ABC transporter compound knockout mice: physiological and pharmacological characterization
Vlaming, M.L.H.

Citation for published version (APA):
Chapter 9

Physiological and pharmacological roles of ABCG2 (BCRP): recent findings in Abcg2 knockout mice

Maria L.H. Vlaming, Jurjen S. Lagas and Alfred H. Schinkel

doi:10.1016/j.addr.2008.08.007
Physiological and pharmacological roles of ABCG2 (BCRP): recent findings in Abcg2 knockout mice

Maria L.H. Vlaming*, Jurjen S. Lagas* and Alfred H. Schinkel

Division of Experimental Therapy, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.

* These authors contributed equally to this work.

The multidrug transporter ABCG2 (BCRP/MXR/ABCP) can actively extrude a broad range of endogenous and exogenous substrates across biological membranes. ABCG2 limits oral availability and mediates hepatobiliary and renal excretion of its substrates, and thus influences the pharmacokinetics of many drugs. Recent work, relying mainly on the use of Abcg2−/− mice, has revealed important contributions of ABCG2 to the blood-brain, blood-testis and blood-fetal barriers. Together, these functions indicate a primary biological role of ABCG2 in protecting the organism from a range of xenobiotics. In addition, several other physiological functions of ABCG2 have been observed, including extrusion of porphyrins and/or porphyrin conjugates from hematopoietic cells, liver and harderian gland, as well as secretion of vitamin B2 (riboflavin) and possibly other vitamins (biotin, vitamin K) into breast milk. However, the physiological significance of these processes has been difficult to establish, indicating that there is still a lot to learn about this intriguing protein.

Contents
1. Introduction
2. Recently established pharmacological functions of ABCG2/Abcg2
   2.1. Functional role of Abcg2 at the blood-brain barrier; value of compound transporter knockout mice.
   2.2. Functional role of ABCG2/Abcg2 in the placenta and fetal membranes.
   2.3. Functional role of ABCG2/Abcg2 at the blood-testis barrier.
3. Physiological functions of ABCG2/Abcg2
   3.1.1. Abcg2 pumps vitamins into milk.
   3.1.2. Secretory function of the multidrug resistance transporter ABCG2/Abcg2 in the mammary gland: a conundrum?
   3.2. Abcg2 is expressed in the harderian gland and involved in transport of conjugated protoporphyrin IX.
   3.3. Abcg2 is expressed at the murine blood-retinal barrier where it might protect the retina from circulating phototoxins.
4. Concluding remarks
1. Introduction
The ATP-binding cassette (ABC) transporter ABCG2 (BCRP) is located at the apical membrane of hepatocytes and epithelial cells of intestine and kidney where it pumps a wide variety of endogenous and exogenous compounds out of the cell. Due to its activity in these excretory organs, ABCG2 can have a profound effect on the pharmacokinetics of many drugs and their metabolites, by enhancing their excretion and limiting their uptake from the intestinal lumen after oral administration. In addition, ABCG2 can confer multidrug resistance to tumor cells (1-5). ABCG2 might further be important for the pharmacological sanctuary properties of several tissues, due to its expression in the blood-brain, blood-placental and blood-testis barriers, where it could limit the penetration of its substrates into these critical tissues (1-7). ABCG2 is also found in stem cell-enriched cell populations and progenitor cells of a number of tissues, where it might potentially protect these important cells from insult by a variety of toxic or carcinogenic xenobiotics (2). A schematic overview of ABCG2 tissue distribution is shown in Figure 1. In general, ABCG2 appears to have a xenobiotic protective function, reducing levels of noxious compounds in individual cells, in certain organs, and in the body as a whole. Nevertheless, recently also high expression of ABCG2 in the lactating mammary gland was demonstrated, in the luminal membrane (Figure 1). Here ABCG2 concentrates its (often toxic) substrates into the milk (8), leading to the question whether ABCG2 may have additional, yet unrecognized physiological functions. The physiological and pharmacological roles of ABCG2/Abcg2 elucidated thus far have been extensively described in earlier reviews (1-5). Characterization of Abcg2 knockout (Abcg2-/-) mice has greatly contributed to the knowledge of in vivo ABCG2 functions (9;10). This review will focus on some of the more recently gained insights into the physiological and pharmacological functions of Abcg2, obtained using these valuable mouse models.

2. Recently established pharmacological functions of ABCG2/Abcg2
2.1 Functional role of Abcg2 at the blood-brain barrier; value of compound transporter knockout mice.

P-glycoprotein (P-gp, MDR1/ABCB1) was the first ABC multidrug transporter that was found to be highly expressed at the blood-brain barrier (BBB), where it efficiently restricts the entry of a wide variety of compounds into the brain (Figure 1) (11;12). More recently, ABCG2 was also identified at the BBB of humans, pigs and rodents, where it colocalizes with P-gp at the luminal side of endothelial cells of brain capillaries (13-17). Mdr1a knockout mice have proven to be a valuable tool to unravel the dominant function of P-gp at the BBB (11). In contrast, for ABCG2 it was not as straightforward to unequivocally establish a functional role at the BBB, despite the availability of mouse models deficient in either P-gp or Abcg2 (9-11). This was illustrated by a number of studies, with
sometimes contradicting outcomes. Shortly after the discovery of ABCG2 expression at the BBB, Cisternino et al. performed in situ brain perfusion experiments with the prototypic ABCG2 substrates prazosin and mitoxantrone (18). To exclude involvement of P-gp, they used Mdr1a<sup>−/−</sup> mutant CF-1 mice and compared these to wild-type mice. Brain uptake of both compounds was not affected by the absence of P-gp, indicating that P-gp does not restrict the uptake of these compounds into the brain. Furthermore, elacridar, a dual P-gp and ABCG2 inhibitor, significantly increased the brain uptake of the studied compounds in wild-type and Mdr1a<sup>−/−</sup> CF-1 mice. Interestingly, elacridar increased the brain uptake of prazosin and mitoxantrone more in Mdr1a<sup>−/−</sup> mutant mice than in wild-type mice, which appeared to correlate with the observation that Mdr1a<sup>−/−</sup> mutant CF-1 mice had a threefold higher Abcg2 mRNA expression in their brain capillaries. From these somewhat indirect experiments it was inferred that Abcg2, and not P-gp, restricts the uptake of prazosin and mitoxantrone into the brain.

Contradicting results, however, were reported by Lee et al. (16), who performed similar in situ brain perfusion experiments with the ABCG2 model substrates mitoxantrone and dehydroepiandrosterone sulfate (DHEAS), using knockout mice in an FVB strain background (16). Mdr1a/b<sup>−/−</sup> mice displayed moderately but significantly higher brain uptake compared to wild-type mice for mitoxantrone and DHEAS, suggesting that brain penetration of both compounds is limited by P-gp. However, when Abcg2<sup>−/−</sup> mice were compared to wild-type mice, no difference in brain uptake of mitoxantrone and DHEAS was found. Furthermore, co-perfusion of the brains with elacridar resulted in a comparable and significantly increased brain uptake of mitoxantrone and DHEAS in all three genotypes. Based on these results, the authors concluded that one or more elacridar sensitive transporters are involved in the efflux of mitoxantrone and DHEAS at the BBB, but that no evidence was found for a functional role of Abcg2 at the BBB. Expression of ABC transporters in the BBB of mice (for instance, Abcc2) can sometimes vary markedly between different mouse strains (19). If this is also true for Abcg2, this could perhaps explain the contradicting outcomes of the described experiments. Whether indeed other ABC transporters than P-gp or Abcg2 are involved in limiting transport of mitoxantrone across the BBB may possibly be investigated in the future using (compound) knockout mice for different ABC transporters.

In another study addressing the functional role of Abcg2 at the BBB, Breedveld et al. used a different approach (20). Imatinib, a tyrosine kinase inhibitor anticancer drug and in vitro substrate of both P-gp and Abcg2 (21), was intravenously applied to wild-type, Mdr1a/1b<sup>−/−</sup> and Abcg2<sup>−/−</sup> mice and brain concentrations were determined 2 hours after administration. Consistent with a previous study (22), Mdr1a/1b<sup>−/−</sup> mice displayed 3.6-fold higher brain penetration compared to wild-type mice. Interestingly, for Abcg2<sup>−/−</sup> mice, which have functional P-gp at the BBB, a 2.5-fold higher imatinib brain uptake was found. Furthermore,
pharmacological inhibition of both P-gp and Abcg2 with the dual inhibitor elacridar resulted in a 4.2-fold increased brain penetration in wild-type mice. In addition, co-administration of imatinib with the Abcg2 inhibitor pantoprazole slightly increased the brain uptake in wild-type and Mdr1a/1b<sup>−/−</sup> mice, but did not affect brain penetration in Abcg2<sup>−/−</sup> mice. Collectively, these observations suggest that, in addition to P-gp, Abcg2 restricts the brain penetration of imatinib.

**Figure 1.** Schematic overview of ABCG2 expression throughout the body. Bold dark grey lines indicate the location of ABCG2. At all expression sites where small arrows indicate the direction of ABCG2-mediated transport, *in vivo* functionality of ABCG2/Abcg2 has been demonstrated. Wide arrows indicate net body excretion of ABCG2 substrates. For testis the situation in humans is depicted, where ABCG2 is found in both myoid cells of the seminiferous tubules and in blood capillary endothelial cells. However, only the Abcg2 barrier function of testis endothelial cells as demonstrated in mice is indicated with arrows. Expression of ABCG2 in endothelial cells of blood capillaries and veins, or in "side population" cells throughout many tissues in the body is not indicated. This figure was modified from reference (2).
The roles of P-gp and Abcg2 in limiting the brain uptake of imatinib were also studied in a recent series of in situ brain perfusion experiments (23). In this study, brain uptake of imatinib was not different between wild-type and Abcg2<sup>+/−</sup> mice when it was perfused at non-saturating concentrations. However, when imatinib was perfused at higher (>1 μM) perfusate concentrations, increased brain penetration was found in both wild-type and Abcg2<sup>+/−</sup> mice, which suggests that saturation of one or more efflux processes occurred. The fact that this saturation phenomenon did not occur in Mdr1a/1b<sup>−/−</sup> mice, pointed towards saturation of P-gp. Interestingly, at imatinib concentrations exceeding ~20 μM, brain uptake was substantially more increased in Abcg2<sup>+/−</sup> than in wild-type mice. This suggests that, when P-gp is saturated in wild-type mice, the contribution of Abcg2 in reducing imatinib passage across the BBB becomes detectable. Saturation of P-gp might also explain the higher imatinib brain uptake that Breedveld et al. found in Abcg2<sup>−/−</sup> mice (20). Initial high plasma concentration in Abcg2<sup>−/−</sup> mice of ~15 mg/L (~25 μM), as applied in this study, indeed exceeded the saturation cut-off of ~20 μM that was found by Bihorel et al. (23).

The above summarized studies, employing mice that are deficient in either P-gp or Abcg2, suggest that P-gp is a dominant transporter at the BBB, which can even restrict brain penetration of comparatively poor P-gp substrates. Therefore, if one uses Abcg2<sup>+/−</sup> mice, in which P-gp is still present, it can be difficult to unequivocally demonstrate a functional role for Abcg2 at the BBB for shared P-gp/ABCG2 substrates. This was recently further supported by Enokizono et al., who found that the brain uptake clearance of the dietary carcinogen and shared ABCG2 and P-gp substrate PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) in brain perfusion experiments was not different between wild-type and Abcg2<sup>+/−</sup> mice (7), whereas the ABCG2 (but not P-gp) substrates dantrolene and daidzein did show increased brain uptake clearance. Interestingly, however, when continuous infusion into the systemic circulation was applied, Abcg2<sup>+/−</sup> mice did show increased brain-to-plasma ratios even for the common ABCG2 and P-gp substrates PhIP and prazosin (7), although the differences were modest (1.5- to 2.5-fold). This discrepancy might be explained by the fact that in the brain perfusion experiments the compounds were perfused for only 1 minute, whereas in the continuous infusion experiments the substrates were applied for 150 minutes before brain concentrations were measured. Consequently, prolonged exposure of the brain to relatively high plasma concentrations of these compounds may result in saturation of P-gp at the BBB and therefore a marked detectable effect on brain penetration of PhIP and prazosin in Abcg2<sup>+/−</sup> mice. Using these same continuous infusion conditions, the drug dantrolene and the phytoestrogens daidzein, genistein and coumestrol (Figure 3), which are ABCG2 but not P-gp substrates, did show very marked (up to 10-fold) increased brain penetration in Abcg2<sup>−/−</sup> mice (7;24).
Considering the complications described above, an elegant model to study the overlapping functions of P-gp and ABCG2 at the BBB for shared substrates is the compound P-gp and Abcg2 knockout mouse (Mdr1a/1b/Abcg2−/−) (25). Recently, de Vries et al. used this model to study the brain uptake of the anticancer drug topotecan, which is a good substrate of ABCG2 and a weaker P-gp substrate (26). Compared to wild-type mice, brain-to-plasma AUC ratios were 2.0-fold higher in Mdr1a/1b−/− mice, not significantly different in Abcg2−/− mice, and 3.2-fold higher in Mdr1a/1b/Abcg2−/− mice. This study shows that, although topotecan appears to be a better ABCG2 substrate in vitro and in the mouse intestine (27), P-gp still dominates at the BBB. Nonetheless, Abcg2 also has a functional role and restricts the brain uptake of topotecan when P-gp is absent. Similarly, Oostendorp et al. recently used the Mdr1a/1b/Abcg2−/− mouse model to establish the restricting roles of P-gp and Abcg2 for the brain penetration of imatinib (28). The brain-to-plasma ratios of imatinib were highly (>10-fold) increased in Mdr1a/1b/Abcg2−/− mice, whereas for Mdr1a/1b−/− mice a modest (~2-fold) increase and for Abcg2−/− mice no increase in brain-to-plasma ratios was found. These latter two studies illustrate the value of compound transporter knockout mouse models to study the overlapping roles of Abcg2 and P-gp in the brain. To further illustrate the usefulness of compound knockout mice, recently also Abcg2/Abcc4−/− mice have been generated and these could be similarly used to elucidate the overlapping functions of both transporters in the disposition of the purine analogue drug PMEA (9-(2-(phosphonomethoxy)ethyl)-adenine) to various tissues (29). However, in this case brain penetration was not affected by the combination knockout in comparison to the single knockouts.

Taken together, Abcg2 appears to play a significant role at the BBB, where it can effectively restrict the brain penetration of potentially harmful compounds, especially those that are good ABCG2 substrates and not P-gp substrates. For shared substrates, Abcg2 and P-gp can (partially) take over each other's function at the BBB, although P-gp often dominates, whereas absence of both transporters can result in a drastically increased brain penetration.

2.2 Functional role of ABCG2/Abcg2 in the placenta and fetal membranes.

One of the main expression sites for ABCG2/Abcg2 is the placenta, where it is, like P-gp and MRP2 (ABCC2), expressed at the apical membrane of syncytiotrophoblasts (Figure 1) (6). The syncytiotrophoblast cellular layer forms the main barrier between the maternal and fetal blood circulations, and virtually all exchange of nutrients and waste products between mother and fetus occurs across these cells. Here ABCG2, which faces the maternal blood, could be involved in transport of its substrates from fetal to maternal blood, likely protecting the fetus from toxic compounds in the maternal circulation (6). Interestingly, the expression of Abcg2 changes with gestational age. In mice and rats Abcg2 mRNA expression
peaks at mid-gestation (day 12-15) and decreases thereafter (6). However, from gestation day 9.5 on, protein levels of murine Abcg2 did not change significantly over time, although there was a tendency of a decrease after mid-gestation (30). Whether this is similar in humans is not completely clear, as contradictory results have been reported (6). The physiological function of the variable RNA expression of Abcg2 during gestation is not known (6). If the protein levels remain similar, it may simply reflect a lower turnover rate of placental Abcg2 protein at later gestational stages.

The first evidence that ABCG2 in the placenta limited fetal penetration of drugs was found in Mdr1a/b<sup>−/−</sup> mice that were treated with the Abcg2 and P-gp inhibitor elacridar. The fetal penetration of the anti-cancer drug topotecan was 2-fold increased in Mdr1a/b<sup>−/−</sup> mice that were treated with elacridar compared to vehicle-treated mice (27). Using Abcg2<sup>−/−</sup> and/or Mdr1a/b/Abcg2<sup>−/−</sup> mice, the effect of Abcg2 on limiting fetal exposure has recently been more directly demonstrated for the drugs topotecan (9), nitrofurantoin (31) and glyburide (32), as well as for the phytoestrogen genistein (24). In these studies absence of Abcg2 led to 2- to 5-fold increased fetal-to-maternal plasma ratios of the tested compounds, indicating the relatively important role for Abcg2 in protecting the fetus from potential toxins. The functional role of placental ABCG2 in humans has been investigated in vitro by several studies on the anti-diabetic drug glyburide, which is used in gestational diabetes. Using right-side out vesicles that were derived from human placental brush border membranes a 1.4-fold increase of glyburide uptake was shown after adding the ABCG2 inhibitor novobiocin (33). Furthermore, an ex vivo placental perfusion study revealed that co-administration of the ABCG2-inhibitor nicardipine led to 2-fold increased fetal-to-maternal concentration ratios of glyburide as measured in the respective perfusates (34). These data suggest a barrier function of ABCG2 in the human placenta as well. However, it cannot be excluded that the used inhibitors may have inhibitory or stimulating effects on other efflux and/or uptake transporters. This complicates the interpretation of these results. Furthermore, ex-vivo studies may not completely reflect what happens in vivo. To address such issues, the complementary use of Abcg2<sup>−/−</sup> mice for studies on Abcg2-mediated transplacental transport remains invaluable.

Recently, expression of Abcg2 and other ABC transporters in murine fetal membranes, in particular the visceral yolk sac, has been detected. mRNA expression was higher than in the placental membrane and, like for placental expression of murine Abcg2, peaked at mid-gestation (35). Protein levels of Abcg2 in the yolk sac could only be measured after gestational day 12 and did not change up to 18 days after gestation (30). Similar high expression of ABCG2 protein and RNA was recently demonstrated in human fetal membranes (36). For the mouse, immunolocalization of Abcg2 in the yolk sac indicated that it faces the maternal side, leading to the speculation that it might have a protective function for the fetus,
in analogy to the placental Abcg2 (30). Interestingly, the multidrug transporter Mrp2/Abcc2 colocalizes with Abcg2 at the apical (maternal) side of the visceral yolk sac membrane (35). However, whether Abcg2/ABCG2 expression at this site has any physiological or pharmacological significance remains to be investigated in functional studies.

Altogether, there is strong evidence that placental ABCG2/Abcg2 can have a marked protective effect for the fetus against numerous xenobiotics, in analogy to the function of placental P-gp. A possible protective contribution of ABCG2/Abcg2 in fetal membranes remains to be established.

2.3 Functional role of ABCG2/Abcg2 at the blood-testis barrier.

The testis is another sanctuary site where ABCG2 is highly expressed (Figure 1). Immunohistochemical analysis of human testis sections revealed that ABCG2, like P-gp, is expressed in the luminal membranes of endothelial cells of blood capillaries (Figure 2). In analogy to endothelial cells at the blood-brain barrier, testis endothelial cells form tight junctions with each other, providing a continuous cellular layer that could contribute to the blood-testis barrier (37). However, testis capillary endothelial cells (at least in the rat) are not as tightly linked as those in the brain, which has raised doubts about an optimal barrier function (38). Obviously, when the physical barrier is not completely closed, the impact of drug transporters may be limited. Furthermore, ABCG2 and P-gp are strongly expressed in the apical membranes of myoid cells surrounding the seminiferous tubules (Figure 2) (39). The transport direction of both transporters in myoid and endothelial cells is outward from the seminiferous tubuli, which suggests that ABCG2 and P-gp protect the developing germ cells by restricting testicular penetration of potentially harmful substrates (Figure 2). It should be noted, though, that myoid cells too are not consistently joined by tight junctions (at least, in the rat), which might hamper their function as an effective barrier (38). In addition, the ABC multidrug transporter MRP1/ABCC1 is expressed in the basal membrane of the Sertoli cells of the seminiferous tubules (Figure 2), where it can protect the seminiferous tubules from drug induced damage, as was shown for the anticancer agent etoposide (40). Sertoli cells are consistently joined by tight junctions. It is noteworthy, however, that the progenitor spermatogenic cells (i.e., germ line cells) are located just outside the Sertoli cells (41) and are therefore not protected by MRP1 (Figure 2). Theoretically there is therefore a need for an additional protective barrier between blood and these critical germline cells, which could be situated in the myoid and/or capillary endothelial cells expressing ABCG2 and P-gp.
Also in the mouse testis, Abcg2 expression was recently demonstrated in the luminal membranes of endothelial cells of blood capillaries (24). In contrast to the human testis, however, Abcg2 expression in the apical membranes of myoid cells was not observed (24). In the same study, expression of ABCG2/Abcg2 in the murine and human epididymis was investigated. The epididymis is part of the male reproductive system that is attached to the testis and used for maturation and storage of spermatozoa after production in the testis (42). In the body region of the mouse epididymis (i.e., somewhat distal from the testis), Abcg2 was observed in the endothelial cells of the blood capillaries, whereas in the epididymis head region (i.e., most proximal to the testis) Abcg2 was expressed in both the luminal and abluminal membranes of the ducts that contain spermatozoa. Expression of Abcg2 in the blood capillaries of the epididymis head and in the abluminal membranes of the ducts in the epididymis body might restrict penetration of potentially harmful Abcg2 substrates and thus protect the spermatozoa in the ducts. However, expression of Abcg2 in the luminal membrane of the ducts in the epididymis head seems paradoxical, because the transport direction is towards the spermatozoa in the lumen. A physiological function cannot be excluded, but male fertility (and hence sperm function) in Abcg2−/− mice does not seem to be compromised (9;10). In the human epididymis, mRNA expression of BCRP was demonstrated, but no immunohistochemical analysis was reported yet (24).
Using \( \text{Abcg}^{2-/-} \) mice, it was recently demonstrated that penetration of a number of exogenous compounds (all Abcg2 substrates) into the testis was indeed efficiently restricted by Abcg2 (7;24). When these compounds were systemically infused under steady state conditions, the testis-to-plasma ratios were markedly (up to 15-fold) higher in \( \text{Abcg}^{2-/-} \) mice compared to WT mice. This was shown for PhIP, two metabolites of PhIP, MeIQx (2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline), dantrolene, prazosin and for the phytoestrogens daidzein, genistein and coumestrol (7;24). In addition, genistein penetration into the epididymis was also higher in \( \text{Abcg}^{2-/-} \) mice (2.5-fold). Together, these data indicate a prominent role of ABCG2 in reducing testis and epididymis exposure to numerous xenobiotics.

As pointed out by Enokizono et al. (7), the pronounced effect of Abcg2 knockout on testis penetration of a range of substrates provides strong evidence for a substantial role of testis capillary endothelial cells in the blood-testis barrier, at least in the mouse, as these cells are the only location where testicular Abcg2 could be detected. The physiological function of Abcg2 at the blood-testis and blood-epididymis-barriers might be particularly important with respect to (phyto)estrogens, many of which have been identified as Abcg2 substrates (Figure 3) (24;43;44), because these compounds are known to influence reproductive functions, e.g. reducing testicular weight and sperm count (45;46).
In conclusion, at all three of the above discussed blood-tissue barriers, ABCG2 appears to play an important role in restricting the uptake of (potentially harmful) substrates. It is interesting to note that for shared substrates of ABCG2 and P-gp, the restricting role of P-gp at the BBB appears more pronounced than that of ABCG2. Even for very good ABCG2 substrates in vitro and in the intestine, such as topotecan (27), penetration into brain is predominantly limited by P-gp and an effect of ABCG2 could only be demonstrated under P-gp deficient conditions (9;26). Nonetheless, deficiency of both P-gp and Abcg2 results in higher brain penetration than observed for P-gp deficient animals (9;26). This illustrates that Abcg2 can be an important backup mechanism for shared substrates, which can partially take over the function of P-gp at the sanctuary site barriers.

3. Physiological functions of ABCG2/Abcg2

3.1.1 Abcg2 pumps vitamins into milk.
In addition to its expression in excretory organs such as liver, kidney and small intestine, and its function in protecting the brain, testis and fetus from xenobiotics, it was recently found that Abcg2 is also expressed in the lactating mammary gland of mice, sheep, cows and humans (Figure 1) (8;47). Abcg2 localizes here to the apical side of alveolar epithelial cells, the main site of milk production. Mammary Abcg2 expression is strongly induced during pregnancy and lactation, and only falls back upon weaning of the pups (8). As a consequence, (potentially toxic) Abcg2 substrates in the maternal circulation are actively pumped into the milk, leading to the exposure of suckling infants and dairy consumers to a wide range of xenobiotics. This intriguing finding therefore has major pharmacological and toxicological consequences for breast-feeding infants and dairy consumers (and producers), which are discussed in more detail in a recent review (2). The fact that Abcg2/ABCG2 is expressed in the lactating mammary gland led to the hypothesis that besides a xenobiotic efflux transporter, Abcg2 may also be involved in the transfer of nutrients from mother to pup via the milk (2).

Recently, it was indeed found that an important nutrient, namely riboflavin (vitamin B$_2$) (Figure 4A), is a transported substrate for mouse and human Abcg2/ABCG2 in vitro (48). Riboflavin is necessary to form the enzyme cofactors FMN and FAD (Figure 4A), which function as electron carriers in many essential redox reactions in the body (49). Using Abcg2$^{+/−}$ mice it was shown that Abcg2 on the one hand limits the tissue distribution and plasma concentrations of dietary riboflavin, but on the other hand also actively pumps riboflavin into the milk. In fact, steady state milk secretion of riboflavin in Abcg2$^{+/−}$ mice was 60-fold reduced compared to wild-type mice. Additionally, the (already low) concentrations of FMN were ~6-fold reduced in milk of Abcg2$^{+/−}$ mice, suggesting that Abcg2 also pumps FMN into the milk. Nevertheless, pups from Abcg2$^{+/−}$ mothers do not display any phenotype associated with riboflavin-deficiency (e.g. growth retardation, skin
lesions or neurodegenerative changes (50)), suggesting that Abcg2-mediated transport of riboflavin into milk is not essential for the health of suckling pups (48). This is most likely explained by the fact that substantial amounts of the cofactor FAD can still enter the milk independently of Abcg2 (48). FAD can be converted to riboflavin in the intestine before being absorbed, and this is probably sufficient to compensate for the riboflavin deficiency in the milk of Abcg2<sup>2<sup>−</sup>-</sup> mice, at least in the protective environment of the lab, with ample supplies of riboflavin-rich food. It could well be that under more natural conditions, such as a variable food supply, reduced dietary intake, or increased need for vitamins due to stress or disease, Abcg2-mediated transport of riboflavin into the milk will be essential for an optimal nutritional and health state of suckling pups.

After the mammary transport of riboflavin by Abcg2 was discovered, the milk of Abcg2<sup>2<sup>−</sup>-</sup> mice was analyzed for levels of other vitamins (48). In addition to riboflavin, the concentration of biotin (vitamin H, vitamin B<sub>7</sub>) (Figure 4B) was reduced in milk of Abcg2<sup>2<sup>−</sup>-</sup> mice, although the difference was smaller than for riboflavin (~3.5-fold decrease). This suggests that Abcg2 may also be involved in the secretion of biotin into the milk. Direct transport of biotin by Abcg2/ABCG2, however, has not been shown yet, so decreased milk concentrations of biotin may also be caused by indirect effects of Abcg2 deficiency. Furthermore, no signs of biotin deficiency (dermatitis, hair loss and neurological signs (51)) in suckling Abcg2<sup>2<sup>−</sup>-</sup> pups have been reported. This may be due to the fact that a significant amount of biotin is still present in the milk of Abcg2<sup>2<sup>−</sup>-</sup> mothers (48), which may be sufficient for the pups, at least in the protective environment of the lab where the mice receive an excess of nutrients through the diet.

The fact that Abcg2/ABCG2 has a prominent effect on the elimination or transport of a variety of exogenous and endogenous porphyrins, such as pheophorbide a (9), PPIX (9;52;53) and PPIX glycoconjugates (53) and its interaction with hemin (54) (see also below), would suggest that vitamin B<sub>12</sub>, a porphyrin-containing vitamin, could be transported into the milk by Abcg2. However, the analysis of milk of Abcg2<sup>2<sup>−</sup>-</sup> mice did not show decreased levels of this vitamin, suggesting that this is not the case, or at least that Abcg2 does not make a quantitatively important contribution to vitamin B<sub>12</sub> secretion into milk (48).

Folic acid (folate, vitamin B<sub>9</sub>) is another vitamin that is directly transported by ABCG2 in vitro (55). It was therefore hypothesized that ABCG2 would transport folic acid into the milk. Nevertheless, studies under both steady-state conditions (48) as well as after i.v. administration of 1 mg/kg [³H]folic acid (8) to lactating dams did not reveal any difference in folate levels between milk of wild-type and Abcg2<sup>2<sup>−</sup>-</sup> mice. This indicates that alternative, and quantitatively more important pathways of folate secretion into milk must exist in the mammary gland. Some other ABC transporters are also capable of folic acid transport, at least in vitro (55), and these might perhaps contribute, but various other transport processes may also be relevant.
In fact, very little is known yet about milk secretion mechanisms of various vitamins, and this may be an interesting area for future research.

Figure 4. Vitamins of which the milk secretion can be affected by Abcg2. (A) Riboflavin (vitamin B_2) (R = R1) and FMN (R = R2), but not FAD (R = R3) are concentrated into milk by Abcg2. (B) Biotin (vitamin B_7) levels in milk are 3.5-fold decreased in Abcg2^{+/−} mice. (C) Vitamin K_1 (R = R1) levels in milk are not affected by Abcg2 and vitamin K_2 (R = R2) or K_3 (R = R3) concentrations in Abcg2^{+/−} milk have not been determined yet. Vitamin K_3 is transported by ABCG2 in vitro and therefore may be concentrated into milk by ABCG2/Abcg2.

It was recently shown that ABCG2 expression in HEK 293 cells conferred resistance to vitamin K_3 (menadione, 2-methyl-1,4-naphtoquinone) (Figure 4C) in vitro, and that ABCG2-dependent efflux of mitoxantrone could be inhibited by vitamin K_3 (56). This suggests that this vitamin is a substrate for ABCG2 as well. Vitamin K_3 is a synthetic precursor of vitamin K_2 (menaquinone) (Figure 4C), and can be converted to K_2 in the body, although the biological significance of this conversion is unclear (57). Vitamin K_1 (phyllloquinone) (Figure 4C) levels in milk were not different between Abcg2^{+/−} and wild-type mice, suggesting that this compound is not a substrate of Abcg2, or that other transport processes dominate its milk secretion (48). Levels of vitamins K_2 and K_3 in milk of Abcg2^{−/−} mice have not been analyzed thus far. Based on the in vitro experiments with vitamin K_3 this may be interesting to investigate, although also vitamin K deficiency (reflected by haemorrhagic disease (57)) has never been reported for Abcg2^{−/−} pups. This might of course be explained by the fact that vitamin K_1 secretion into milk is normal in Abcg2^{−/−} mothers, which could be sufficient to meet the required vitamin K intake of pups, at least in the favorable environment of the lab (see above).
Although these recent reports indicate that Abcg2 contributes to transport of some important nutrients into the milk, the question remains why pups fed by Abcg2+/− mothers do not show any abnormalities. Challenging Abcg2−/− mice with different types of diets containing low amounts of nutrients may shed more light on this. It appears that for many of these nutrients multiple (possibly redundant) pathways are available for transport into the milk. Investigation of other mechanisms of nutrient secretion into milk may also provide more insight into the relative contribution of Abcg2 to this process.

3.1.2. Secretory function of the multidrug resistance transporter ABCG2/Abcg2 in the mammary gland: a conundrum?

What is the biological meaning of ABCG2 function in the mammary gland? Clearly, the use of a multispecific xenobiotic transporter to secrete a number of vitamins into milk poses problems, both biological and conceptual. Based on the tissue distribution of ABCG2 (Figure 1), its extremely wide substrate specificity, and extensive functional studies in Abcg2−/− mice, there can be no doubt that one of the main biological functions of ABCG2 is protection from naturally occurring dietary xenobiotics. It limits oral uptake and bioavailability, mediates hepatobiliary and renal excretion, and restricts penetration of its substrates into critical tissues such as brain, testis, and fetus, as well as individual cells (e.g. hematopoietic progenitors) expressing ABCG2. It thus limits exposure to its substrates at the organismal (systemic), organ and cellular levels. This is all well and good for noxious xenobiotics, but at the same time ABCG2 will also limit uptake and availability of transported vitamins such as riboflavin, folate, and vitamin K3. In theory this might compromise vitamin supply to the organism, to critical tissue and cell compartments, and to the developing fetus. We indeed observed higher levels of riboflavin in Abcg2−/− than in wild-type mice (48), but the differences were modest. It is therefore reasonable to assume that for critical nutrients that are ABCG2 substrates, efficient uptake and retention mechanisms exist in all relevant barriers (intestine, placenta and other blood-tissue barriers, cell membranes) that largely overrule the extruding function of ABCG2. This would make the vitamin extrusion function of ABCG2 tolerable for the organism.

Conversely, the active secretion of potentially noxious xenobiotics into the milk by ABCG2 seems difficult to reconcile with optimal protection of the newborn offspring. Altogether, the combination of properties of ABCG2 (tissue distribution, substrate specificity, established functions) seems highly paradoxical. Here we will consider some hypotheses on ABCG2 function in the mammary gland that might begin to explain this paradox.

A. ABCG2 expression in mammary gland is coincidental. This hypothesis assumes that there is no positive biological need for mammary gland ABCG2. This would be
consistent with the lack of obvious deficiency phenotypes observed so far in pups
nursed by $Abcg2^{-/-}$ mothers. The mammary gland expression of ABCG2 during
pregnancy and lactation would merely be a consequence of the presence of
transcriptional regulation elements that are necessary for proper expression and
regulation of ABCG2 at other developmental stages or elsewhere in the body. The
possible negative effects of pumping noxious dietary xenobiotics into the milk are
mitigated by the fact that the nursing mother can itself prevent high systemic
accumulation of dietary ABCG2 substrates through her own ABCG2 functions (in
liver, intestine, kidney). Only a limited amount of noxious ABCG2 substrates would
therefore be secreted into the milk. The riboflavin and other vitamin secretion into
milk would be a coincidental consequence of the very wide substrate specificity of
ABCG2. Although we cannot exclude this coincidental hypothesis, we feel it is less
likely. Mammary gland ABCG2 is consistently induced during pregnancy and
lactation in at least four widely divergent mammalian species (mouse, man, sheep,
cow) (8;47). This evolutionary conservation suggests some functional need.
Moreover, Abcg2 expression elsewhere in the mouse body (liver, kidney, small
intestine) is not altered during pregnancy and lactation (58), so there is also at least
one tissue-specific factor in the mammary gland that allows specific induction of
ABCG2. Altogether, the conserved presence of so many regulatory factors that
allow specific mammary gland induction of ABCG2 in a variety of mammalian
species suggests there is some biological need for it.

B. ABCG2 activity in the mammary gland is useful as a xenobiotic clearance
mechanism for the mother. This hypothesis assumes that mammary gland Abcg2 is
not necessary for transport of nutrients, but functions primarily as an “overflow
mechanism”. Lactating mothers are in general more vulnerable and may therefore
need extra elimination routes for potentially toxic compounds. Abcg2 expression in
the mammary gland may be used for this. Still then the question remains why it
would be acceptable to expose suckling pups to these potentially toxic compounds.
Abcg2 protein expression has been detected in the intestinal submucosa of the fetus
recently (30). Abcg2 in the intestinal wall of the suckling pups may therefore be
sufficient to restrict intestinal uptake of Abcg2 substrates from the milk. Even
though we have observed that sometimes substantial fractions (up to 15% of the
dose) of i.v. administered drugs are cleared via the milk (59), we consider this
hypothesis unlikely as well. If the mother is compromised by xenobiotic exposure,
so will the pup, and it is likely that the rapidly growing and developing pup is more
susceptible to adverse effects of toxins than the mother. Moreover, there are highly
efficient alternative clearance pathways available to the mother (liver, kidney,
intestine). If need be, it would make more biological sense for the mother to
optimize those clearance pathways, for instance by upregulating ABCG2 in these
tissues, rather than endangering the well-being of the pups through the milk.
C. Xenobiotic exposure of suckling pups prepares them for the switch to solid food. This hypothesis assumes that deliberate transfer of moderate levels of dietary xenobiotics through the milk will induce proper detoxification systems in the pup (48). Very likely, upon weaning the pups will be eating the same solid food as the mother, and therefore be exposed to the same dietary xenobiotics. It would make biological sense to preemptively upregulate the detoxification systems in the pup that are suited to handling these xenobiotics (e.g., xenobiotic-metabolizing, -conjugating and -transporting proteins), instead of challenging a "virgin" detoxification system. The preinduction through the milk xenobiotics, for instance via activation of the nuclear xenobiotic receptors PXR and CAR, will help the pups in dealing immediately with the solid food xenobiotics, and thus improve their overall fitness. We feel this hypothesis is conceptually attractive, and pilot experiments to test it are ongoing. A limitation that we have encountered here is, however, that there are not yet many ABCG2 substrates known that are also good activators of PXR and CAR, which would seem to be the most obvious mediators for preemptive upregulation of detoxification systems in pups (60).

D. Milk transfer of xenobiotics may reduce the chance of developing allergy against these molecules later in life. This hypothesis is based on the recent demonstration that, upon milk transfer of an allergen to which the lactating mother is exposed, the chance that the suckling pups will develop allergy against this compound later in life is reduced (61). This oral tolerance induction in the pups was dependent on the presence of TGF-β, which is normally present in breast milk. The presence of this protein suggests that oral tolerance induction is a natural function of milk. Milk transfer by ABCG2 of potentially allergenic compounds (either exogenous or endogenous) to which the mother is exposed might thus reduce the chance for the pups of developing allergy against these compounds later in life. A limitation of this hypothesis is that it would mainly apply to relatively small molecules (i.e., transported by ABCG2) whereas the experimental demonstration concerned a protein (ovalbumine). Proteins and protein conjugates are more usual allergens than small molecules. However, small molecules can also be highly allergenic (penicillin is a well-known example) (62), and true allergies to various other drugs (cephalosporins, sulfa drugs, anticonvulsants, neuromuscular blocking agents, novocaine) have been described (62-64). This hypothesis could be tested when a suitable allergenic small molecule is identified as an ABCG2 substrate.

E. Mammary gland ABCG2 is necessary for nutrient transfer in the milk. This assumes that we have not yet applied the proper conditions to nursing Abcg2+/− mice to reveal a strong need for ABCG2 in providing vitamins and perhaps other nutrients in milk. Testing this hypothesis may depend on finding the proper conditions in the
lab, or perhaps identifying additional nutrients that might be transported by ABCG2 into the milk. Nevertheless, this hypothesis still begs the question why mammals didn't evolve dedicated transport systems to pump the various nutrients into milk, rather than a multispecific xenobiotic transporter. This would circumvent the risk of xenobiotic exposure of pups through milk. Still, nature tends to be pragmatic, and it may have been relatively easy (in an evolutionary sense), sufficiently efficient and with acceptable risk to the pups to apply a multidrug transporter for simultaneously pumping a variety of nutrients into milk.

Of course other, even more tentative hypotheses can be formulated, but here we restrict ourselves to the most obvious ones. We further note that some possibilities are not mutually exclusive. For instance, it is quite possible that mammary gland ABCG2 is important for both preemptively inducing detoxification mechanisms in suckling pups (C), and in providing them with some nutrients (E). Even the allergen hypothesis (D) might apply in parallel to hypotheses C and E. We finally note that there may still be other, unrecognized functions for mammary gland ABCG2 that would clarify its presence in the breast. Clearly, much more work will need to be done to resolve these complex but intriguing questions.

3.2 *Abcg2* is expressed in the harderian gland and involved in transport of conjugated protoporphyrin IX.

The tubulo-alveolar epithelial cells of the harderian gland have recently been identified as another site of high Abcg2 expression in the mouse (53). The harderian gland is a lipid-secreting exocrine gland located behind the eye, which is especially well developed in rodents and many other vertebrates (occupying about one third of the eye socket in mice), but appears to be absent in humans and other primates. Perhaps related to its absence in humans, the physiological function of the harderian gland is still unclear. Situated next to the tear gland, its secreta are released at the inner corner of the eye. In rodents it secretes lipids as well as large amounts of porphyrins, which are, amongst others, suggested to function as phototransducers capable of absorbing UV-light (65). Due to its high concentration and excretion of porphyrins, the harderian gland is an interesting organ for studies on porphyrin excretion mechanisms. It may be worth observing that substantial amounts of these porphyrins, which have a reddish colour, end up in the coat of rats through grooming. In white-coated animals they are responsible for the typical reddish coat glow that is often seen. Conversely, in sick animals harderian gland secreta accumulate in the eye corner due to lack of grooming, causing typical red crusts. A possible function of harderian gland secreta in rodent coat care or function should not be excluded.

One of the main porphyrins, protoporphyrin IX (PPIX), is a direct precursor of heme (Figure 5), which is an important cofactor in many essential biological
processes (O₂ transport by hemoglobin, electron transfer in cytochrome P450s, etc). Nevertheless, porphyrins are highly efficient photosensitizers and an excess can cause severe cellular damage (66), so their synthesis and distribution must be carefully regulated. When harderian glands of Abcg2^{−/−} mice were macroscopically investigated, it was found that they had a deep red-brown colour instead of the pale colour seen in wild-type mice. Fluorescence microscopy showed that this could be attributed to a vast increase in intracellular porphyrin levels, which suggested that Abcg2 is normally involved in secretion of porphyrins into the tubulo-alveolar lumen of the harderian gland (53).

The involvement of Abcg2/ABCG2 in endogenous porphyrin transport has previously been suggested based upon the fact that Abcg2^{−/−} mice had increased erythrocyte PPIX levels (9). Subsequently, it was shown that progenitor cells from Abcg2^{−/−} mice were more affected by hypoxic conditions (leading to increased intracellular heme concentrations) compared to wild-type progenitor cells, and that ABCG2 specifically could bind to hemin, a heme analogue (Figure 5) (54). This binding could be intensified by the presence of ABCG2 substrates. Presence of hemin on the other hand also increased ABCG2-mediated transport of estrone-3-sulfate (54). Furthermore, Zhou et al. (52) showed that ABCG2 overexpression in K562 cells led to reduced cellular PPIX levels, which could be reversed by the ABCG2 inhibitor Ko143. Also treatment of mature erythrocytes expressing ABCG2 with Ko143 could increase PPIX accumulation (52). These studies clearly suggested that ABCG2 is capable of transporting PPIX and that it can interact with heme under excess conditions. However, they did not distinguish between PPIX and its various conjugates.

Analysis of harderian gland extracts from Abcg2^{−/−} mice showed that levels of the PPIX-glycoconjugates protoporphyrin-1-O-acyl-β-xyloside (penta) and to a minor extent protoporphyrin-1-O-acyl-β-glucoside (hexa) (Figure 5) were dramatically increased. No unconjugated PPIX was detected in harderian glands of Abcg2^{−/−} mice, which suggested that PPIX is mainly excreted from harderian gland as a penta conjugate (53). Indeed, only the penta conjugate of PPIX (but not unconjugated PPIX) was detectable in tear fluid collected from wild-type mice. Furthermore, although Abcg2^{−/−} mice still showed penta secretion in tear fluid, the tear/gland ratio of penta was 9-fold lower than in wild-type. Subsequent in vitro cellular accumulation experiments with MDCKII cells expressing murine Abcg2 or human ABCG2 suggested that the harderian gland PPIX glycoconjugates are indeed transported efficiently by Abcg2/ABCG2 (53).

Also in the liver of Abcg2^{−/−} mice an accumulation of protoporphyrin-1-O-acyl-β-xyloside (penta) was found, but under normal conditions the total amounts of PPIX and its conjugates were only slightly increased in livers of Abcg2^{−/−} mice. However, when a high dose of PPIX was administered i.v. to wild-type and Abcg2^{−/−} mice, the biliary excretion of total PPIX was dramatically reduced in the Abcg2^{−/−}
mice compared to wild-type (53). This suggests that although Abcg2/ABCG2 is likely an efficient transporter of PPIX-glycoconjugates, it is probably also a low-affinity (but high capacity) transporter for PPIX, which may primarily be important under excess PPIX conditions.

**Figure 5.** Physiological and exogenous porphyrins that interact with and/or are transported by ABCG2/Abcg2. Hemin, an analogue of heme, interacts with ABCG2 in vitro (54). The heme-precursor PPIX is likely transported by ABCG2 in vitro (52) and PPIX levels in erythrocytes of Abcg2-/- mice are elevated (9). The PPIX-glycoconjugates “penta” and “hexa” are likely transported in vitro and in vivo by Abcg2/ABCG2 (53). The exogenous porphyrin pheophorbide a is transported by Abcg2/ABCG2 in vitro and causes severe phototoxicity in Abcg2-/- mice (9).

The physiological function of Abcg2-mediated transport of PPIX(-conjugates) remains unclear, also as no functional abnormalities have been described in Abcg2-/- mice that could be directly attributed to the altered PPIX levels. One possibility is that Abcg2 helps to reduce porphyrin toxicity in liver and other cells in situations of PPIX excess. Also, the PPIX-conjugates that are excreted from the harderian gland by Abcg2 will likely have a (so far unknown) exocrine or
endocrine function. Considering that the whole heme biosynthetic pathway up till PPIX (i.e., the last step before insertion of Fe to generate heme, Figure 5) must be highly activated in the harderian gland in order to obtain the normal levels of PPIX conjugate secretion, it is difficult to imagine that there would not be a biological need for it. Be that as it may, the actual biological function of PPIX conjugate secretion (and thus of Abcg2) in the harderian gland remains a mystery.

3.3 Abcg2 is expressed at the murine blood-retinal barrier where it might protect the retina from circulating phototoxins.
Abcg2 is expressed in blood capillary endothelial cells of many so-called tissue sanctuaries (see above). Recently, also the murine blood-retinal barrier (BRB) has been identified as a site of Abcg2 expression, where it was detected at the luminal membrane of retinal capillary endothelial cells (67). Intrinsic to its function as a light-sensing organ, the retina is highly photosensitive and vulnerable to damage caused by circulating phototoxic compounds such as pheophorbide a and PPIX, which are both Abcg2 substrates (see also above) (9;53;68). Abcg2 expression was also detected in conditionally immortalized rat retinal capillary endothelial cells (TR-iBRB2 cells), in which the accumulation of pheophorbide a increased significantly by treatment with the Abcg2-inhibitor Ko143, suggesting that Abcg2 expression in these cells is functional (67). The physiological function of the Abcg2 expression in the BRB may be to protect the retina from phototoxicity induced by circulating PPIX(-conjugates), or phototoxins derived from the diet. It should be noted, though, that ABCG2 is generally expressed in endothelial cells of small veins and capillaries throughout the body, also in blood vessels where there are no tight junctions between endothelial cells (69). It is therefore uncertain whether endothelial ABCG2 expression always has to indicate a blood-tissue barrier function. Whether Abcg2 really limits the penetration of drug or phototoxic substrates into the retina should therefore be determined in future studies, possibly using Abcg2-/- mice.

4. Concluding remarks
The last 4-5 years have seen a significant increase in insights into possible pharmacological and physiological functions of ABCG2, many based on analyses of Abcg2-/- mice. These include a definitive demonstration of the impact of ABCG2 in protecting brain, testis, and fetus from various xenobiotics, and elucidation of the function of mammary gland ABCG2 in pumping various drugs, carcinogens and toxins into milk. These findings can have a wide range of clinical applications. For example, when pregnant women are treated with drugs that are potentially harmful to the fetus, it might be preferable to select drugs that are ABCG2 substrates, so the fetus is protected. Conversely, if a drug should enter the fetus for therapeutic purposes, it might be better to select non-, or poor ABCG2 substrates. A similar consideration would apply to drugs that should or should preferably not enter the
brain. On the other hand, when drugs that are potentially dangerous to suckling infants are used for treatment of lactating mothers, it would be wise to select drugs that are not ABCG2 substrates, in order to reduce the amount of drug that is secreted into the milk. Of course, whether ABCG2 functions as efficiently at these sites in humans as in mice will first have to be investigated in more detail, before extrapolating directly from the mouse to humans. Nevertheless, we consider that Abcg2-/- and combination knockout mice are valuable tools to determine the in vivo effects of ABCG2 for many drugs that are currently used in the clinic, as well as for the characterization of newly discovered drugs.

As for the physiological functions of ABCG2, even though we have learnt many new aspects of both physiological ABCG2 substrates and new expression sites and functions, it seems that a straightforward picture is still lacking. Although we are convinced that one main physiological function of ABCG2 is protection from noxious dietary xenobiotics, there are many indications that there must be various additional physiological functions. Given the multispecificity of ABCG2, it may simply be that the body has applied this protein for a multitude of different functions, without an essential underlying common theme, except for the transport function. However, it would not surprise us if we are still lacking many pieces of the ABCG2 puzzle, which may one day come together to yield a comprehensive picture of the functions of this intriguing protein. We are convinced that Abcg2-/- mice will provide very useful tools in this process.

**Acknowledgements**

We thank Evita van de Steeg and Robert van Waterschoot for critical reading of the manuscript. This work was supported by grant NKI 2003-2940 from the Dutch Cancer Society.

**References**


In vivo functions of Abcg2


(28) Oostendorp RL, Buckle T, Beijnen JH, van Tellingen O, Schellens JH. The effect of P-gp (Mdr1a/1b), BCRP (Bcrp1) and P-gp/BCRP inhibitors on the in vivo absorption, distribution, metabolism and excretion of imatinib. Invest New Drugs 2008.


In vivo functions of Abcg2


(49) Powers HJ. Riboflavin (vitamin B-2) and health. Am J Clin Nutr 2003;77:1352-60.


(57) Shearer MJ. Vitamin K. Lancet 1995;345:229-34.


