Prenatal exposure to flavonoids Short- and long-term effects

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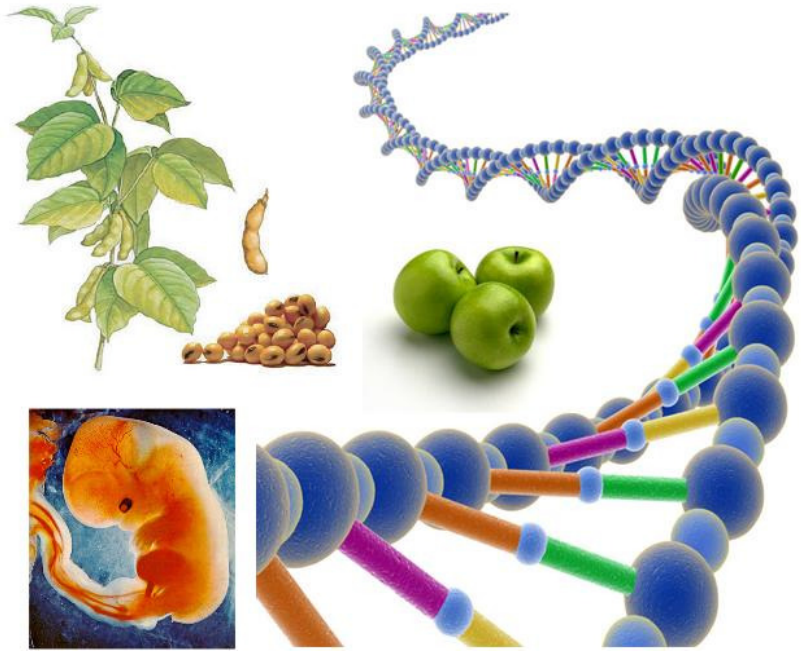

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List of abbreviations

SERM	Selective estrogen receptor modulator
ROS	Reactive oxygen species
BFU-E	Burst-forming unit-erythroid
CFU-E	Colony-forming unit-erythroid
HSC	Hematopoietic stem cell
AGM	Aorta-gonad-mesonephros
RBC	Red blood cell
EPO	Erythropoietin
HIF-1	Hypoxia-inducible factor
TopoII	Topoisomerase II
MLL	Mixed-lineage leukemia
Hox	Homeobox
ALL	Acute lymphoblastic leukemia
AML	Acute myologenous leukemia
DSB	DNA double strand break
E14.5	Embryonic day 14.5
RT-QPCR	Quantitative real-time polymerase chain reaction
GPA	Glycophorin A
HO-1	Heme-oxygenase 1
iPCR	Inverse polymerase chain reaction
WBC	White blood cell
MCV	Mean corpuscular volume
RDW	Red blood cell distribution width
CHr	Hemoglobin content of reticulocytes

Preface

The past eight months of hard work and writing this thesis have gone by so fast, it's hard to believe the end of my last master year is nearly here. I would like to take the opportunity to show my upmost gratitude to several people who have helped me along the way.

First of all I would like to thank Dr. Sahar Khosrovani for giving me the opportunity to do my senior internship with her. Thank you for always leaving your door open so I could pop in countless times with questions and problems. You were always prepared to listen and gave me helpful advice. Furthermore, you gave me the freedom to explore on my own and come up with my own ideas, which greatly helped me to become independent and confident.

I would also like to thank Kimberly Vanhees because she helped me to initiate my experiments in the lab, showed me around and made me feel right at home. Thank you for your tips and suggestions when it came to writing this thesis.

Furthermore, I would like to thank one of my fellow students and best friend Liesbeth. Thank your for your support not only during this last year, but during the past five years! The other interns at GRAT must not be forgotten – Danielle, Astrid, Ingrid, Lore, Najat, Anke, Katrien, Erik, Bakir – thank you for letting me pour my heart out when something went wrong! We had some great times together in the 'computer room' and could always depend on each other.

Finally, I would like to thank the people closest to me; my boyfriend Pieter-Jan, my parents, my brother and sister, for supporting me during the past five years, keeping me motivated and believing in me.

Abstract

Flavonoids are plant polyphenols and widely distributed in fruits and vegetables. They are well-known antioxidants and due to claimed health benefits they are also commercially available as dietary supplements. Unfortunately, the potentially toxic effects of an excessive flavonoid intake are largely ignored. Since flavonoids readily cross the placenta and are even able to accumulate, the unborn fetus may be particularly at risk when the maternal diet contains an excessive amount of flavonoids. Moreover, during this critical and sensitive period of early life, imbalances in maternal nutrition and exposure to certain compounds (e.g. flavonoids) can lead to short-, as well as long-term effects, a phenomenon known as fetal programming.

Preliminary data from our department indicated that prenatal exposure to flavonoids could induce long-term hematological changes. This led us to further investigate the short- and long-term effects of a prenatal exposure to the ubiquitous flavonoids quercetin and genistein. Because flavonoids are more than just dietary antioxidants and are also able to inhibit topoisomerase II, we investigated whether prenatal exposure to flavonoids was associated with an increased cancer risk later in life.

In this study, we observed that a prenatal exposure to flavonoids lead to an accelerated maturation of the erythroid lineages during fetal development and an altered blood composition in adult mice, with differences between the two flavonoids. This underlines the heterogeneity of the plant polyphenols and their pleiotropic effects. Furthermore, only mice that were prenatally exposed to genistein were observed to have a higher risk for developing tumours.

These data warrant further investigations into the role of dietary flavonoids during pregnancy and the mechanisms by which they affect developmental and postnatal erythropoiesis and their role in cancer. Ultimately, since we observed potentially harmful effects of a prenatal flavonoid exposure and because they are being increasingly consumed either via the diet or supplementation, it is important to raise public awareness about the detrimental effects of flavonoids.

1. Introduction

1.1 Flavonoids: too much of a good thing?

Flavonoids are natural compounds (plant polyphenols) and are widely distributed in fruits, vegetables, grains, tea, coffee and red wine[1-3]. It has been estimated that an average western diet contains between 100-1000 mg flavonoids per day[4]. Flavonoids are mostly known for their antioxidant properties, but they have a much wider range of biochemical and pharmacological effects. Flavonoids have been shown to have cytostatic effects in tumerogenesis, antiallergic and antiinflammatory effects, (anti)estrogenic properties and the ability to inhibit/modulate several enzymes such as protein kinase C and topoisomerase II[1 2]. Due to these potentially beneficial properties, flavonoids are being increasingly investigated as anticarcinogens and cardioprotective agents. In addition, due to claimed health benefits, they are marketed as dietary supplements containing high concentrations of flavonoids and recommended in doses that far exceed the amount received from a daily vegetarian diet[1 2]. Manufacturers frequently exaggerate their therapeutic effects, most of which are not confirmed by regulated clinical trials and little regulations exist for these supplements because flavonoids are considered to be 'natural compounds'[5]. It is therefore possible that individuals ingest extremely high levels of flavonoids because of the misconception that if a little of something is good, then more is even better.

Unfortunately, the potentially toxic effects of an excessive flavonoid intake are largely ignored. At higher doses, flavonoids may act as mutagens, pro-oxidants and inhibitors of key enzymes involved in DNA repair (e.g. topoisomerase II), drug-metabolism and hormone balance[1 6]. Additionally, since flavonoids readily cross the placenta, the unborn fetus may be particularly at risk when the maternal diet contains an excessive amount of flavonoids. Furthermore, it has been indicated that flavonoids can even accumulate in the fetus (slower elimination) allowing the fetus to be exposed to very high levels of flavonoids[1 7].

More research is therefore necessary to investigate the toxicological properties of flavonoids, including their effects during pregnancy. Especially because many epidemiological studies exploring the role of flavonoids in human health are inconclusive and because flavonoids are being increasingly consumed either via the diet or supplementation[1 2].

1.1.1 Quercetin – detrimental as an iron-chelator?

Quercetin (3,3',4',5,7-pentahydroxyflavone) is the most frequently-occurring flavonoid in the diet and has potent free radical-scavenging (antioxidant) and iron-chelating properties[8-10]. It is present in high concentrations in onions, apples and many other fruits

and vegetables and is also sold over-the-counter as a dietary supplement[2 8]. Since quercetin is ubiquitously present in foods and it is able to cross the placenta and accumulate in fetal tissues, the prenatal exposure to quercetin was investigated.

As mentioned above, quercetin is an iron-chelator. Iron is essential to life (and during pregnancy[11]) but its metabolism is tightly regulated because excessive iron (iron-overload) leads to detrimental effects. These effects are due to iron's ability to generate harmful oxygen radicals which can induce oxidative stress[9]. Additionally, iron is crucial for erythropoiesis because it is required in large amounts for the production of hemoglobin and an iron-deficiency can lead to anaemia[12]. Previous research demonstrates that the iron-chelating capacity of quercetin is potentially harmful[13]. Polyphenols such as quercetin are able to penetrate the cytoplasm of erythrocytes and react with hemoglobin due to their iron-chelating capacity and oxidize the heme iron. This results in the formation of methemoglobin, which is incapable of binding and transporting O_2. Increased methemoglobin amounts can result in tissue hypoxia[13 14].

1.1.2 Genistein – harmful due to endocrine disrupting capacity?

Genistein (4',5,7-trihydroxyisoflavone) is another well-known flavonoid belonging to the subclass of the isoflavones[1 15]. The effects of a prenatal exposure to this flavonoid were also investigated. Genistein is mainly found in soybeans and other soy-based products such as tofu[15]. Just like quercetin, genistein is a strong antioxidant and is able to cross the placenta[7 16]. However, unlike quercetin which has been shown to act like a pure anti-estrogen, genistein is a well-known phytoestrogen[1 15 17].

Phytoestrogens are defined as "plant compounds with estrogen-like biological activity"[18]. Genistein shares common features with the major endogenous estrogen 17β-estradiol, allowing it to bind to the estrogen receptors and sex hormone binding proteins. Of all the isoflavones, genistein has the strongest estrogenic activity. However, like other phytoestrogens, genistein also has antiestrogenic activity[6 15]. Due to this double mode of action on the estrogen receptor, genistein is sometimes referred to as a selective estrogen receptor modulator (SERM).

A high dietary consumption of genistein (especially prominent in Asian countries) has been linked to a number of potential health benefits. Genistein has been associated with decreased prostate cancer and breast cancer risk. On the contrary, some studies suggest that genistein has the potential to have the opposite effect and increase the risk for cancer (including childhood leukemia; see section 1.3)[2 15 19]. In the case of breast cancer, genistein has been shown to enhance the proliferation of estrogen-dependent tumours, illustrating the complexity of the effects of flavonoids on human health[20]. Genistein has also been linked to improved plasma lipids in cardiovascular disease and reduced menopause-related complaints such as osteoporosis and hot flashes[15 21]. Due to these

potential beneficial health effects, genistein is also found in dietary supplements, especially targeted towards menopausal women. Still, the use of such isoflavone supplements remains controversial[6 21].

Moreover, epidemiological data suggest that during the past five decades there has been a progressive decline in human male reproductive health and fertility. This decrease in fertility led to the 'estrogen hypothesis', first proposed by Sharpe et al[22] in 1993. It was hypothesised that the observed decline in male fertility was associated to exposure to endocrine disrupting chemicals in the environment (also during fetal life), more specifically those chemicals that mimic the action of natural estrogens[6 23 24]. Therefore, due to their phytoestrogenic potential, the safety of genistein and other isoflavones in soy-based infant formulas has been recently questioned due to possible hormonal effects. Even so, until now exposure to soy-based infant formulas does not appear to lead to different reproductive outcomes than exposure to cow milk formulas. Nevertheless, more studies investigating the effects of exposure to phytoestrogen-containing products, particularly soy-based infant formulas, are necessary[2 6 25].

1.2 Prenatal exposure to flavonoids and fetal programming

A developing mammalian embryo undergoes an accumulation of critical developmental events from conception through organogenesis. These developmental events encompass numerous changes, all of which are tightly controlled by a large number of molecular processes. Since the developing embryo is dependent on its mother for all nutritional requirements, it is not surprising that imbalances in maternal nutrition and exposure to certain compounds during this critical and sensitive period of early life, can lead to short-, as well as long-term effects, a phenomenon known as fetal or developmental programming[26]. Fetal programming can also be described as "long-term adaptive changes that an organism undergoes in response to an intra-uterine insult"[27]. Although these fetal changes may be beneficial in the womb, in postnatal life – when the circumstances are changed – this fetal programming may have harmful long-term effects. Many different stimuli have been identified as being capable of inducing fetal programming (e.g. maternal nutrition, fetal hypoxia and oxidative stress) in both human and animal studies. These different stimuli can induce a variety of alterations in multiple organ systems[26 27].

Having summarised the properties of the flavonoids under investigation, it is generally hypothesised that prenatal exposure to either quercetin or genistein will induce changes in the developing embryo (fetal programming). Moreover, we assume that these alterations will be related to the antioxidant/pro-oxidant capacity of these compounds and their ability to change the prenatal antioxidant/pro-oxidant balance in such a way that oxygen-dependent or ROS-dependent processes will be programmed differently (Fig.

1) and that this will have consequences in later life. The two processes that were investigated in this study are erythropoiesis and globin switching because these processes are (partly) regulated by ROS- and oxygen-levels. Evidence in both *in vitro* and *in vivo* studies suggests that oxidative stress (ROS) contributes to the regulation of hematopoietic cell homeostasis through its effects on gene expression, transcription factor signalling and cell cycle alterations. Moreover, regulation of oxidative stress is particularly important in erythropoiesis and unregulated ROS-levels have a significant impact on the survival of erythrocytes [28 29]. Furthermore, little is known about the factors that influence the globin switch, but it is now commonly accepted that oxygen tension plays a role in this process. Additionally, there is in vitro evidence that oxygen tension can indeed modulate β-globin switching in embroid bodies[30]. The following sections will discuss these two processes in more detail.

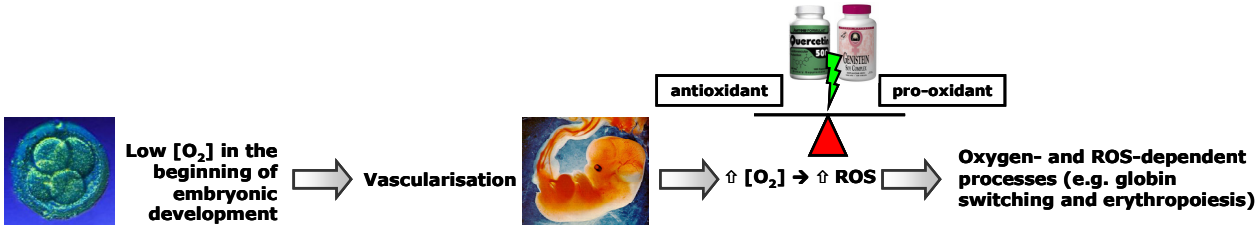

Figure 1. Depiction of the general hypothesis. The early embryo develops in a low oxygen environment to protect the fetal tissues and developmental processes from oxidative damage. Furthermore, hypoxia coordinates the development of e.g. the blood, vasculature and placenta. As vascularisation increases and the oxygen tension rises, so do the amount of reactive oxygen species (ROS). It is hypothesised that prenatal exposure to quercetin or genistein will induce changes in the developing embryo. Moreover, it is assumed that these alterations will be related to the antioxidant/pro-oxidant capacity of these compounds and their ability to change the prenatal antioxidant/pro-oxidant balance in such a way that oxygen-dependent or ROS-dependent processes will be programmed differently, with effects in later life (fetal programming).

1.2.1 Developmental biology of erythropoiesis

Erythropoiesis is crucial for the survival and growth of the developing embryo[12 31]. The newly formed erythrocytes are essential for delivering oxygen to the growing body tissues and are a major component of the antioxidant capacity of the blood[32].

Blood cell development in the mammalian embryo is a very complex process because it occurs in many sites that are separated both spatially and temporally[33]. During development, mammals have two distinct erythroid lineages (Fig. 2). The 'primitive' erythroid lineage originates in the yolk sac from hemangioblast precursors soon after implantation in blood islands at embryonic day 7.5 (E7.5). These large and nucleated, 'primitive' erythroblasts (megaloblasts) mature in the bloodstream. Here they undergo various changes including a limited number of cell divisions, the accumulation of hemoglobin, nuclear condensation, a progressive decrease in cell size and eventually

enucleation. This loss of the nucleus results in the formation of a pyrenocyte and a reticulocyte[31 34 35].

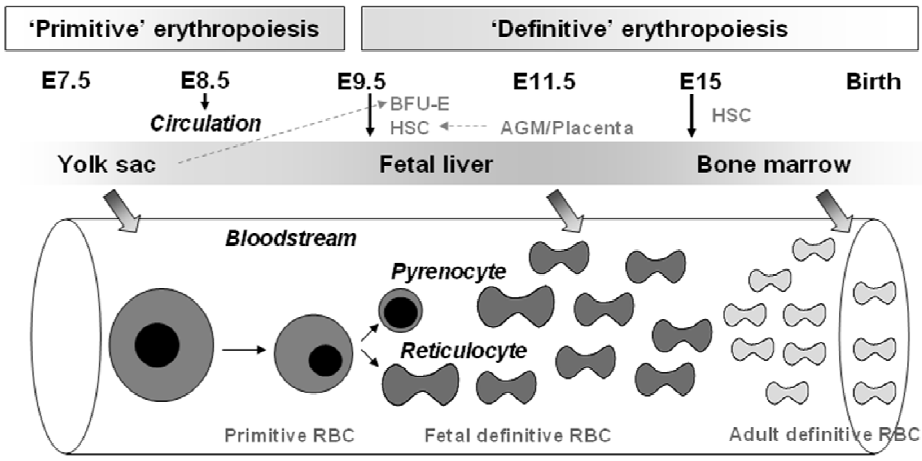

Figure 2. Erythroid lineages during developmental erythropoiesis. The 'primitive' erythroid lineage originates in the yolk sac from hemangioblast precursors (E7.5). When the fetal liver is seeded at E9.5, it serves as the major hematopoietic organ (E11.5) generating the 'definitive' erythroid lineage. Eventually, late in fetal life and after birth, the spleen (E12.5) and bone marrow (E15) will become colonized by hematopoietic stem cells (HSCs) and will be responsible for the adult 'definitive' erythropoiesis. [This figure was adapted from[33 35].]

Later, when circulation is established at E8.5, the fetal liver is seeded (E9.5) by 'definitive' hematopoietic progenitors (BFU-E) and hematopoietic stem cells (HSCs) from the yolk sac and the aorta-gonad-mesonephros (AGM) region/placenta[36], respectively[33 35 37]. From this point, the fetal liver serves as the major hematopoietic organ (E11.5) generating the 'definitive' erythroid lineage (Fig. 3) which matures extravascularly in erythroblast islands in close association with macrophages.

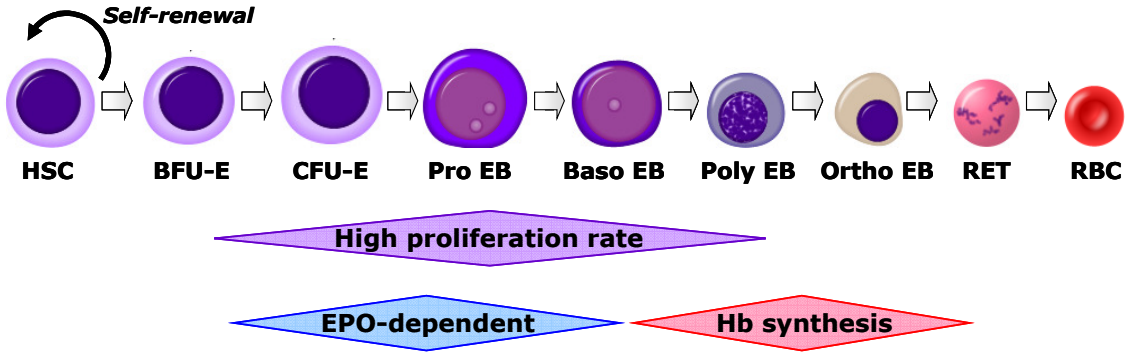

Figure 3. Stages of erythroid differentiation. Specific stages of erythroid differentiation beginning with the pluripotent hematopoietic stem cell, followed by the burst-forming unit-erythroid (BFU-E) and ending with the mature red blood cell (RBC). Multiple cell divisions occur between stages prior to the polychromatophilic erythroblast (poly EB) stage after which the cells do not divide. Periods of high cellular proliferation, erythropoietin (EPO) dependence, and hemoglobin (Hb) synthesis are demarcated. Other abbreviations: CFU-E, colony-forming unit-erythroid; Pro EB, proerythroblast; Baso EB, basophilic erythroblast; Ortho EB, orthochromatic erythroblast; and RET, reticulocyte.

The fetal liver produces smaller, enucleated erythrocytes and these 'typical' biconcave red blood cells (RBC) ultimately become predominant in the fetal and postnatal circulation. Eventually, late in fetal life and after birth, the spleen (E12.5) and bone marrow (E15) will become colonized by HSCs and will be responsible for the adult 'definitive' erythropoiesis[31 35 38].

The primary regulator of definitive erythropoiesis is erythropoietin (EPO) but its role in prenatal erythropoiesis is less well understood. In definitive erythropoiesis, EPO binds a transmembrane receptor expressed by erythroid precursors (Fig. 3) and provides a proliferative signal to early erythroid progenitors (BFU-E, burst-forming unit-erythroid) and a differentiation signal to late erythroid progenitors (CFU-E, colony-forming unit-erythroid)[31]. EPO is produced by the kidneys and its levels are inversely related to the oxygen availability. The transcription factor hypoxia-inducible factor-1 (HIF-1) is a hypoxia sensor and is the major regulator of EPO[39].

1.2.2 Globin switching

Hemoglobin is a tetrameric molecule consisting of two α- and two β-subunits, encoded by the α- and β-globin gene loci. Each subunit is a polypeptide chain folded around a heme group which contains an iron atom that is able to bind oxygen in its ferrous state[14]. The two erythroid lineages present during development have distinct globin expression patterns to produce stage-specific hemoglobins that meet the oxygen demand of the developing fetus.

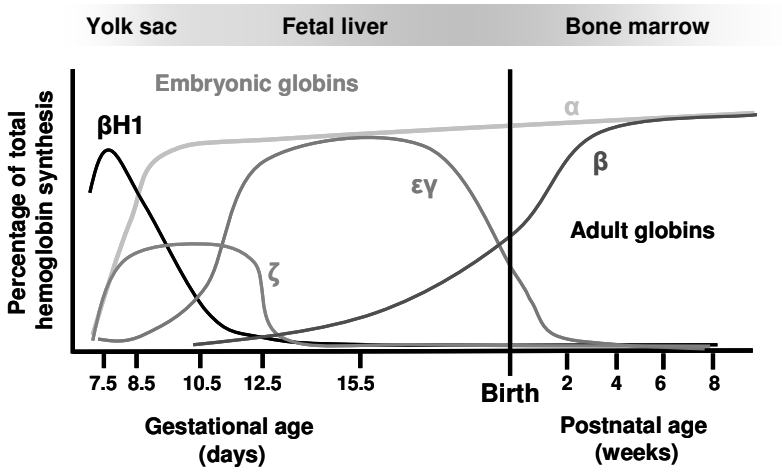

Figure 4. Globin switching in mice. The first 'maturational' switch from βH1- to εγ-globin occurs as primitive erythroid cells mature in the bloodstream. The embryonic globins are only expressed in the primitive erythroid lineage and have a higher O_2-affinity to facilitate O_2-transfer across the placenta. Later, adult β-globins are upregulated. Additionally, there is a switch from embryonic ζ-globins to adult α-globins. [Reproduced with kind permission from K. Vanhees.]

The 'switch' in globin expression coincides temporally with the transition from the 'primitive' to the 'definitive' erythroid lineage. In mice, the β-globin locus contains four

functional genes (εγ-βH1-β1-β2) and in contrast to humans, they have no fetal hemoglobins. Hence, the 'hemoglobin-switch' occurs slightly different in mice (Fig. 4). First there is a 'maturational' switch from βH1- to εy-globin which occurs as primitive erythroid cells mature in the bloodstream. These 'embryonic' globins are only expressed in the 'primitive' erythroid lineage and have a higher oxygen affinity than adult globins which facilitates the transfer of oxygen across the placenta from the maternal to the fetal circulation. Later, adult β1- and β2-globins are upregulated. Furthermore, there is a switch from embryonic ζ-globins to adult α-globins (α1, α2). It should be noted that all of the globin genes in the α- and β-clusters are expressed in 'primitive' RBCs. Conversely, in 'definitive' RBCs only the adult globin genes are expressed[40].

Overall there is a good understanding of the molecular mechanisms controlling globin switching, especially for the β-globin gene[41 42]. However, little is known about the factors that influence the hemoglobin switch and it is assumed that changes in oxygen tension and other environmental factors are regulators of the hemoglobin switch[30 42].

1.3 Flavonoids as topoisomerase II inhibitors and infant leukemia

As previously emphasized, flavonoids are not 'just' dietary antioxidants and have a wide range of biochemical and pharmacological properties that might be beneficial in adults but could be harmful for the unborn fetus. One property, namely their ability to inhibit topoisomerase II (topoII), is particularly interesting because it is believed that exposure to topoII inhibitors in utero is related to infant leukemia risk.

Infant leukemia, diagnosed in the first 12 months of life, is associated with chromosomal abnormalities that originate during fetal development[43 44] and often involves rearrangements in the mixed-lineage leukemia (MLL) gene[45 46]. MLL is a histone methyltransferase that is involved in the positive regulation of homeobox (hox) gene expression and plays a crucial role in hematopoiesis[47]. MLL is affected in 80% of infant acute lymphoblastic leukemia (ALL) and 65% of infant acute myelogenous leukemia (AML) and is associated with a poor to intermediate prognosis[46]. Interestingly, these typical MLL rearrangements have also been observed in therapy-related leukemias in children and adults that were treated with topoII inhibitors (e.g. etoposide)[48 49]. This finding led to the hypothesis that maternal exposure to topoII inhibitors during pregnancy could induce infant leukemias[50].

TopoII is an important enzyme that regulates DNA under- and over-winding, and removes tangles and knots from the genome by inducing transient double strand breaks (DSBs) in the DNA which are subsequently religated. However, in the presence of topoII inhibitors, the topoII-DNA cleavage complex is stabilised and increase the risk for chromosomal translocations[51]. Quercetin and genistein are known dietary topoII inhibitors[52 53] and have been shown to induce DSBs and MLL translocations in primary human $CD34^+$ cells[54]. Additionally, Ross et al. have reported an approximately 10-fold

higher risk of infant AML with increasing maternal consumption of topoII inhibitor containing foods and research is ongoing[55]. Furthermore, the incidence of infant leukemia is almost 2-fold higher in several Asian cities (e.g. Hong Kong), than in Western countries. This difference might be explained by the high food intake of bioflavonoids in many regions of Asia, especially by consumption of soybeans and soybean products[53].

1.4 Aim of this study

The aim of this study was to investigate the short- and long-term effects of a prenatal flavonoid exposure in a mouse model. Since preliminary data from our department indicated that prenatal exposure to flavonoids could induce hematological changes, we investigated the short- and long-term effects of a prenatal exposure on the developmental programming of erythropoiesis. To this end, the short- and long-term effects on blood composition and globin gene expression were investigated. However, because quercetin and genistein are also topoII inhibitors, we investigated whether prenatal exposure to these dietary flavonoids was associated with an increased postnatal cancer (including leukemia) risk. Since MLL translocations are commonly present in leukemias, the effect of DNA repair was investigated too and is indicated by the Atm-genotype of the mice (see section 2.1 Mouse model and experimental design).

2. Materials & Methods

2.1 Mouse model and experimental design

Long-term experiment

ATM-ΔSRI mice carry a human mutation in the Atm (ataxia-telangiectasia-mutated) gene which is involved in DNA repair[56]. Heterozygous ATM-ΔSRI mice (129/SvJ:C57BL/6J background) were mated to obtain wild-type, heterozygous and homozygous mutant offspring with different susceptibility to cancer (Figure **5**. 5A). PCR genotyping was performed as previously described by Spring et al[56].

From three days before conception until the end of the pregnancy, the dams were put on either a normal diet scarce in flavonoids (Sniff®, Soest, Germany), or this chow supplemented with quercetin (338,27 ppm; Sigma-Aldrich, Zwijndrecht, The Netherlands) or genistein (270,24 ppm; LC laboratories, Woburn, USA). These concentrations of flavonoids are based on a dietary intake in humans of approximately 20 mg/kg. After delivery, all mothers and pups received a normal diet regardless of their prenatal diet. Pups were weaned at day 45 and sacrificed twelve weeks after birth, when blood was collected by cardiac puncture and organs were isolated (Figure **5**. 5B). The

collected blood was sent to the National Institute for Public Health and Environment (RIVM, Bilthoven, The Netherlands) to determine blood composition.

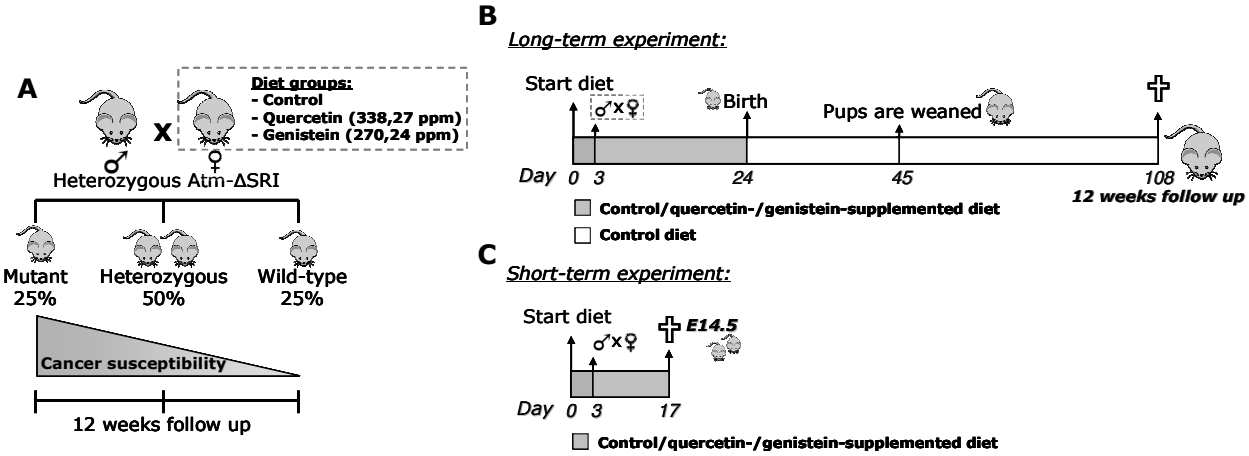

Figure 5. Mouse model and experimental setup. A: Heterozygous ATM-ΔSRI mice are mated to obtain offspring with different genotypes and different cancer susceptibility. The Atm gene is involved in DNA repair and a mutation in this gene predisposes mice for DNA damage and cancer development. **B:** Time-line for long-term experiment. **C:** Time-line for short-term experiment.

Short-term experiment

Wild-type mice (129/SvJ:C57BL/6J background) were mated overnight and vaginal plugs were checked the next morning. Like the long-term experiment, from three days before conception until the end of the pregnancy the dams were put on one of the three diets. Fetuses were sacrificed two weeks after conception, at embryonic day 14.5 (E14.5), when the fetal liver was isolated (Fig. 5C). Fetuses, fetal liver and placentas were washed in 0,9% NaCl and weighed.

2.2 Erythropoietin, bilirubin and iron determination in amniotic fluid

EPO levels were determined in the amniotic fluid of the fetuses using the Immulite® 1000 EPO kit (Siemens AG, Erlangen, Germany) according to manufacturer's protocol. This is a solid-phase chemiluminescent immunometric assay.

Bilirubin levels and total amount of iron were determined in amniotic fluid using the Synchron LX20 autoanalyzer (Beckman Coulter, Woerden, The Netherlands).

2.3 Fetal blood cytology – May-Grünwald staining

Blood smears were made of fetal blood (E14.5) immediately after sacrifice. After initial fixation of the blood smears with methanol, the samples were stained with May-Grünwald solution (Sigma) for 5 minutes. Next, smears were washed with tap water and stained with Giemsa solution (Merck, Darmstadt, Germany) for 20 minutes. Subsequently, smears were again washed in tap water. Blood smears were examined using a Leica DMRXA microscope (Leica, Rijswijk, The Netherlands) under a 620× oil-immersion

objective and analysed using the Leica IM50 image manager (Leica). Two independent and blinded examiners assessed the blood smears and counted approximately 500 cells on each slide.

2.4 Gene expression analysis by quantitative real-time PCR (RT-QPCR)

2.4.1 Analysis of globin gene expression

RNA was isolated from fetal livers and adult bone marrow using respectively TRIzol® reagent (Invitrogen, Breda, The Netherlands) and TRIzol® LS reagent (Invitrogen) as described by the manufacturer's protocol. cDNA was synthesized from 1 µg RNA using the iScript™ cDNA synthesis kit (Bio-Rad, Veenendaal, The Netherlands) and RT-QPCR's were performed using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad). cDNA was diluted 3 or 30 times for RT-QPCR experiments studying the expression of embryonic globins and adult globins, respectively.

RT-QPCR was carried out in 96-well plates containing a reaction volume of 25 µl which consisted of 12.5 µl iQ supermix (Bio-Rad), 300 nM primers (forward and reverse), 200 nM Taqman probe, 2 µl cDNA and sterile water. 5' to 3' sequences of the primers and probes (Eurogentec, Liege, Belgium) are listed in table 1. The β-primer and the α-primer plus probes were designed to measure the expression of both β1/β2 and α1/α2, respectively. Taqman probes were labelled with the 5' reporter 6-Carboxyfluorescein (FAM) and the 3' quencher tetramethylrhodamine (TAMRA) and were designed to span exon-exon junctions to prevent amplification of contaminating genomic DNA. The expression of glycophorin A (GPA) was used as an internal control to ensure that changes in the amount of globin mRNA was not due to changes in the number of viable red blood cells. GPA is an erythroid-specific membrane marker and is expressed in differentiating primitive erythroid cells, as well as definitive erythroid cells[57].

Table 1. RT-QPCR primer and probe sequences

Gene	Forward primer	Reverse primer	Probe
α	AATATGGAGCTGAAGCCCTGG	ATCAAAGTGAGGGAAGTAGGTCT	AAGGATGTTTGCCAGCTTCCCCACTACT
β	GTGAGCTCCACTGTGACAAGCT	GGTGGCCCAGCACAATCACGATC	CATGTGGATCCTGAGAACTTCAGGCTCCT
εγ	CAAGCTACATGTGGATCCTGAGAA	TGCCGAAGTGACTAGCCAAA	TCAAACTCTTGGGTAATGTGCTGGTGATTG
ζ	GCGAGCTGCATGCCTACAT	GCCATTGTGACCAGCAGACA	TGGATCCGGTCAACTTCAAGCTCCTGT
βH1	AGGCAGCTATCACAAGCATCTG	AACTTGTCAAAGAATCTCTGAGTCCAT	AGAAACTCTGGGAAGGCTCCTGATTGTTTACC
GPA	GCCGAATGACAAAGAAAAGTTCA	TCAATAGAACTCAAAGGCACACTGT	TTGACATCCAATCTCCTGAGGGTGGTGA
HO-1	TCCAGAGTTTCCGCATAC	CGGACTGGGCTAGTTCA	/
β-actin	ACGGCCAGGTCATCACTATTG	CAAGAAGGAAGGCTGGAAAAGA	/

Amplification was performed using the following RT-QPCR program; initial heating at 95ºC for 3 minutes, 45 cycles of 95ºC for 15 seconds and 1 minute at 60ºC, followed by

heating for 1 minute at 95 ºC, 1 minute at 65 ºC and steadily increasing temperature from 65 ºC in steps of 0.5 ºC per 10 seconds for 60 cycles.

2.4.2 Analysis of heme-oxygenase 1 (HO-1) expression

RNA was isolated from fetal livers and adult bone marrow as described above. RNA was additionally isolated from adult spleens using TRIzol® reagent (Invitrogen). cDNA synthesis and RT-QPCR were performed as described in section 2.3.1. cDNA was diluted 30 times. RT-QPCR was carried out in 96-well plates containing a reaction volume of 25 µl which consisted of 12.5 µl iQ SYBRgreen supermix (Bio-Rad), 300 nM primers (forward and reverse), 2 µl cDNA and sterile water. 5' to 3' sequences of the primers (Eurogentec) are listed in table 1. The expression of β-actin was used as an internal control. Amplification was performed using the same RT-QPCR programme as described in 2.3.1.

2.4.3 Data analysis and statistics

Each sample was subjected to 3 independent RT-QPCR experiments for the target gene and the internal control. The mean per duplo measurement was used to calculate ΔCT (cycle threshold) values and expression levels ($2^{-\Delta CT}$). For statistical analysis, one-way ANOVA was performed using the SPSS statistical software (v.16 SPSS Inc., Chicago, USA). Differences were considered significant at P-values of less than 0.05.

2.5 Blood composition

Blood composition was determined in duplicate, using the ADVIA® 120 Hematology System (Siemens AG) as described by the manufacturer's protocol. This flow cytometry-based system uses light scatter, differential white blood cell lysis, and peroxidase staining to provide a complete blood cell count, a white blood cell differential, and a reticulocyte count[58]. All samples were submitted to the automatic determination of the following blood cell parameters: total amount of red blood cells (10^{12}/l), hemoglobin level (mmol/l), hematocrit (l/l), mean corpuscular volume (fl), mean corpuscular hemoglobin (fmol), mean cell 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), reticulocyte hemoglobin content (fmol), absolute amount of reticulocytes (10^9/l), number of white blood cells (10^9/l), total amount (10^9/l) and percentage of neutrophils, lymphocytes, monocytes, eosinophils, large unstained cells and basophils.

Statistical analysis

Analysis of blood composition results was done using the SPSS statistical software. One-way ANOVA was used to compare hematological characteristics between different diet

groups. Multiple regression analysis of blood composition parameters was performed to identify confounding variables such as sex and genotype. Chi-square tests were performed to compare the distribution of sex, genotype and tumour development in the diet groups.

2.6 Inverse PCR (iPCR) assay to detect MLL translocations

2.6.1 Bone marrow isolation and cell culture

Bone marrow cells were isolated from 12-week old wild-type, heterozygous and homozygous mutant ATM-ΔSRI mice by flushing the femurs with Iscove's Modified Dulbecco's Medium (IMDM; Invitrogen) supplemented with 10% fetal calf serum (FCS). The cells were washed by centrifuging them for 3 minutes at 1130 rpm and resuspending them in sterile IMDM.

2.6.2 Treatment with quercetin, genistein or etoposide and DNA harvest

The isolated bone marrow cells were plated in 24-well plates and treated with either 50 µM of quercetin (Sigma-Aldrich), genistein (LC Laboratories), etoposide (Sigma-Aldrich) or 0.05% dimethylsulfoxide (DMSO; vehicle control). The cells were then incubated for 24h at 37ºC in a 5% CO_2 humidified incubator. After exposure the cells were washed twice with IMDM and incubated again to recover for 24h. After that, genomic DNA was extracted from the bone marrow cells using the DNeasy Blood & Tissue Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. Cell counts and viability were determined by hemocytometer and 0.4% trypan blue exclusion.

2.6.3 Inverse PCR assay and sequencing

400 ng DNA was incubated with 1 U shrimp alkaline phosphatase (SAP; Promega, Madison, USA) for 1 h at 37°C and subsequently inactivated at 75°C for 20 minutes. Next, the DNA was digested with 5 U PciI (New England Biolabs, Leusden, The Netherlands) for 1 h at 37°C and inactivated at 75°C for 20 minutes. Then, the DNA was circularized overnight at 4°C using T4 DNA ligase (Promega) in a final volume of 50 µl. The reaction was terminated by incubation at 20°C for 2 hours, followed by incubation at 75°C for 20 minutes. Approximately, 32 ng of circularized DNA was used for the first PCR. First and nested PCRs were performed in PCR-tubes containing a reaction volume of 50 µl which consisted of 0.7 µl expand long template DNA polymerase mix (Roche, Mannheim, Germany), 5 µl system 2 reaction buffer (Roche), 400 nM primers (forward and reverse), 2 mM dNTP mix (Roche) and sterile water. 5' to 3' sequences of the first and nested primers (Eurogentec) are listed in Table 2. The first and nested reaction was performed using a Biometra TProfessional thermocycler (Biometra, Leusden, The Netherlands) and the following cycles were used: 3 min at 94°C, 10 cycles: 30 sec at

94°C, 45 sec at 56°C, 3 min at 68°C; 25 cycles: 30 sec at 94°C, 45 sec at 56°C, 3 min (+20 s/cycle) at 68°C; followed by 6 min at 72°C. The amplified DNA fragments from the first PCR were diluted (1/50) and 2 µl was used for the nested PCR. The nested PCR products were separated by gel electrophoresis on a 1% agarose gel with 5 µl ethidium bromide (10 mg/ml; Invitrogen), excised and purified by using the QIAquick Gel Extraction Kit (Qiagen). The purified PCR products were subsequently sequenced using approximately 50-100 ng DNA, 1 µl BigDye v1.1 (Applied Biosystems, Foster City, USA), 1,5 µl 5X sequencing buffer (Applied Biosystems), 2,5 pmol sequencing primer (Table 2) and sterile water. The following cycling conditions were used: 1 min at 96°C, 25 cycles: 30 sec at 96°C, 15 sec at 50°C, 4 min at 60°C, followed by 10 min at 72°C. After purification with Sephadex, the products were sequenced by ABI 3730 Automatic DNA Sequencer (Applied Biosystems). Nucleotide sequences were analyzed using the 'BLAST tool' of NCBI and Ensembl.

Table 2. iPCR and sequencing primers

Name	Forward primer	Reverse primer
First PCR MLL primers	5'- CCAGAGTAGTGTGCTTTCTC -3'	5'- AGGTGGCTTCTCCTGAGACAG -3'
Nested PCR MLL primers	5'- AGTGGGCATGTAGAGGTAAG -3'	5'- AACAATGACTTGCCCTCATATTG -3'
Sequencing MLL primers	5'- CAGCTTGACGTACAGAGTGC -3'	/

2.7 Histology of the thymus

Thymuses were isolated from 12-week old mice and placed in a mould with Tissue-Tek® (Sakura, Zoeterwoude, The Netherlands) to provide a specimen matrix for cryostat sectioning. The thymuses were stored at -80°C until frozen thymus sections of 5 µm were made using the Microm HM560 CryoStar cryo-microtome (Thermo Scientific, Waltham, USA). Hereafter, the sections were allowed to dry for 2 days after which they were stained with hematoxylin-eosin (H&E) staining. Stained thymus (and thymoma) sections were analysed using a Leica DMRXA microscope (Leica) and photomicrographs were made (20X magnification).

2.8 Statistical analysis

All statistical analysis was done using the SPSS statistical software. Exact statistical procedures have been discussed earlier in the different sections above.

3. Results

Short-term effects of a prenatal exposure to flavonoids

3.1 Fetuses exposed to flavonoids during prenatal development have an increased fetal weight at E14.5

To investigate whether prenatal exposure to flavonoids (quercetin: 338.27 ppm and genistein: 270.24 ppm) had an effect on the growth and survival of the developing fetuses, litter size, weight of the fetus and placenta and the percentage of necrotic fetuses was measured (Table 3). The average weight of the placentas showed no significant difference amongst the different diet groups. Average litter size and percentage of necrotic fetuses showed no significant differences amongst the diet groups. Fetal weight on the other hand was significantly increased for the genistein-supplemented ($p=0.008$) diet compared to the control diet. Fetal weight was also increased in the quercetin group, however this increase was not significant ($p=0.216$).

Table 3. Characteristics of the fetal mice at E14.5 within the different diet groups

	Control	Quercetin	Genistein
Litter size	5.5±1.71	7.33±1.04	6±0.71
Weight placenta (mg)	85.97±6.94	84.25±3.63	85.56±6.02
Weight fetus (mg)	163.14±4.67	199.38±11.80	222.52±17.80**
% necrotic fetuses	13	13	4

General characteristics of the fetuses prenatally exposed to a quercetin- (N=22) or genistein-supplemented (N=24) diet or normal diet (N=22). Results represent the mean ± the standard error. Significant differences are depicted by "**" = $p<0.01$.

3.2 Prenatal exposure to flavonoids affects the percentage of primitive and definitive erythroid cells in fetal blood

To investigate the effect of a prenatal exposure to flavonoids on the cells from the two erythroid lineages, blood smears of fetuses (E14.5) were investigated. Prenatal exposure to either quercetin or genistein resulted in a significant decrease ($p=0.001$ and $p=0.0001$, respectively) in the percentage of primitive erythroid cells (Figure 6. 6). Accordingly, the percentage of definitive erythroid cells was significantly increased in both flavonoid exposed groups (quercetin: $p=0.026$; genistein: $p=0.003$). For the quercetin group the increase in definitive erythroid cells was mostly due to the increase in the percentage of basophilic erythroblasts. For the genistein group on the other hand, this increase was mainly due to the increase in the percentage of orthochromic erythroblasts. Generally, the impact on both erythroid cell types was more prominent in the genistein-supplemented group.

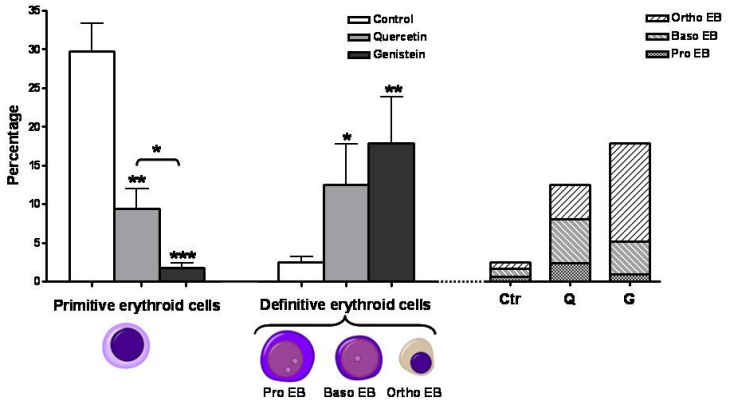

Figure 6. Effect of prenatal exposure to quercetin or genistein on the percentage of primitive and definitive erythroid cells in the fetal blood (E14.5). The percentage of definitive erythroid cells constitutes proerythroblasts (Pro EB), basophilic erythroblasts (Base EB) and orthochromic erythroblasts (Ortho EB). Bars on the right show the contribution of each cell type to the definitive lineage. Error bars represent the standard error. Statistically significant differences are depicted by "*" = $p<0.05$, "**" = $p<0.01$ and "***" = $p<0.001$

To make sure differences in the blood composition were not due to increased age (reflected in the increased fetal weight) in the flavonoid exposed groups, single and multiple linear regression analysis was performed (Table 4). For the quercetin group, the decrease in the percentage of primitive erythroid cells was independent of the fetal and placental weight. For the genistein group, primitive erythrocytes and fetal weight were slightly correlated in the single linear regression analysis. However, when both factors were taken into account in the multiple regression model, only diet was a significant variable ($p=0.000$). For the definitive erythrocytes there was no effect of fetal weight in the univariate model. In the multiple regression model however, when both factors were taken into account, fetal weight was a confounding variable. However, this was due to effect of the genistein-supplemented diet on the fetal weight (Table 3).

Table 4. Single and multiple linear regression analysis of the short-term effects of genistein and quercetin on the blood composition of fetuses at E14.5

	Control vs. Quercetin					**Control vs. Genistein**				
	Univariate			Multivariate		Univariate			Multivariate	
	Diet	*Fetus*[#]	*Placenta*[#]	*Diet*	*Fetus*[#]	*Diet*	*Fetus*[#]	*Placenta*[#]	*Diet*	*Fetus*[#]
Primitive erythrocytes	***0.001***	0.050	0.571	***0.006***	0.552	***0.000***	***0.014***	0.500	***0.000***	0.661
Definitive erythrocytes	***0.026***	***0.012***	0.816	0.206	0.090	***0.003***	0.889	0.624	***0.000***	***0.000***
Proerythroblast	0.059	0.150	0.873	0.177	0.532	0.403	0.600	0.458	0.096	0.124
Basophilic erythroblast	***0.018***	***0.018***	0.849	0.143	0.139	***0.006***	0.524	0.829	***0.002***	0.069
Ortochromic erythroblast	0.055	***0.004***	0.494	0.447	***0.028***	***0.007***	0.767	0.482	***0.000***	***0.000***
Nucleus	0.888	0.129	0.697	0.313	0.074	0.323	0.604	0.749	0.396	0.831

Values in the table represent the p-values of the single and multiple regression coefficients. ***Significant p-values*** are typed bold italic. [#] Fetal or placental weight.

3.3 Prenatal exposure to flavonoids reduces expression of the murine embryonic globins ζ, βH1 and εγ in the fetal blood

Given that prenatal exposure to flavonoids changed the amount of primitive and definitive erythroid cells in fetal blood, the effect on the globin-switch was investigated as well. The expression of the different embryonic and adult globin genes was studied using quantitative real-time PCR to measure the amount of globin transcripts in the fetal blood. Prenatal exposure of fetuses to the different flavonoids resulted in an altered embryonic globin gene expression in fetal blood (Figure 7A).

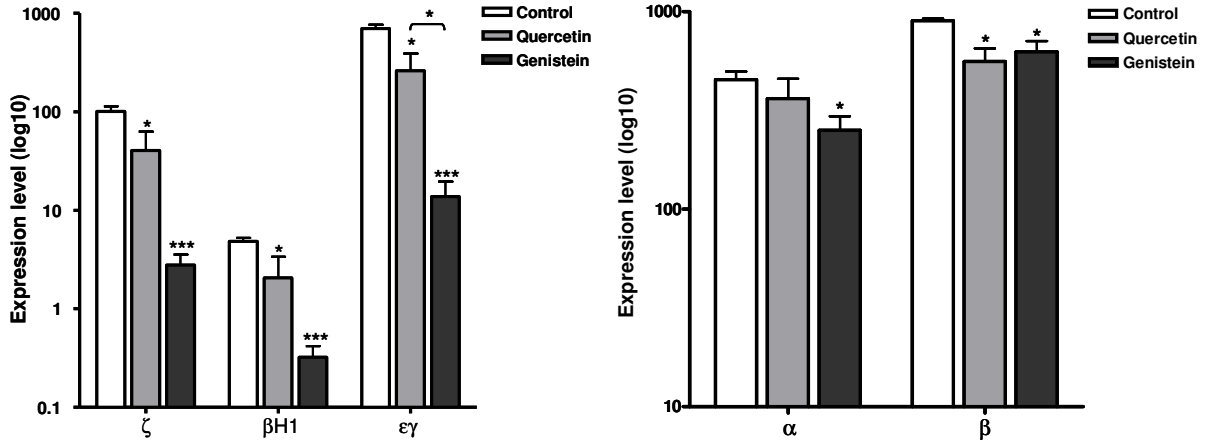

Figure 7. Effect of prenatal exposure to quercetin or genistein on the murine embryonic and adult globin gene expression in fetal blood (E14.5). A: Expression of embryonic globins in the control (N=5), quercetin (N=2) and genistein (N=4) diet groups. **B:** Expression of adult globins in the control, quercetin and genistein diet groups. GPA was used as an internal control. Expression levels were determined by RT-QPCR and calculated for each globin as $2^{-\Delta ct}$ and log10 scale was used for the graphs. Error bars represent the standard error. Statistically significant differences are depicted by "*" = p<0.05, "**"= p< 0.01 or "***"= p<0.001.

Compared to the control group, mRNA levels of all embryonic globin genes (ζ, βH1 and εγ) were significantly reduced in the genistein-supplemented group (p<0.001). For the quercetin-supplemented group there was also a significant decrease (p<0.05) in the gene expression of the different embryonic globin genes, however this reduction was less pronounced compared to the genistein group and for the εγ-globin gene expression there was a significant difference (p=0.037) between the two flavonoid groups

3.4 Prenatal exposure to flavonoids also affects expression of the murine adult globins in the fetal blood

The effect of prenatal flavonoid exposure on adult globin gene expression was studied in fetal blood (Figure 7. 7B). In both the quercetin and genistein exposed group, there was a significant reduction (p<0.05) in the β-globin expression level.

However, the α-globin gene expression was only significantly reduced in the genistein group, compared to either the control diet (p=0.018).

3.5 Prenatal exposure to flavonoids decreases expression of the murine embryonic globins ζ and εγ in the fetal liver

Since the fetal liver is the major site of definitive erythropoiesis during development, the amount of globin mRNA was also measured in the fetal liver. Prenatal exposure of fetuses to the different flavonoids resulted in an altered embryonic globin gene expression in the fetal liver (Figure 8. 8A). Compared to the control group, significantly reduced levels of ζ-globin gene expression were observed in both the quercetin (p=0.035) and genistein group (p=0.027). Similarly, a significant reduction of εγ-globin gene expression in both the quercetin (p=0.012) and genistein (p= 0.020) fetal livers was observed. There was no significant difference in the expression of the murine βH1-globin gene, although its expression was slightly increased in the genistein group.

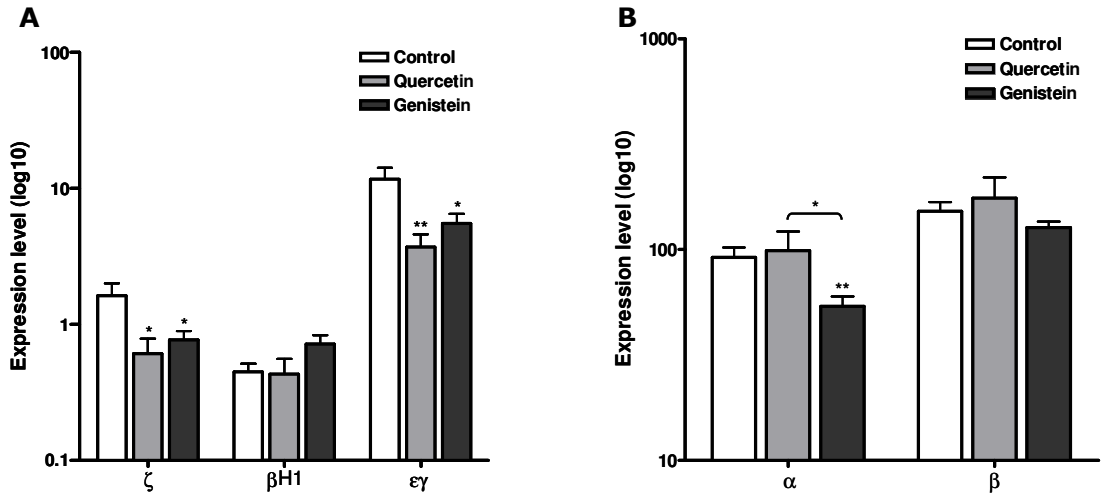

Figure 8. Effect of prenatal exposure to quercetin or genistein on the murine embryonic and adult globin gene expression in the E14.5 fetal liver. A: Expression of embryonic globins in the control (N=9), quercetin (N=8) and genistein (N=12) diet groups. **B:** Expression of adult globins in the control, quercetin and genistein diet groups. GPA was used as an internal control. Expression levels were determined by RT-QPCR and calculated for each globin as $2^{-\Delta ct}$ and log10 scale was used for the graphs. Error bars represent the standard error. Statistically significant differences are depicted by "*" = p<0.05 or "**"= p< 0.01.

3.6 Prenatal exposure to genistein affects expression of the murine adult α-globin gene in the fetal liver

Adult globin gene expression was also studied in the fetal liver (Figure 8. 8B). No significant differences in the expression of the murine β-globin gene were observed in either diet group compared to the control group. However, the α-globin gene expression

was significantly reduced in the genistein group, compared to either the control diet or the quercetin diet (p=0.005 and p=0.038 respectively).

Moreover, when comparing the embryonic and adult globin gene expression in fetal blood and liver, it is observed that the absolute expression of all globin genes is highest in the fetal blood.

3.7 Subtle effect on erythropoietin, bilirubin and iron levels in amniotic fluid after prenatal flavonoid exposure

Since prenatal exposure to flavonoids changed the amount of primitive and definitive erythroid cells in fetal blood and affected globin gene expression, EPO-levels (hormone that controls erythropoiesis), bilirubin levels (breakdown product of heme) and iron levels (component of heme) were measured in amniotic fluid (Fig. 9A-C). No significant changes were observed for either EPO, bilirubin or iron levels in the flavonoid-supplemented groups. Nonetheless, a slight increase in bilirubin levels was observed for both flavonoid exposed groups. Additionally, quercetin's iron chelating capacity was visible as a reduction in the total amount of iron.

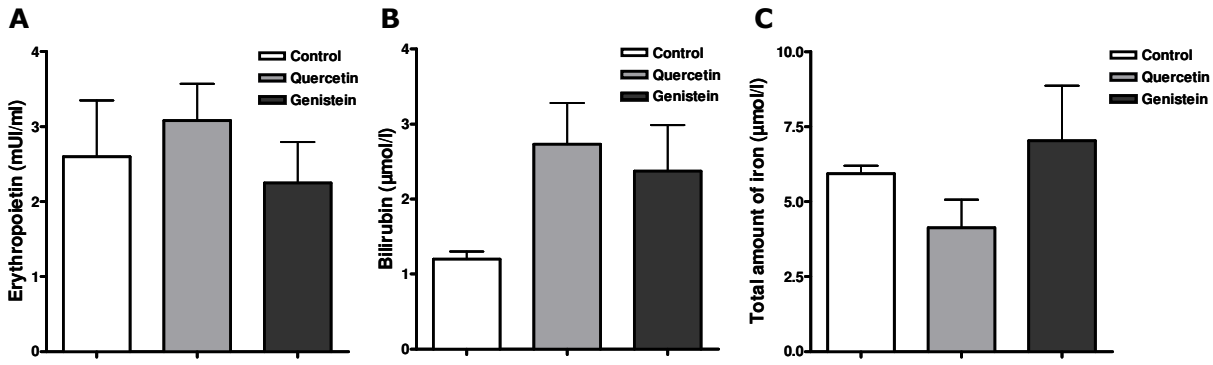

Figure 9. Effect of prenatal exposure to quercetin or genistein on erythropoietin, bilirubin and iron levels. Erythropoietin (A), bilirubin (B) and iron levels (C) were determined in amniotic fluid of fetuses at E14.5. Amniotic fluid was pooled per nest due to minute amounts of amniotic fluid obtained per fetus. This was done for 3 control nests, 3 quercetin nests and 4 genistein nests. Error bars represent the standard error.

Long-term effects of a prenatal exposure to flavonoids

3.8 Prenatal exposure to either quercetin or genistein affects genotype and sex ratio, average litter size and survival rate

To investigate the effects of a prenatal flavonoid exposure, three groups of heterozygous Atm-ΔSRI mice were placed on either the normal diet or a diet supplemented with quercetin (338.27 ppm) or genistein (270.24 ppm), from 3 days prior to conception until the end of the pregnancy. After delivery all mice were placed on the normal diet. The

average weight of the offspring (Table 5), measured five days after birth, was not significantly changed in the different diet groups.

Table 5. Characteristics of the offspring within the different diet groups

	Control	**Quercetin**	**Genistein**
Litter size	6.38 ± 0.73	5.13 ± 0.81*	5.33 ± 0.78*
Genotype			
% wt	27	34	36
% hetero	47	56	36*
% mut	26	10*[c]	28
Weight pup (g)[a]	3.32 ± 0.10	3.16 ± 0.06	3.25 ± 0.10
% males	57	46*	45*
% deceased pups[b]	10	8	7

General characteristics of the litters prenatally exposed to a quercetin- (N=8) or genistein-supplemented diet (N=9) or normal diet (N=8). Results represent the mean ± the standard error. "*" = $p<0.05$. [a] Average pup weight in grams at day 5. [b] Percentage of deceased pups after birth but before sacrifice at 12 weeks. [c] Significant difference between quercetin and both the control and genistein group.

On the contrary, the prevalance of the different genotypes appeared to be affected by the prenatal diet. Theoriticaly, wild-type and homozygous mutant offspring should each count for approximatly 25% of the offspring. Accordingly, wild-type and mutant offspring showed equal prevalence (respectively 27% and 26%) in the control diet group (Table 5). Conversely, in the quercetin-supplemented diet group, the prevalence of homozygous mutant offspring was significantly lower ($p<0.05$) than in both the control and genistein group. The quercetin group demonstrated a 3.4:1 ratio of wild-type to homozygous mutant mice. The lower prevalence of homozygous mutant offspring, is possibly due to high prenatal lethality in the quercetin group which also resulted in a significantly reduced litter size ($p<0.05$). While the distribution of the different genotypes was not significantly different for the genistein group compared to the control group, the percentage of heterozygous animals was significantly lower than in the quercetin group ($p<0.05$). Furthermore, when comparing the genistein group to the control group, a significant decrease in litter size ($p<0.05$) was also observed.

Since genistein is a known phytoestrogen, it was investigated whether prenatal exposure to this flavonoid could influence sex ratio. Notably, a significant decrease ($p<0.05$) was observed in the number of male offspring in both quercetin and genistein diet groups.

Long-term effects of a prenatal flavonoid exposure on erythropoiesis

3.9 Prenatal exposure to flavonoids does not affect the postnatal expression of globin genes in the bone marrow

To study whether a prenatal exposure to flavonoids resulted in long-term effects on the expression of embryonic and adult globin genes, their expression was determined in the 12-week old wild-type offspring. Using RT-QPCR the amount of globin mRNA was measured in the bone marrow, the site of definitive erythropoiesis. Prenatal exposure of mice to the different flavonoid-supplemented diets did not affect long-term embryonic or adult globin gene expression, compared to the control diet group (Figure 10. 10A and 10B, respectively).

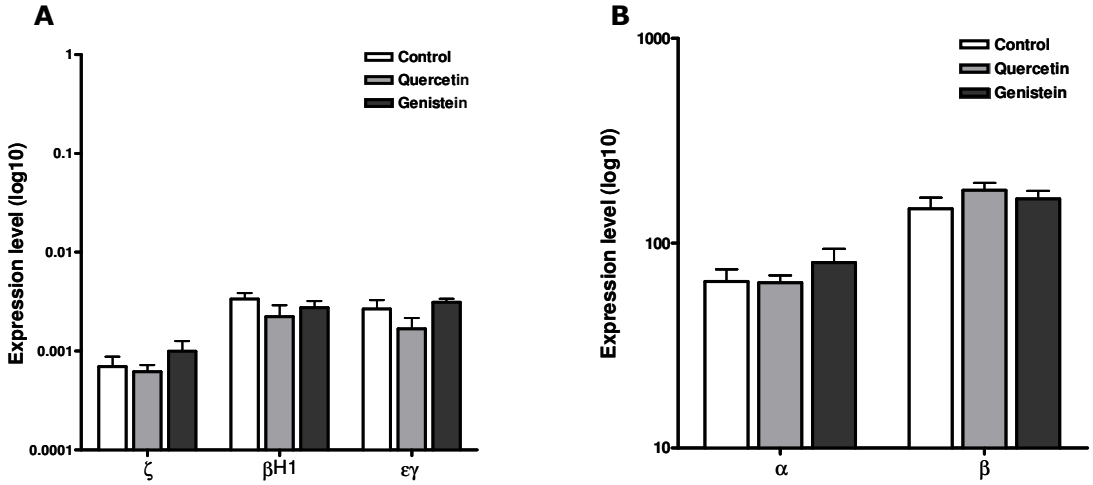

Figure 10. Effect of prenatal exposure to quercetin or genistein on the murine embryonic and adult globin gene expression in the bone marrow of 12-week old mice. A: Expression of embryonic globins in the control (N=6), quercetin (N=6) and genistein (N=6) diet groups. **B:** Expression of adult globins in the control, quercetin and genistein diet groups. GPA was used as an internal control. Expression levels were determined by RT-QPCR and calculated for each globin as $2^{-\Delta ct}$ and log10 scale was used for the graphs. Error bars represent the standard error. Statistically significant differences are depicted by "*" = $p<0.05$.

3.10 Prenatal flavonoid supplementation induces long-term changes in the blood composition

Complete blood counts were obtained of 12-week old mice in duplicate for 41 control mice, and 39 quercetin and 41 genistein exposed mice, using the ADVIA 120 Hematology system. Three mice suffering from leukemia were excluded from the analysis due to extreme outlying measurements. The results (mean±standard error) of the complete blood count are summarized in Table 6. Multiple regression analysis was carried out to adjust for confounding factors such as gender and genotype (Table 7). Generally it was found that both gender and genotype were not confounding variables. Only in the case of

absolute amount of eosinophils was the effect of diet less significant by considering gender in the regression model (Table 7).

Table 6. Summary of blood composition in 12-week old mice

	Control	**Quercetin**		**Genistein**	
	Mean±SEM	*Mean±SEM*	*P-value*	*Mean±SEM*	*P-value*
Red blood cells (10^{12}/l)	7.03±0.13	7.17±0.13	0.450	7.44±0.12	***0.026***
Hemoglobin (mmol/l)	6.78±0.10	6.97±0.14	0.282	7.25±0.09	***0.001***
Hematocrit (l/l)	0.32±0.01	0.33±0.01	0.163	0.35±0.01	***0.004***
Mean corpuscular volume (fl)	44.96±0.45	46.33±0.50	***0.044***	46.31±0.34	***0.018***
Mean corpuscular hemoglobin (fmol)	0.96±0.005	0.97±0.01	0.508	0.99±0.01	0.129
Mean cell hemoglobin concentration (mmol/l)	21.46±0.21	21.06±0.33	0.315	21.23±0.37	0.575
Red blood cell distribution width (%)	13.35±0.12	13.19±0.14**	0.373	13.92±0.19**	***0.013***
Hemoglobin distribution width (mmol/l)	1.42±0.03	1.42±0.04	0.980	1.55±0.06	0.054
Reticulocyte hemoglobin content (fmol)	0.96±0.01	1.03±0.01*	***0.001***	0.94±0.03*	0.640
Absolute amount of reticulocytes (10^9/l)	106.8±15.9	108.1±11.3*	0.946	157.6±17.5*	0.056
White blood cells (10^9/l)	1.45±0.09	1.72±0.15	0.132	1.79±0.16	0.064
Absolute amount of neutrophils (10^9/l)	0.23±0.01	0.25±0.03	0.466	0.32±0.03	***0.021***
Absolute amount of lymphocytes (10^9/l)	1.10±0.08	1.31±0.12	0.169	1.28±0.12	0.218
Absolute amount of eosinophils (10^9/l)	0.06±0.005	0.08±0.01	0.145	0.09±0.02	***0.041***

Values in the table represent the mean±standard error of mean and the p-values from the ONE-way ANOVA analysis. ***Significant p-values*** are typed in bold italic when compared to the control group. Statistically significant p-values between the two flavonoid-supplemented diets is depicted by "*" = p<0.05 or "**" = p<0.01.

Table 7. Multiple regression analysis of blood composition parameters of 12-week old mice

	Control vs. Quercetin			**Control vs. Genistein**			**Genistein vs. Quercetin**		
	Gender	*Genotype*	*Diet*	*Gender*	*Genotype*	*Diet*	*Gender*	*Genotype*	*Diet*
Red blood cells (10^{12}/l)	0.219	0.503	0.318	0.496	0.784	***0.026***	0.376	0.955	0.140
Hemoglobin (mmol/l)	0.804	0.947	0.279	0.847	0.289	***0.002***	0.849	0.281	0.095
Hematocrit (l/l)	0.175	0.906	0.126	0.563	0.804	***0.004***	0.283	0.871	0.174
Mean corpuscular volume (fl)	0.376	0.557	***0.047***	0.457	0.507	***0.023***	0.771	0.856	0.977
Mean corpuscular hemoglobin (fmol)	0.069	0.273	0.796	0.067	0.439	0.225	***0.032***	0.342	0.496
Mean cell hemoglobin concentration	***0.016***	0.903	0.197	0.236	0.608	0.444	***0.035***	0.621	0.733
Red blood cell distribution width (%)	0.064	0.480	0.346	0.820	0.956	***0.014***	0.155	0.999	***0.002***
Hemoglobin distribution width (mmol/l)	0.186	0.445	0.975	0.189	0.302	0.051	0.718	0.937	0.069
Reticulocyte hemoglobin content (fmol)	0.152	0.638	***0.003***	0.642	0.105	0.496	0.918	***0.042***	***0.008***
Absolute amount of reticulocytes (10^9/l)	***0.034***	0.096	0.906	0.269	0.183	0.125	0.141	0.207	***0.035***
White blood cells (10^9/l)	0.068	0.705	0.111	0.648	0.599	0.083	0.710	0.230	0.633
Absolute amount of neutrophils (10^9/l)	0.990	0.129	0.312	0.621	0.306	***0.021***	0.563	0.810	0.143
Absolute amount of lymphocytes (10^9/l)	***0.031***	0.469	0.148	0.845	0.339	0.261	0.873	0.143	0.989
Absolute amount of eosinophils (10^9/l)	0.806	0.669	0.130	0.505	0.813	0.054	0.552	0.405	0.292

Values in the table represent the p-values of the partial regression coefficients. ***Significant p-values*** are typed in bold itallic.

The total number of RBCs (Fig. 11A) was significantly elevated in the genistein group (p=0.026). Accordingly, the hemoglobin concentration (Fig. 11B) and hematocrit level (Fig. 11C) were also significantly increased in this diet group (p=0.001 and 0.004, respectively).

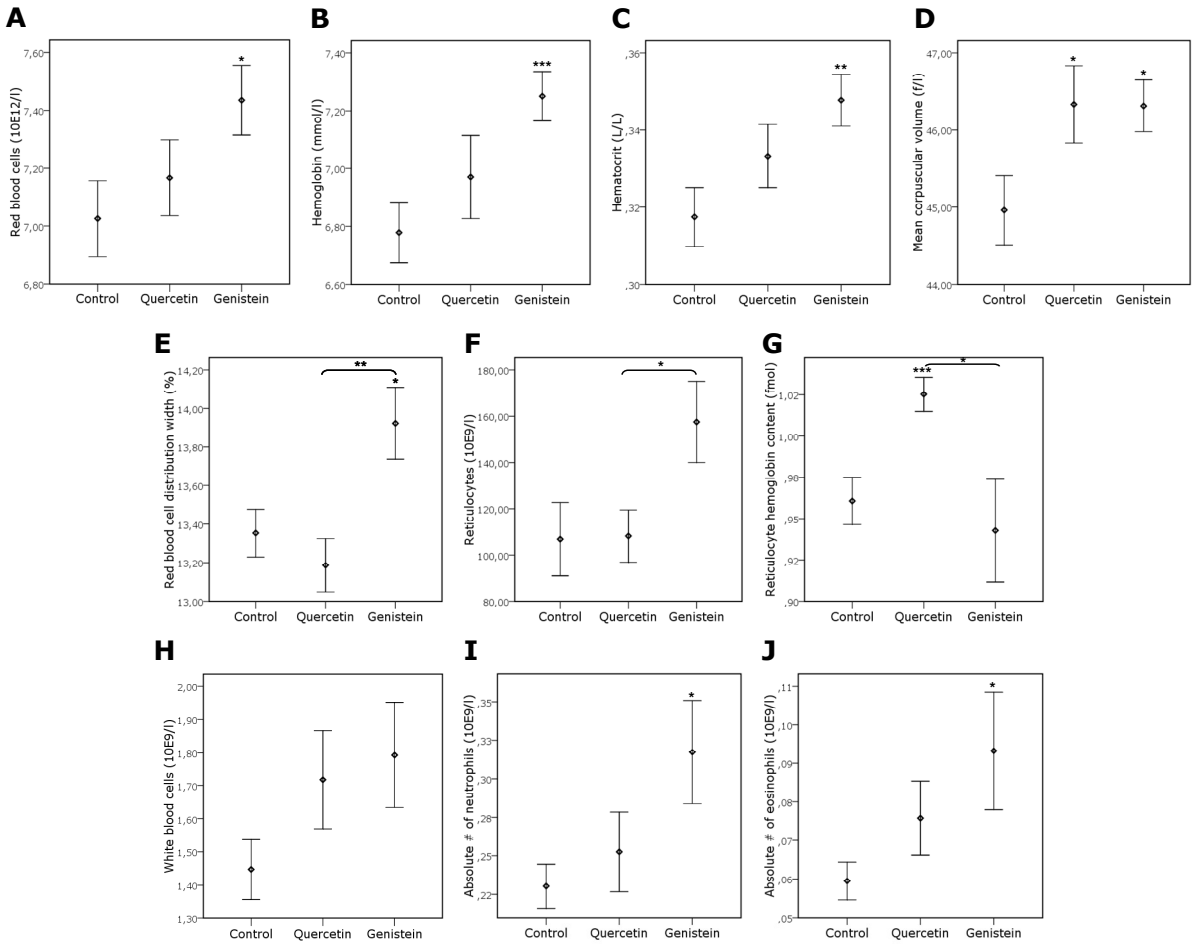

Figure 11. Long-term effects of flavonoid-supplementation during pregnancy on different blood composition parameters in offspring. Complete blood count of 12-week old mice, prenatally exposed to a quercetin- (N=39) or a genistein-supplemented (N=41) or control diet (N=41). **A-J**: Error bar graphs show the mean of the different blood parameters ± the standard error of mean. Statistically significant differences were analysed using ONE-way ANOVA and are represented by "*" = $p<0.05$; "**"= $p<0.01$ and "***"=$p<0.001$.

Mean corpuscular volume (MCV; Fig. 11D) was significantly increased in both the quercetin ($p=0.044$) and genistein ($p=0.018$) group, compared to the control group. Red blood cell distribution width (RDW; Fig. 11E) on the other hand, was only significantly increased in the genistein group compared to the control group ($p=0.013$) and to the quercetin group ($p=0.002$). A combined elevated MCV and RDW could be an indication of increased erythropoiesis and thus a higher reticulocyte count. Correspondingly, the total amount of reticulocytes (Fig. 11F) was significantly elevated in the genistein group compared to the quercetin group ($p=0.021$) but not to the control group ($p=0.056$). While prenatal exposure to a quercetin-supplemented diet did not result in an increased reticulocyte count, the reticulocyte hemoglobin content (CHr; Fig. 11G) was significantly higher than the control ($p=0,001$) and genistein group ($p=0.013$). In addition, white blood cell (WBC) parameters were affected by prenatal genistein exposure. The number

of WBC (Fig. 11H) was slightly, but not significantly (p=0.064) increased by prenatal genistein exposure compared to the control diet. This increase in WBC was due to a higher absolute number of granulocytes in the peripheral blood. Both the absolute amount of neutrophils (Fig. 11I) and eosinophils (Fig. 11J) were significantly elevated (p=0.021 and p=0.041, respectively) in the group that was the prenatally exposed to genistein.

3.11 Heme-oxygenase 1 expression

To investigate whether prenatal exposure to the flavonoids quercetin and genistein induced oxidative stress, the expression of the inducible heme-oxygenase 1 (HO-1) was investigated. HO-1 is a cytoprotective enzyme that catalyzes the rate-limiting step in the catabolism of heme (a pro-oxidant), yielding bilirubin (an antioxidant), carbon monoxide (a second messenger) and free iron (recycled for the synthesis of heme proteins), and is involved in the degradation of erythrocytes by splenic macrophages[59 60].

Since HO-1 is regulated at the level of gene transcription[61], RT-QPCR was used to measure HO-1 mRNA levels in different organs; namely the fetal liver (E14.5) and spleen and bone marrow from 12-week old mice (Figure 12 12). These different organs were selected due to their involvement in either RBC production (fetal liver and bone marrow) or degradation (spleen).

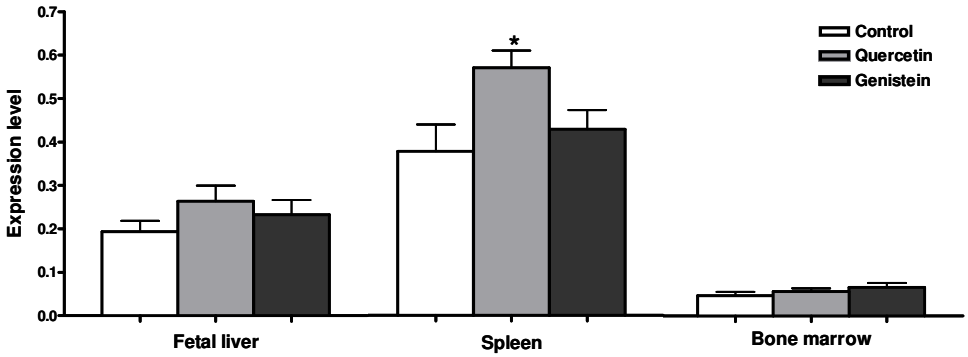

Figure 12. Heme-oxygenase 1 mRNA expression level in the fetal liver (E14,5) and in the spleen and bone marrow from 12-week old mice. N=4 per diet group in every organ. β-actin was used as an internal control. Expression levels were determined by RT-QPCR and calculated as $2^{-\Delta ct}$. Error bars represent the standard error. "*" = p<0.05 and indicates statistically significant difference between corresponding control diet group.

In the fetal liver and bone marrow, no significant difference in the expression level of HO-1 was observed. In the spleen however, prenatal exposure to quercetin resulted in a significant increase in HO-1 expression level compared to control (p=0.040). Furthermore, it was observed that the absolute expression of HO-1 in the spleen was higher than in the bone marrow of the same 12-week old mice, indicating its role in the

breakdown of RBCs. It was also observed that the expression of HO-1 in the fetal liver followed a similar 'pattern' as in the adult spleen; HO-1 was slightly increased in the prenatal quercetin-supplemented diet group, more than in the genistein-supplemented group. Additionally, the slightly increased HO-1 expression in the fetal liver was reflected in the subtle increase in bilirubin levels in amniotic fluid (Fig. 9B).

Long-term effects of a prenatal flavonoid exposure and cancer risk

3.12 Cells with Atm mutation are more sensitive for Mll aberrations after *in vitro* exposure to 50 µM quercetin or genistein

To examine whether exposure to dietary topoII inhibitors could induce chromosomal aberrations, an inverse PCR method (Fig. 14A) was set up to detect murine Mll translocations. The assay was validated by detecting Mll-translocations in murine bone marrow cells (from homozygous wild-type and mutant Atm-ΔSRI mice) that were exposed for 24h to the chemotherapeutic agent and known topoII inhibitor etoposide (50 µM) or dietary topoII inhibitors quercetin or genistein (50 µM).

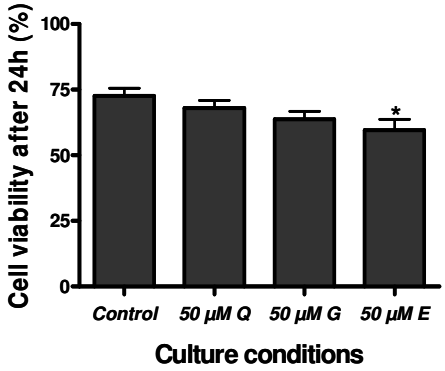

Figure 13. Cell viability of bone marrow cells. Viability of cells was determined after 24-hour exposure to 50 µM quercetin (Q), genistein (G), etoposide (E) and the vehicle 0.05% DMSO (Control) by trypan blue exclusion.

Cell viability was assessed after 24h exposure (Fig. 13) and no significant increase in cell death was observed after exposure to either quercetin or genistein. However, viability was significantly decreased in the cells that were exposed to etoposide. After exposure, cells were left to recover for another day and DNA was isolated to screen for Mll aberrations. 24h exposure to either 50 µM quercetin or genistein induced possible Mll rearrangements, regardless the genotype of the mice. Notably, when sequencing analysis was performed, it was observed that some Mll translocations in the wild-type cells were actually artefacts and not true Mll translocations (Table 8 gives two examples of observed

translocations). In the Atm mutant cells more chromosomal translocations were observed than in the wild-type cells (Fig. 14B).

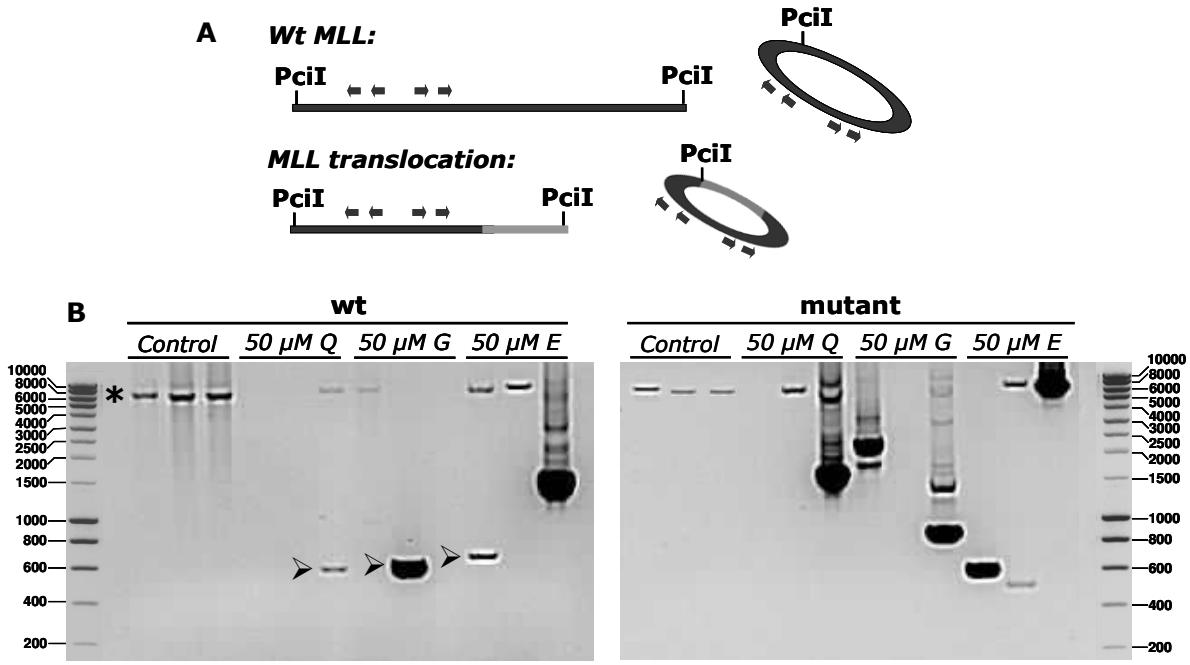

Figure 14. Mll rearrangements determined by iPCR after 24h exposure to 50 µM quercetin (Q), genistein (G) or etoposide (E). Control cells were only exposed to the vehicle DMSO 0.05%. **A:** Genomic DNA from bone marrow cells from wild-type and Atm mutant mice was digested with PciI restriction enzyme and circularized by T4-ligation. The circularized ligation products were used for first PCR and nested PCR using primer pairs that point away from each other. Amplification of the wt (native) Mll by inverse PCR generates a 6.0 kb product (indicated by "*"). Mll translocations, however, generate alternative sized PCR products. **B:** The iPCR products of bone marrow cells were visualized by gel electrophoresis. Artefacts are indicated by "➢".

Table 8. Partner chromosomes and breakpoint sequences of translocations

Treatment (24h)	Genotype	Mll-Partner chromosome	Breakpoint sequence[a]
50 µM quercetin	wt	Mll-chromosome 6	**TGACAGTGAGAGAAGTCTGA**TGCGTCGTAGACGAATTGGT
50 µM etoposide	wt	Mll-chromosome 5	**TGTTGGTAGTTAGCAGGTCT**GTCTTCGGCTTAGCAGCAAG

[a] Bold text: Mll sequence. Grey underlined: partner sequence.

3.13 Mice prenatally exposed to genistein have more malignancies

In total 130 mice (45 in the control group, 40 in the quercetin- and 45 genistein-supplemented group) reached the age of 12 weeks. The percentage of deceased offspring before this time point was not significantly different for all three diet groups (Table 5). At autopsy no tumour was detected and it was not possible to determine the cause of death. The mice that did reach 12 weeks of age were sacrificed and their internal organs were examined for visual signs of abnormalities and presence of solid tumours (Table 9).

Table 9. Malignancies detected in 12-week old mice in the different diet groups

Tumour type	Control	Quercetin	Genistein	Sites involved	Gender	Genotype
Thymoma and Leukemia						
Acute lymphoblastic leukemia			2	bone marrow, thymus	f/m	mutant
Erythroleukemia	1			bone marrow, thymus, spleen	f	mutant
Thymoma		1	1	thymus	f/m	mutant
Fallopian tube cancer			1	ovarian ducts	f	heterozygote

One mouse in the control group (1/45) demonstrated splenomegaly with concomitant erythroleukemia and thymoma. Similarly, a thymoma was detected in only one of the 40 mice that were prenatally exposed to quercetin. In the genistein-supplemented group however, three mice (3/45, 7%) were diagnosed with thymomas.

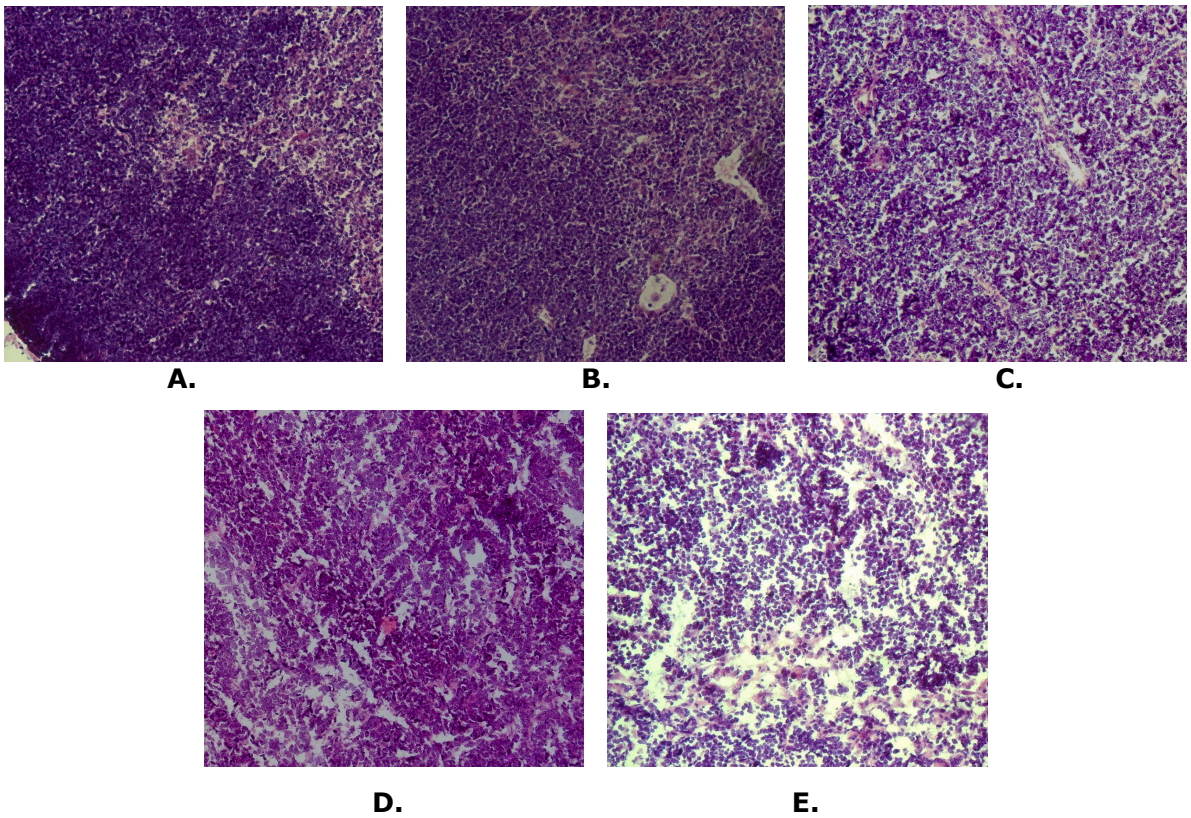

Figure 15. Histological examination of the thymus stained with hematoxylin-eosin. A-C: Photomicrographs of the thymus (20X magnification) of mice from the control, quercetin- and genistein-supplemented group respectively that showed no apparent abnormalities. The thymus shows the cortex, mainly composed of lymphocytes (thymocytes) and the medulla, mainly composed of epithelial cells which are functionally essential for the maturation of T-lymphocytes and often called 'nurse cells'[62]. **D-E:** Photomicrograph of thymoma (20X magnification) in a homozygous mutant mouse from the control group and genistein-supplemented group respectively.

Histological examination of the thymus (Figure 15 15) showed marked structural differences between normal thymuses and thymomas. More 'white gaps' were visible in

the thymoma (Fig. 15D-E) sections, indicating increased apoptosis. Interestingly, when comparing normal thymuses in the different diet groups, the structure of the thymus in the genistein group (Fig. 15C) also seemed to slightly altered. Blood count and bone marrow examination showed concomitant acute lymphoblastic leukemia in two out of the three cases. 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 (ovarian tumour) belonged to the genistein group. Altogether, mice that were prenatally exposed to genistein were observed to have a higher risk for developing tumours than the control mice.

4. Discussion

Preliminary data from our department indicated that prenatal exposure to flavonoids could induce long-term hematological changes. This led us to further investigate the short- and long-term effects of a prenatal exposure to the flavonoids quercetin and genistein.

Flavonoids are mostly known for their antioxidant properties, but they have a much wider range of biochemical and pharmacological properties and an excessive intake could lead to detrimental effects. Since flavonoids readily cross the placenta, and are even able to accumulate, the unborn fetus may be particularly at risk when the maternal diet contains an excessive amount of flavonoids. Furthermore, since the developing embryo is dependent on its mother for all nutritional requirements, it is not surprising that imbalances in maternal nutrition and exposure to certain compounds (e.g. flavonoids) during this critical and sensitive period of early life, can lead to short-, as well as long-term effects, a phenomenon known as fetal programming[26].

Short-term effects of a prenatal exposure to flavonoids

Our results show that prenatal exposure to quercetin or genistein leads to an accelerated maturation of the erythroid lineages, indicated by the decrease in primitive erythroid cells and increase in definitive erythroid cells. There are few studies investigating the potential role of flavonoids and erythroid maturation. However, one *in vitro* study in K562 leukemic cells by Csokay et al.[63] also demonstrated that 5,5 µM quercetin was able to moderately induce erythroid differentiation, indicating that there is indeed a potential for flavonoids to influence erythroid maturation.

For the quercetin group the increase in definitive erythroid cells was mostly due to the increase in the percentage of basophilic erythroblasts. For the genistein group on the other hand, this increase was mainly due to the raise in the percentage of orthochromic erythroblasts, showing that the definitive lineage in the genistein group is even more advanced in development than the quercetin group. Generally, the impact on both

primitive and definitive erythroid cell types was more prominent in the genistein-supplemented group. This accelerated maturation could be explained by a change in ROS-levels due to prenatal flavonoid exposure. ROS-levels have been thought to be of major importance in the regulation of erythroid cell maturation. Moreover, the forkhead box O3 (FoxO3) transcription factor has been assumed to play a role in the ROS-coordinated erythroid cell cycle and differentiation and determines the rate of erythroid cell maturation[28 53 64] (Fig. 16A). How exactly flavonoids target this ROS-coordinated erythroid maturation (Fig. 16B) remains to be investigated.

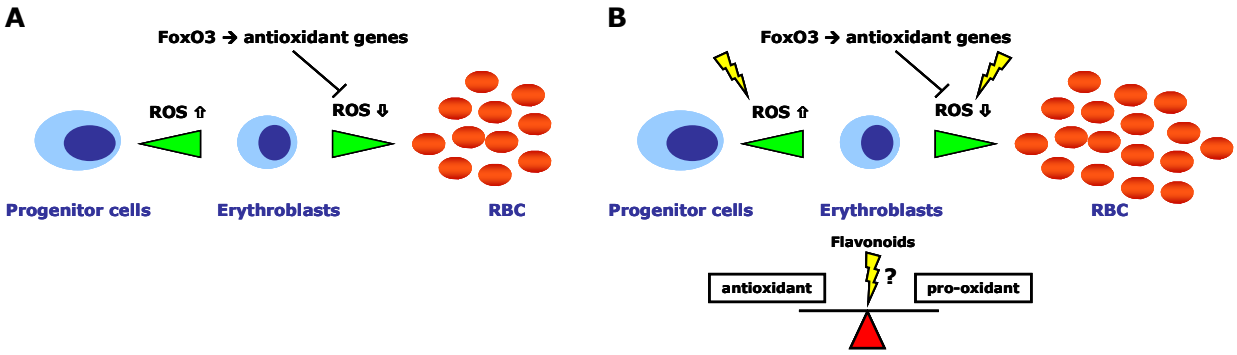

Figure 16. ROS coordinates the erythroid cell cycle and differentiation and determines the rate of erythroid maturation. A: Under normal conditions, as progenitor cells mature, more hemoglobin is produced leading to an increase in ROS. Nuclear FoxO3 activity in erythroid precursors represses ROS to coordinate the erythroid cell cycle and maturation. **B:** Prenatal flavonoid exposure somehow leads to an accelerated maturation. It is assumed that flavonoids change ROS-levels and that this leads to an altered maturation in erythroid cells. [This figure was adapted from[28].]

Since prenatal exposure to flavonoids changed the amount of primitive and definitive erythroid cells in fetal blood, the effect on the globin-switch was also investigated (fetal blood and liver). RT-QPCR allowed a precise measurement of the different globin mRNA's. Compared to the control group, mRNA levels of all embryonic globin genes (ζ, βH1 and εγ) were significantly reduced in fetal blood in the quercetin- and genistein-supplemented group. In the fetal liver (major site of definitive erythropoiesis during development) prenatal flavonoid exposure also resulted in reduced mRNA levels of embryonic globins (ζ and εγ). However, no significant changes were seen for the βH1-globin gene. Generally, the reduction in embryonic globins is consistent with the earlier observation of accelerated maturation of erythroid cells. Accordingly, the reduction in embryonic globins was more pronounced in the genistein-supplemented group.

Paradoxically, adult globins mRNA levels were decreased as well. In fetal blood, a decrease in β-globin expression was observed in both the quercetin- and genistein supplemented group. No significant changes were seen for the β-globin expression in the fetal liver. However, the α-globin gene expression was significantly reduced in the genistein group in both fetal blood and liver. The observation that adult globin expression

was reduced was unanticipated because one would expect an increase in adult globin mRNA levels if embryonic globin mRNA levels are decreased. A possible explanation for this could be that adult globin gene expression takes longer to increase, possibly because there is a greater need for the limited supply of heme and iron (essential components of hemoglobin). Furthermore, GPA expression which was used as an internal control does not make a distinction between the different erythroid cells. GPA is already present from the proerythroblast stage even though globin production starts later at the basophylic erythroblast stage[65] and this could lead to the observed decrease in adult globin mRNA levels present in the different cells. It is also possible that hemoglobin production has already reached its maximum level and that mRNA molecules have begun to be degraded. More research is nevertheless needed to give an appropriate explanation for this decrease in adult globin mRNA levels.

Long-term effects of a prenatal exposure to flavonoids

Long-term effects of a prenatal flavonoid exposure was investigated in 12-week old mice (wild-type, heterozygous and mutant Atm-ΔSRI) that were prenatally exposed to either quercetin (338.27 ppm) or genistein (270.24 ppm). When the 12-week old mice from the different prenatal diets were compared, it became clear that prenatal exposure to the flavonoids significantly reduced litter size. Since this reduction was not observed in the short-term experiment for which only wild-type mice were used, it could be explained by the lethality of flavonoids for littermates carrying an Atm mutation. Accordingly, for the quercetin-supplemented group there was a prominent reduction in the percentage of homozygous Atm mutants. For the genistein group on the other hand the genotype ratio was not significantly altered compared to the control group. Since genistein is a well-known phytoestrogen, we investigated whether prenatal exposure to this flavonoid could influence sex ratio. A significant reduction in the number of male offspring was observed in both the genistein and quercetin group which could be related to a hormonal imbalance induced by the phytoestrogic properties of both these flavonoids.

Long-term effects of a prenatal exposure to flavonoids on erythropoiesis

Since short-term effects were observed in the expression of embryonic and adult globin genes, we also determined their expression in the bone marrow of 12-week old wild-type offspring. However, no long-term effects of a prenatal flavonoid exposure were observed in the distinct globin mRNA levels. As expected, embryonic globins were expressed in extremely low levels. This corresponds to the normal situation in human adults in whom fetal hemoglobins are normally present in very small amounts (<0,6%) and are only increased in hemoglobinopathies[66].

Furthermore, a complete blood count was performed to investigate the effects of a prenatal exposure to flavonoids on adult hematopoiesis. Blood composition was significantly altered in both the quercetin and genistein groups, with differences between the two flavonoids (Fig. 17).

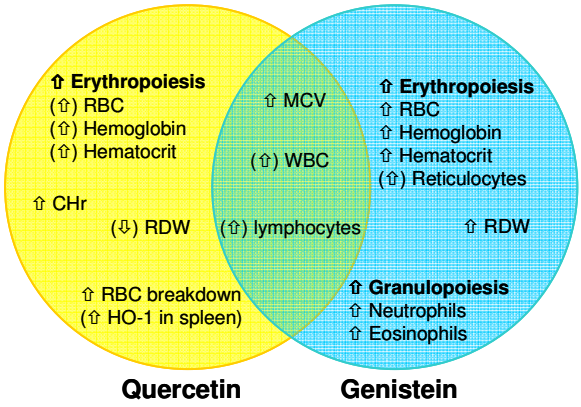

Figure 17. Comparison of the blood composition between the two flavonoid-supplemented groups. Arrows between brackets indicate changes that were not significant (p>0,05) compared to the control diet group. RBC= red blood cell; CHr= hemoglobin content of reticulocytes; RDW= red blood cell distribution width; HO-1= heme-oxygenase 1; MCV= mean cell volume.

Only genistein exposed mice, showed a significant increase in the absolute amount of neutrophils and eosinophils (granulocytes). Granulopoiesis is regulated by granulocyte colony-stimulating factor (G-CSF) which induces the growth and maturation of granulocytic precursor cells. G-CSF mediates its biological activities by binding to and activating its transmembrane receptor that belongs to the cytokine receptor superfamily. The G-CSF receptor has no intrinsic kinase activity in its cytoplasmic domain and relies on the cytoplasmic protein tyrosine kinase signalling cascade[67 68]. We assume that the observed increase in granulopoiesis is related to the protein tyrosine kinase inhibiting capacity of genistein which leads to a suppression of the granulopoiesis in utero. It has been shown in previous studies that genistein is anti-inflammatory and that it is able to suppress granulocyte-mediated inflammation in vivo[69 70] and in vitro[67]. Postnatally, the mice are no longer exposed to genistein and this possibly took off the 'brake' on granulopoiesis (Figure 18). More research is however needed to investigate this 'phenomenon'.

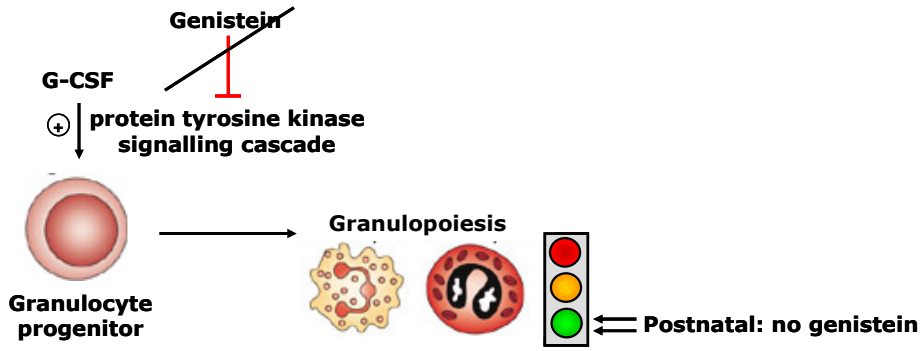

Figure 18. Model to explain the increased postnatal granulopoiesis. The observed increase in granulopoiesis is assumed to be related to the protein tyrosine kinase inhibiting capacity of genistein which leads to a suppression of the granulopoiesis in utero. Postnatally, the mice are no longer exposed to genistein and this leads to a green light for granulopoiesis.

Both in the quercetin and genistein exposed diet group increased erythropoiesis was observed. This increase in erythropoiesis could be related to hypoxia (low oxygen levels) and oxidative stress, since erythropoiesis is a very dynamic process that responds promptly to the need for more oxygen delivery. Furthermore, oxidative stress induces damage to the erythrocytes which can lead to a decreased RBC survival and life span and thus a decreased erythrocyte function (decreased O_2-transporting capacity and antioxidant capacity)[12 28 32].

When comparing the two flavonoids, it was observed that in the genistein exposed group the balance between RBC breakdown (survival) and production appeared to by unregulated and RBCs were able to accumulate. This was apparent by the increase in RDW indicating that there is a diverse range of RBC sizes (large young RBCs and smaller older RBCs). For the quercetin group on the other hand, the increase in erythropoiesis was not as pronounced as in the genistein group. In the quercetin group the increase in erythropoiesis (evident by the increase in mean cell volume and subtle increase in RBC) was balanced by an increased breakdown of RBCs in the spleen (evident by an increase in HO-1 and decrease in RDW). Furthermore, even though no increased reticulocyte count was observed in the quercetin exposed group, a significant increase in the hemoglobin content of reticulocytes (CHr) was observed. Measurement of the CHr gives an indication of the iron directly available for hemoglobin synthesis and is an early indicator of the body's iron status[71]. Since the CHr is increased in the quercetin group, this points to a possible increase in the amount of iron available for hemoglobin synthesis. Given that quercetin is an iron-chelator, it is possible that this capacity changed the fetal programming of the iron balance (notably iron was slightly decreased during pregnancy at E14.5) and that this led to increased postnatal iron-levels that induced symptoms of oxidative stress and a shortened life span of erythrocytes which in turn lead to increased erythropoiesis to sustain enough erythrocytes in the blood and increased breakdown of old RBCs.

Long-term effects of a prenatal exposure to flavonoids and cancer risk

Since quercetin and genistein are dietary topoII inhibitors, we investigated whether prenatal exposure to these compounds was associated with an increased cancer risk. To this end, three groups of heterozygous Atm-ΔSRI mice were placed on either normal diet or a diet supplemented with genistein (270.24 ppm) or quercetin (338.27 ppm), from 3 days before conception until the end of the pregnancy. After delivery all mice were placed on the normal diet. Since the Atm kinase is involved in DNA repair, mice with a mutation in the Atm gene have a decreased DNA repair capacity and a higher susceptibility for cancer. When the mice were 12 weeks old, they were sacrificed and their internal organs were examined for visual signs of abnormalities and presence of

solid tumours (complete blood counts also revealed several mice with leukemia). Overall, tumours were found most in Atm mutant mice. Additionally, mice that were prenatally exposed to genistein were observed to have a higher risk for developing tumours (predominantly thymomas) than the control mice. Prenatal exposure to quercetin did not result in more malignancies than the control group. Again, prenatal exposure to genistein lead to more pronounced effects, including increased cancer risk. Possibly, prenatal exposure genistein lead to more abnormalities due to its diverse range of actions including its phytoestrogenic capacity, its topoII inhibiting capacity and its ability to induce epigenetic changes (data not shown).

We also investigated whether a 24h *in vitro* exposure to the dietary topoII-inhibitors could induce chromosomal aberrations in bone marrow cells and an inverse PCR method was set up to detect murine Mll translocations. As a positive control we used etoposide, a well-known topoII inhibitor that has been shown to induce Mll translocations[72]. More Mll translocations were observed after exposure to quercetin, genistein or etoposide in the bone marrow cells of Atm mutant mice compared to wild-type mice. This was expected since the Atm-mutant cells have an impaired DNA repair system. Whether prenatal exposure to dietary flavonoids leads to Mll translocations *in vivo*, remains to be investigated.

5. Conclusion & Synthesis

In this study, we observed that a prenatal exposure to quercetin or genistein lead to an accelerated maturation of the erythroid lineages during fetal development (E14.5) and an altered blood composition in adult mice (12 weeks old), with differences between the two flavonoids. Erythropoiesis was increased in both flavonoid exposed groups, but the effects were more prominent in the genistein group. Furthermore, in the latter group we observed a significant increase in granulopoiesis. Generally, the cause for this observed increase in hematopoiesis due to prenatal flavonoid exposure is still unknown and in the view of the current data, it is not possible to refute or accept the hypothesis. Furthermore, the differences that were observed between the two flavonoids, underline the heterogeneity of the plant polyphenols and their pleiotropic effects. Whether the observed changes in erythropoiesis in the flavonoid-exposed groups are caused by an underlying change in the programming of the hematopoietic stem cells or due to a defect in the RBCs themselves leading to a decreased O_2-transport and antioxidant capacity, remains to be elucidated. However, since not only the amount of erythrocytes was increased but also the amount of white blood cells and lymphocytes (though not significantly), it is possible that the origin of the problem is situated in an altered developmental programming of the HSCs. Furthermore, since we already observed

changes in the rate of maturation of erythroid cells in the short-term experiment, it is likely that this induced effects later in life.

Since quercetin and genistein are dietary topoII inhibitors, we investigated whether prenatal exposure to these compounds was associated with an increased cancer risk. Overall, only mice that were prenatally exposed to genistein were observed to have a higher risk for developing tumours than the control mice. This again emphasizes a more pronounced effect of a prenatal exposure to genistein.

These data warrant further investigations into the role of dietary flavonoids during pregnancy and the mechanisms by which they affect developmental and postnatal erythropoiesis and their role in cancer. Future research should focus on the pro-oxidant/antioxidant balance in pre- and postnatal conditions and on the effect of flavonoids on Mll translocations in vivo (both in fetuses and adult mice).

Ultimately, since we observed potentially harmful effects of a prenatal flavonoid exposure and because they are readily available in high doses in dietary supplements, it is important to raise public awareness about the detrimental effects of flavonoids.

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