatie kan uitschakelen, met het laatste resulterend in hypomethylatie tijdens de foetale ontwikkeling (72). Daarbij neemt het niveau van blootstelling aan genisteïne en quercetine ook enkel toe doorheen de dracht, waardoor hun effect enkel versterkt wordt. Eerder is al aangetoond dat prenatale blootstelling aan genisteïne de gen expressie kan veranderen door het methylatie patroon te beïnvloeden. Meer bepaald gele agouti muizen die prenataal blootgesteld waren aan genisteïne vertoonden een verandering in vachtkleur en lichaamsgewicht doordat een repetitief element stroomopwaarts van de transcriptie start site van het *Agouti* gen gehypermethyleerd was (31). Daarbij is voor zowel genisteïne als quercetine aangetoond dat ze in staat zijn DNA methyltransferases te remmen om zo hypomethylatie te veroorzaken, meer bepaald van gehypermethyleerde tumor suppressor genen, waaraan hun beschermend effect tegen kanker ook wordt toegeschreven (73, 74). Er is echter ook een weefsel effect zichtbaar wat de methylatie betreft. Hypermethylatie komt voor in het geval van het beenmerg terwijl de lever en long hypomethylatie ondergaan, met name de SINE transcripten. In het begin van de embryogenese worden SINE en LINE repetitieve elementen normaal uitgedrukt, waarna hun expressie snel afneemt. Naderhand wordt de expressie van SINEB1, SINEB2 en LINE1 geassocieerd met cellulaire stress. Dit kan veroorzaakt worden door DNA beschadigende stoffen (75, 76) zoals genisteïne en quercetine, aangezien zij een topoïsomerase II remmende werking hebben, wat aangetoond werd in Hoofdstuk 2. Aangezien SINE en LINE expressie geassocieerd is met kanker (75, 76) kan dit betekenen dat een flavonoïden stimulus *in utero* SINEB1, SINEB2 en LINE1 expressie kan onderdrukken om zo te beschermen tegen genoom instabiliteit op latere leeftijd.

Implicaties en verder onderzoek

Omdat dit slechts het begin is van inzicht te verkrijgen in hoe genisteïne en quercetine inname door de moeder een effect kan hebben op het risico van de nakomelingen om kanker te ontwikkelen op latere leeftijd, moeten verdere studies uitgevoerd worden:

- Betreffende het epigenetische mechanisme dat betrokken is bij de genisteïne en quercetine veroorzaakte foetale programmering. De 'critical window' van de blootstelling moet achterhaald worden.
- Ondanks dat genisteïne en quercetine beide flavonoïden zijn, oefenen ze toch verschillende effecten uit. Daarom moet hun specifieke werking verder onderzocht worden.
- Als de muizen 12 weken oud zijn, lijkt het alsof prenatale blootstelling aan genisteïne en quercetine over het algemeen voordelige effecten heeft veroorzaakt ten opzichte van de gevoeligheid voor kanker. Deze muizen zijn echter nog relatief jong, waardoor de werkelijke bijdrage van de aanpassingen gemaakt door de foetus als reactie op de *in utero* blootstelling aan genisteïne en quercetine misschien nog niet zichtbaar zijn op deze leeftijd. Daarom dat toekomstige studies op oudere muizen uitgevoerd moeten worden. Onderzoek naar het effect van foetale programmering zou ook op latere leeftijd moeten gebeuren, wanneer deze muizen te maken krijgen met chronische ziektes. Deze chronische ziektes zouden ook geïnduceerd kunnen worden aan de hand van pro-inflammatoire stimuli.
- Het zou ook interessant zijn te kijken naar het effect van levenslange blootstelling aan genisteïne en quercetine met betrekking tot de gevoeligheid voor het ontwikkelen van kanker.
- Het zou ook waardevol zijn te kijken naar het effect van prenatale blootstelling aan genisteïne en quercetine in volgende generaties. Echter, om de werkelijke erfelijkheid te bepalen, zou de derde generatie, de eerste niet blootgestelde generatie, onderzocht moeten worden. De eerste generatie is namelijk direct blootgesteld geweest aan genisteïne of quercetine, terwijl de tweede generatie ontstaat uit voortplantingscellen die blootgesteld zijn aan genisteïne of quercetine (Figuur 1).

Moeder: ouderlijke generatie
Inname van flavonoïden via de voeding

Gameten: **2de** generatie
Blootgesteld aan flavonoïden tijdens gametogenese

Foetus: **1ste** generatie
Blootgesteld aan flavonoïden tijdens foetale ontwikkeling

Figuur 1. Overzicht van hoe de effecten geïnduceerd door genisteïne of quercetine blootstelling overgeërfd kan worden door volgende generaties.

Wanneer alle resultaten beschreven in deze thesis opgesomd worden, kan geconcludeerd worden dat prenatale blootstelling aan quercetine en genisteïne de foetale programmering veranderd in vergelijking met een niet-gesupplementeerd dieet tijdens de zwangerschap.

Tijdens de dracht, wanneer de foetussen direct blootgesteld zijn aan flavonoïden, treden veranderingen op in de gen expressie van genen die betrokken zijn bij het antioxidante verdedigingssysteem en bij het carcinogeen metabolisme. Dit doet vermoeden dat foetussen zich aanpasten aan de *in utero* omgeving. Eens geboren viel de blootstelling aan genisteïne en quercetine weg. Sommige veranderingen in gen expressie bleven echter behouden tot op volwassen leeftijd, terwijl andere verkregen werden doorheen het leven, wat erop wijst dat de nakomelingen zich probeerden aan te passen aan de nieuwe 'onverwachte' omgeving.

Op de leeftijd van 12 weken lijkt het dat prenatale blootstelling aan genisteïne en quercetine voordelig blijkt te zijn, aangezien de muizen minder oxidatieve stress geïnduceerde DNA schade hadden ten gevolge van een verhoogde enzymatische antioxidante verdedigingssysteem en omdat ze op een meer efficiëntere manier ijzer opsloegen. Het lijkt ook dat ze onder deze condities beter overweg konden met B[a]P geïnduceerde DNA schade door de verhoogde expressie van fase I en II enzymen. Daarbij vertoonden deze muizen ook een verhoogde methylatie van bepaalde repetitieve elementen dat erop wijst dat deze dieren meer beschermd waren tegen genoom instabiliteit, waardoor ze minder gevoelig leken voor het ontwikkelen van kanker. Aan de andere kant veroorzaakte prenatale blootstelling aan genisteïne en quercetine ook *MII* translocaties waardoor het risico op leukemie verhoogd was, zeker voor muizen met een beperkt DNA herstel. Daarbij was ook de basale expressie van een aantal inflammatoire cytokines verhoogd, wat de inflammatoire respons kan beïnvloeden.

Een algemeen overzicht van het effect van prenatale blootstelling aan genisteïne en quercetine op het risico voor de nakomeling om kanker te ontwikkelen, wordt weergegeven in Figuur 2.
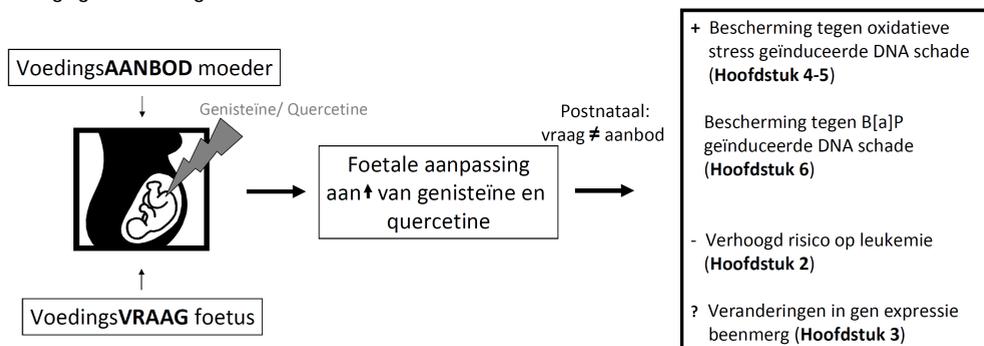
Figuur 2. Algemeen overzicht van het effect van prenatale blootstelling aan genisteïne en quercetine op het risico voor de nakomelingen om kanker te ontwikkelen beschreven in deze thesis.

Referenties

1. Darnton-Hill, I., Nishida, C., and James, W. P. (2004) A life course approach to diet, nutrition and the prevention of chronic diseases. *Public Health Nutr* 7, 101-121
2. Khan, T. H., Jahangir, T., Prasad, L., and Sultana, S. (2006) Inhibitory effect of apigenin on benzo(a)pyrene-mediated genotoxicity in Swiss albino mice. *J Pharm Pharmacol* 58, 1655-1660
3. Muhammad, S., Bierhaus, A., and Schwaninger, M. (2009) Reactive oxygen species in diabetes-induced vascular damage, stroke, and Alzheimer's disease. *J Alzheimers Dis* 16, 775-785
4. Tabner, B. J., Turnbull, S., El-Agnaf, O., and Allsop, D. (2001) Production of reactive oxygen species from aggregating proteins implicated in Alzheimer's disease, Parkinson's disease and other neurodegenerative diseases. *Curr Top Med Chem* 1, 507-517
5. Touyz, R. M. (2004) Reactive oxygen species and angiotensin II signaling in vascular cells -- implications in cardiovascular disease. *Braz J Med Biol Res* 37, 1263-1273
6. Weinberg, F., and Chandel, N. S. (2009) Reactive oxygen species-dependent signaling regulates cancer. *Cell Mol Life Sci* 66, 3663-3673
7. Arts, I. C., and Hollman, P. C. (2005) Polyphenols and disease risk in epidemiologic studies. *Am J Clin Nutr* 81, 317S-325S
8. Hollman, P. C., and Katan, M. B. (1999) Dietary flavonoids: intake, health effects and bioavailability. *Food Chem Toxicol* 37, 937-942
9. Ross, J. A., and Kasum, C. M. (2002) Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annu Rev Nutr* 22, 19-34
10. Yao, L. H., Jiang, Y. M., Shi, J., Tomas-Barberan, F. A., Datta, N., Singanusong, R., and Chen, S. S. (2004) Flavonoids in food and their health benefits. *Plant Foods Hum Nutr* 59, 113-122
11. Erdman, J. W., Jr., Balentine, D., Arab, L., Beecher, G., Dwyer, J. T., Folts, J., Harnly, J., Hollman, P., Keen, C. L., Mazza, G., Messina, M., Scalbert, A., Vita, J., Williamson, G., and Burrowes, J. (2007) Flavonoids and heart health: proceedings of the ILSI North America Flavonoids Workshop, May 31-June 1, 2005, Washington, DC. *J Nutr* 137, 718S-737S
12. Aherne, S. A., and O'Brien, N. M. (2002) Dietary flavonols: chemistry, food content, and metabolism. *Nutrition* 18, 75-81
13. Diplock, A. T., Charleux, J. L., Crozier-Willi, G., Kok, F. J., Rice-Evans, C., Roberfroid, M., Stahl, W., and Vina-Ribes, J. (1998) Functional food science and defence against reactive oxidative species. *Br J Nutr* 80 Suppl 1, S77-112
14. Skibola, C. F., and Smith, M. T. (2000) Potential health impacts of excessive flavonoid intake. *Free Radic Biol Med* 29, 375-383
15. Ferrali, M., Signorini, C., Caciotti, B., Sugherini, L., Ciccoli, L., Giachetti, D., and Comporti, M. (1997) Protection against oxidative damage of erythrocyte membrane by the flavonoid quercetin and its relation to iron chelating activity. *FEBS Lett* 416, 123-129
16. Kitagawa, S., Sakamoto, H., and Tano, H. (2004) Inhibitory effects of flavonoids on free radical-induced hemolysis and their oxidative effects on hemoglobin. *Chem Pharm Bull (Tokyo)* 52, 999-1001
17. Marik, R., Allu, M., Anchoori, R., Stearns, V., Umbricht, C. B., and Khan, S. (2011) Potent genistein derivatives as inhibitors of estrogen receptor alpha-positive breast cancer. *Cancer Biol Ther* 11, 883-892
18. Miodini, P., Fioravanti, L., Di Fronzo, G., and Cappelletti, V. (1999) The two phyto-oestrogens genistein and quercetin exert different effects on oestrogen receptor function. *Br J Cancer* 80, 1150-1155
19. Espin, J. C., Garcia-Conesa, M. T., and Tomas-Barberan, F. A. (2007) Nutraceuticals: facts and fiction. *Phytochemistry* 68, 2986-3008
20. Harwood, M., Danielewska-Nikiel, B., Borzelleca, J. F., Flamm, G. W., Williams, G. M., and Lines, T. C. (2007) A critical review of the data related to the safety of quercetin and lack of evidence of in vivo toxicity, including lack of genotoxic/carcinogenic properties. *Food Chem Toxicol* 45, 2179-2205
21. Sullivan, K. M., Ford, E. S., Azrak, M. F., and Mokdad, A. H. (2009) Multivitamin use in pregnant and nonpregnant women: results from the Behavioral Risk Factor Surveillance System. *Public Health Rep* 124, 384-390
22. Baggott, J. E., Oster, R. A., and Tamura, T. (2011) Meta-analysis of cancer risk in folic acid supplementation trials. *Cancer Epidemiol*
23. Bonacasa, B., Siow, R. C., and Mann, G. E. (2011) Impact of dietary soy isoflavones in pregnancy on fetal programming of endothelial function in offspring. *Microcirculation* 18, 270-285
24. Schroder-van der Elst, J. P., van der Heide, D., Rokos, H., Morreale de Escobar, G., and Kohrle, J. (1998) Synthetic flavonoids cross the placenta in the rat and are found in fetal brain. *Am J Physiol* 274, E253-256

25. Ross, J. A., Potter, J. D., Reaman, G. H., Pendergrass, T. W., and Robison, L. L. (1996) Maternal exposure to potential inhibitors of DNA topoisomerase II and infant leukemia (United States): a report from the Children's Cancer Group. *Cancer Causes Control* 7, 581-590

26. Spector, L. G., Xie, Y., Robison, L. L., Heerema, N. A., Hilden, J. M., Lange, B., Felix, C. A., Davies, S. M., Slavin, J., Potter, J. D., Blair, C. K., Reaman, G. H., and Ross, J. A. (2005) Maternal diet and infant leukemia: the DNA topoisomerase II inhibitor hypothesis: a report from the children's oncology group. *Cancer Epidemiol Biomarkers Prev* 14, 651-655

27. Barjesteh van Waalwijk van Doorn-Khosrovani, S., Janssen, J., Maas, L. M., Godschalk, R. W., Nijhuis, J. G., and van Schooten, F. J. (2007) Dietary flavonoids induce MLL translocations in primary human CD34+ cells. *Carcinogenesis* 28, 1703-1709

28. Spring, K., Cross, S., Li, C., Watters, D., Ben-Senior, L., Waring, P., Ahangari, F., Lu, S. L., Chen, P., Misko, I., Paterson, C., Kay, G., Smorodinsky, N. I., Shiloh, Y., and Lavin, M. F. (2001) Atm knock-in mice harboring an in-frame deletion corresponding to the human ATM 7636del9 common mutation exhibit a variant phenotype. *Cancer Res* 61, 4561-4568

29. Spring, K., Ahangari, F., Scott, S. P., Waring, P., Purdie, D. M., Chen, P. C., Hourigan, K., Ramsay, J., McKinnon, P. J., Swift, M., and Lavin, M. F. (2002) Mice heterozygous for mutation in Atm, the gene involved in ataxia-telangiectasia, have heightened susceptibility to cancer. *Nat Genet* 32, 185-190

30. Barker, D. J., and Clark, P. M. (1997) Fetal undernutrition and disease in later life. *Rev Reprod* 2, 105-112

31. Dolinoy, D. C., Weidman, J. R., Waterland, R. A., and Jirtle, R. L. (2006) Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. *Environ Health Perspect* 114, 567-572

32. Zhou, Y., and Mi, M. T. (2005) Genistein stimulates hematopoiesis and increases survival in irradiated mice. *J Radiat Res (Tokyo)* 46, 425-433

33. Reuter, S., Gupta, S. C., Chaturvedi, M. M., and Aggarwal, B. B. (2010) Oxidative stress, inflammation, and cancer: how are they linked? *Free Radic Biol Med* 49, 1603-1616

34. Boots, A. W., Li, H., Schins, R. P., Duffin, R., Heemskerk, J. W., Bast, A., and Haenen, G. R. (2007) The quercetin paradox. *Toxicol Appl Pharmacol* 222, 89-96

35. Choi, E. J., Chee, K. M., and Lee, B. H. (2003) Anti- and prooxidant effects of chronic quercetin administration in rats. *Eur J Pharmacol* 482, 281-285

36. Kaspar, J. W., and Jaiswal, A. K. (2011) Tyrosine phosphorylation controls nuclear export of Fyn, allowing Nrf2 activation of cytoprotective gene expression. *FASEB J* 25, 1076-1087

37. Cao, G., Sofic, E., and Prior, R. L. (1997) Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free Radic Biol Med* 22, 749-760

38. Galati, G., Sabzevari, O., Wilson, J. X., and O'Brien, P. J. (2002) Prooxidant activity and cellular effects of the phenoxyl radicals of dietary flavonoids and other polyphenolics. *Toxicology* 177, 91-104

39. Ullah, M. F., Shamim, U., Hanif, S., Azmi, A. S., and Hadi, S. M. (2009) Cellular DNA breakage by soy isoflavone genistein and its methylated structural analogue biochanin A. *Mol Nutr Food Res* 53, 1376-1385

40. Vargas, A. J., and Burd, R. (2010) Hormesis and synergy: pathways and mechanisms of quercetin in cancer prevention and management. *Nutr Rev* 68, 418-428

41. Boots, A. W., Drent, M., de Boer, V. C., Bast, A., and Haenen, G. R. (2011) Quercetin reduces markers of oxidative stress and inflammation in sarcoidosis. *Clin Nutr* 30, 506-512

42. Siow, R. C., and Mann, G. E. (2010) Dietary isoflavones and vascular protection: activation of cellular antioxidant defenses by SERMs or hormesis? *Mol Aspects Med* 31, 468-477

43. Munoz, M., Villar, I., and Garcia-Erce, J. A. (2009) An update on iron physiology. *World J Gastroenterol* 15, 4617-4626

44. Zhang, A. S., and Enns, C. A. (2009) Molecular mechanisms of normal iron homeostasis. *Hematology Am Soc Hematol Educ Program*, 207-214

45. Kingsley, P. D., Malik, J., Emerson, R. L., Bushnell, T. P., McGrath, K. E., Bloedorn, L. A., Bulger, M., and Palis, J. (2006) "Maturational" globin switching in primary primitive erythroid cells. *Blood* 107, 1665-1672

46. Palis, J. (2008) Ontogeny of erythropoiesis. *Curr Opin Hematol* 15, 155-161

47. Dennery, P. A. (2007) Effects of oxidative stress on embryonic development. *Birth Defects Res C Embryo Today* 81, 155-162

48. Jauniaux, E., Gulbis, B., and Burton, G. J. (2003) The human first trimester gestational sac limits rather than facilitates oxygen transfer to the foetus--a review. *Placenta* 24 Suppl A, S86-93

49. Amoyal, I., and Fibach, E. (2007) Hemoglobin switch in the newborn: a flow cytometry analysis. *Neonatology* 91, 61-68

50. Purdie, A., Wells, R. M., and Brittain, T. (1983) Molecular aspects of embryonic mouse haemoglobin ontogeny. *Biochem J* 215, 377-383

51. Comporti, M., Signorini, C., Buonocore, G., and Ciccoli, L. (2002) Iron release, oxidative stress and erythrocyte ageing. *Free Radic Biol Med* 32, 568-576

52. Koury, M. J., and Ponka, P. (2004) New insights into erythropoiesis: the roles of folate, vitamin B12, and iron. *Annu Rev Nutr* 24, 105-131

53. Weiss, G. (2009) Iron metabolism in the anemia of chronic disease. *Biochim Biophys Acta* 1790, 682-693

54. Ohgami, R. S., Campagna, D. R., McDonald, A., and Fleming, M. D. (2006) The Steap proteins are metalloreductases. *Blood* 108, 1388-1394

55. Alexandrov, K., Rojas, M., and Satarug, S. (2010) The critical DNA damage by benzo(a)pyrene in lung tissues of smokers and approaches to preventing its formation. *Toxicol Lett* 198, 63-68

56. Delgado, M. E., Haza, A. I., Arranz, N., Garcia, A., and Morales, P. (2008) Dietary polyphenols protect against N-nitrosamines and benzo(a)pyrene-induced DNA damage (strand breaks and oxidized purines/pyrimidines) in HepG2 human hepatoma cells. *Eur J Nutr* 47, 479-490

57. Ciolino, H. P., Daschner, P. J., and Yeh, G. C. (1999) Dietary flavonols quercetin and kaempferol are ligands of the aryl hydrocarbon receptor that affect CYP1A1 transcription differentially. *Biochem J* 340 (Pt 3), 715-722

58. Makaji, E., Ho, S. H., Holloway, A. C., and Crankshaw, D. J. (2011) Effects in rats of maternal exposure to raspberry leaf and its constituents on the activity of cytochrome p450 enzymes in the offspring. *Int J Toxicol* 30, 216-224

59. Moon, Y. J., Wang, X., and Morris, M. E. (2006) Dietary flavonoids: effects on xenobiotic and carcinogen metabolism. *Toxicol In Vitro* 20, 187-210

60. Kang, Z. C., Tsai, S. J., and Lee, H. (1999) Quercetin inhibits benzo[a]pyrene-induced DNA adducts in human Hep G2 cells by altering cytochrome P-450 1A1 gene expression. *Nutr Cancer* 35, 175-179

61. Sergent, T., Dupont, I., Van der Heiden, E., Scippo, M. L., Pussemier, L., Larondelle, Y., and Schneider, Y. J. (2009) CYP1A1 and CYP3A4 modulation by dietary flavonoids in human intestinal Caco-2 cells. *Toxicol Lett* 191, 216-222

62. Walle, T., and Walle, U. K. (2007) Novel methoxylated flavone inhibitors of cytochrome P450 1B1 in SCC-9 human oral cancer cells. *J Pharm Pharmacol* 59, 857-862

63. Nelson, D. R., Koymans, L., Kamataki, T., Stegeman, J. J., Feyereisen, R., Waxman, D. J., Waterman, M. R., Gotoh, O., Coon, M. J., Estabrook, R. W., Gunsalus, I. C., and Nebert, D. W. (1996) P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* 6, 1-42

64. Santini, S. E., Basini, G., Bussolati, S., and Grasselli, F. (2009) The phytoestrogen quercetin impairs steroidogenesis and angiogenesis in swine granulosa cells in vitro. *J Biomed Biotechnol* 2009, 419891

65. Abbott, B. D., Birnbaum, L. S., and Perdew, G. H. (1995) Developmental expression of two members of a new class of transcription factors: I. Expression of aryl hydrocarbon receptor in the C57BL/6N mouse embryo. *Dev Dyn* 204, 133-143

66. Kohle, C., and Bock, K. W. (2007) Coordinate regulation of Phase I and II xenobiotic metabolisms by the Ah receptor and Nrf2. *Biochem Pharmacol* 73, 1853-1862

67. Niestroy, J., Barbara, A., Herbst, K., Rode, S., van Liempt, M., and Roos, P. H. (2011) Single and concerted effects of benzo[a]pyrene and flavonoids on the AhR and Nrf2-pathway in the human colon carcinoma cell line Caco-2. *Toxicol In Vitro* 25, 671-684

68. Ten Have-Opbroek, A. A. (1991) Lung development in the mouse embryo. *Exp Lung Res* 17, 111-130

69. Zaret, K. S. (2002) Regulatory phases of early liver development: paradigms of organogenesis. *Nat Rev Genet* 3, 499-512

70. Uno, S., Dalton, T. P., Dragin, N., Curran, C. P., Derkenne, S., Miller, M. L., Shertzer, H. G., Gonzalez, F. J., and Nebert, D. W. (2006) Oral benzo[a]pyrene in Cyp1 knockout mouse lines: CYP1A1 important in detoxication, CYP1B1 metabolism required for immune damage independent of total-body burden and clearance rate. *Mol Pharmacol* 69, 1103-1114

71. Waterston, R. H., Lindblad-Toh, K., Birney, E., Rogers, J., Abril, J. F., Agarwal, P., Agarwala, R., Ainscough, R., Alexandersson, M., An, P., Antonarakis, S. E., Attwood, J., Baertsch, R., Bailey, J., Barlow, K., Beck, S., Berry, E., Birren, B., Bloom, T., Bork, P., Botcherby, M., Bray, N., Brent, M. R., Brown, D. G., Brown, S. D., Bult, C., Burton, J., Butler, J., Campbell, R. D., Carninci, P., Cawley, S., Chiaromonte, F., Chinwalla, A. T., Church, D. M., Clamp, M., Clee, C., Collins, F. S., Cook, L. L., Copley, R. R., Coulson, A., Couronne, O., Cuff, J., Curwen, V., Cutts, T., Daly, M., David, R., Davies, J., Delehaunty, K. D., Deri, J., Dermitzakis, E. T., Dewey, C., Dickens, N. J., Diekhans, M., Dodge, S., Dubchak, I., Dunn, D. M., Eddy, S. R., Elnitski, L., Emes, R. D., Eswara, P., Eyras, E., Felsenfeld, A., Fewell, G. A., Flicek, P., Foley, K., Frankel, W. N., Fulton, L. A., Fulton, R. S., Furey, T. S., Gage, D., Gibbs, R. A., Glusman, G., Gnerre, S., Goldman, N., Goodstadt, L., Grafham, D., Graves, T. A., Green, E. D., Gregory, S., Guigo, R., Guyer, M., Hardison, R. C., Haussler, D., Hayashizaki, Y., Hillier, L. W., Hinrichs, A., Hlavina, W., Holzer, T., Hsu, F., Hua, A., Hubbard, T., Hunt, A., Jackson, I., Jaffe, D. B., Johnson, L. S., Jones, M., Jones, T. A., Joy, A., Kamal, M., Karlsson, E. K., Karolchik, D., Kasprzyk, A., Kawai, J., Keibler, E., Kells, C.,

Kent, W. J., Kirby, A., Kolbe, D. L., Korf, I., Kucherlapati, R. S., Kulbokas, E. J., Kulp, D., Landers, T., Leger, J. P., Leonard, S., Letunic, I., Levine, R., Li, J., Li, M., Lloyd, C., Lucas, S., Ma, B., Maglott, D. R., Mardis, E. R., Matthews, L., Mauceli, E., Mayer, J. H., McCarthy, M., McCombie, W. R., McLaren, S., McLay, K., McPherson, J. D., Meldrim, J., Meredith, B., Mesirov, J. P., Miller, W., Miner, T. L., Mongin, E., Montgomery, K. T., Morgan, M., Mott, R., Mullikin, J. C., Muzny, D. M., Nash, W. E., Nelson, J. O., Nhan, M. N., Nicol, R., Ning, Z., Nusbaum, C., O'Connor, M. J., Okazaki, Y., Oliver, K., Overton-Larty, E., Pachter, L., Parra, G., Pepin, K. H., Peterson, J., Pevzner, P., Plumb, R., Pohl, C. S., Poliakov, A., Ponce, T. C., Ponting, C. P., Potter, S., Quail, M., Reymond, A., Roe, B. A., Roskin, K. M., Rubin, E. M., Rust, A. G., Santos, R., Sapojnikov, V., Schultz, B., Schultz, J., Schwartz, M. S., Schwartz, S., Scott, C., Seaman, S., Searle, S., Sharpe, T., Sheridan, A., Shownkeen, R., Sims, S., Singer, J. B., Slater, G., Smit, A., Smith, D. R., Spencer, B., Stabenau, A., Stange-Thomann, N., Sugnet, C., Suyama, M., Tesler, G., Thompson, J., Torrents, D., Trevaskis, E., Tromp, J., Ucla, C., Ureta-Vidal, A., Vinson, J. P., Von Niederhausern, A. C., Wade, C. M., Wall, M., Weber, R. J., Weiss, R. B., Wendl, M. C., West, A. P., Wetterstrand, K., Wheeler, R., Whelan, S., Wierzbowski, J., Willey, D., Williams, S., Wilson, R. K., Winter, E., Worley, K. C., Wyman, D., Yang, S., Yang, S. P., Zdobnov, E. M., Zody, M. C., and Lander, E. S. (2002) Initial sequencing and comparative analysis of the mouse genome. *Nature* 420, 520-562

72. Waterland, R. A., Kellermayer, R., Rached, M. T., Tatevian, N., Gomes, M. V., Zhang, J., Zhang, L., Chakravarty, A., Zhu, W., Laritsky, E., Zhang, W., Wang, X., and Shen, L. (2009) Epigenomic profiling indicates a role for DNA methylation in early postnatal liver development. *Hum Mol Genet* 18, 3026-3038

73. Duthie, S. J. (2011) Epigenetic modifications and human pathologies: cancer and CVD. *Proc Nutr Soc* 70, 47-56

74. Li, Y., and Tollefsbol, T. O. (2010) Impact on DNA methylation in cancer prevention and therapy by bioactive dietary components. *Curr Med Chem* 17, 2141-2151

75. Kramerov, D. A., and Vassetzky, N. S. (2005) Short retroposons in eukaryotic genomes. *Int Rev Cytol* 247, 165-221

76. Stribinskis, V., and Ramos, K. S. (2006) Activation of human long interspersed nuclear element 1 retrotransposition by benzo(a)pyrene, an ubiquitous environmental carcinogen. *Cancer Res* 66, 2616-2620