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Impact of Abcg2 (Bcrp1) expression in mother and pup on riboflavin levels in suckling pups


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Impact of Abcg2 (Bcrp1) expression in mother and pup on riboflavin levels in suckling pups

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The multidrug transporter ABCG2 (BCRP) is expressed in excretory organs and transports its substrates into bile, feces and urine. Furthermore, ABCG2 is strongly induced in the mammary gland during lactation and pumps riboflavin (vitamin B₂) into milk. However, pups nurtured by Abcg2²⁻/⁻ dams did not suffer from riboflavin deficiency. We now investigated the separate effects of Abcg2 in mammary glands of lactating mothers and Abcg2 in the suckling pups on riboflavin distribution in pups. Newborn Abcg2²⁻/⁻ pups were fostered with wild-type dams and vice versa. We found that stomach riboflavin levels were solely dependent on Abcg2 of the lactating mother. Small intestinal riboflavin levels, however, were mainly determined by Abcg2 of the mother and to a minor extent by the genotype of the pups, suggesting that Abcg2 in suckling pups excretes riboflavin into the intestinal lumen. Furthermore, in plasma, cecum and colon of pups the riboflavin levels were mainly determined by Abcg2 of the pup. We show here for the first time that Abcg2 in newborn pups appears to reduce the systemic exposure to a substrate - in this case, surprisingly, a vitamin. Furthermore, Abcg2 of mother and pup appear to co-operate in increasing riboflavin concentrations in the intestinal lumen of pups. We therefore hypothesize that Abcg2 in mother and pup might be somehow beneficial for the optimal gastrointestinal development of suckling pups by increasing intestinal riboflavin levels.

INTRODUCTION
The ATP binding cassette transporter ABCG2 (BCRP) is a multidrug transporter that can actively transport a wide range of endogenous and exogenous compounds over biological membranes. It is present in the apical membrane of hepatocytes and epithelial cells of kidney and small intestine, where it excretes its substrates into bile, urine and feces. Furthermore, ABCG2 is localized in brain, testis and placenta, where it limits the penetration of its substrates into these so-called tissue sanctuaries (1;2). One main function of ABCG2 is thought to be the protection of cells, tissues and the systemic circulation from accumulation of potentially toxic endogenous and
exogenous compounds. Because of its broad substrate specificity, ABCG2 influences the pharmacokinetics of a wide range of drugs (1;2).

ABCG2 has recently also been detected in the lactating mammary gland, where it pumps its substrates into milk (3). The physiological function of this expression site is not clear yet, raising the intriguing question of why a xenotoxin efflux system would be localized in the lactating mammary gland, thereby exposing vulnerable pups to potentially toxic compounds (1-3). Interestingly, besides many toxic compounds, ABCG2 has recently been shown to transport a range of nutrients, amongst which one of the main vitamins present in milk, riboflavin (vitamin B$_2$) (1;4). ABCG2 can transport this vitamin in vitro and in vivo, and endogenous levels of riboflavin in the milk of Abcg2$^{-/-}$ dams were dramatically lower than in wild-type milk (4). However, although riboflavin is an essential vitamin, pups nurtured by Abcg2$^{-/-}$ dams did not show any signs of riboflavin deficiency (4). Furthermore, although the milk concentrations of riboflavin were 63-fold decreased, the plasma levels of (Abcg2$^{-/-}$) pups fed by Abcg2$^{-/-}$ dams were only 1.6-fold lower than plasma levels of Abcg2$^{+/+}$ pups fed by wild-type dams.

The finding that pups fed by Abcg2$^{-/-}$ dams do not suffer from riboflavin deficiency could be explained by the fact that another biologically active form of riboflavin, the co-factor FAD (flavin adenine dinucleotide) (5), is still present in milk of Abcg2$^{-/-}$ dams (4). FAD can be converted to riboflavin in the intestine of the pup and subsequently taken up into the body (6). Puzzlingly, Abcg2 expression in the intestine and/or liver of the pups may reduce riboflavin plasma levels by pumping this vitamin into bile and the intestinal lumen. Abcg2 expression in intestinal submucosa and liver of the fetus has recently been detected (7), but the effect of Abcg2 on the pharmacokinetics of its substrates in newborn pups has not been investigated yet. So far, the biological significance of riboflavin transport by Abcg2 into mother milk, as well as the influence of Abcg2 expression in pups remains unclear (1).

We wanted to investigate the effect of Abcg2 on endogenous riboflavin levels in newborn pups (as well as the levels of its biologically active forms FAD and FMN (flavin mononucleotide)) in more detail and to thus obtain more insight into the physiological role of Abcg2. We therefore compared the relative influence of Abcg2 expression in mammary glands of lactating mothers versus the influence of Abcg2 expression in suckling pups.

**MATERIALS AND METHODS**

**Animals.** Mice were housed and handled according to institutional guidelines complying with Dutch legislation. Animals used were Abcg2$^{-/-}$ (8) and wild-type mice, all of >99% FVB background. Animals were kept in a temperature-controlled environment with a 12-hour light/12-hour dark cycle. They received a standard diet containing 12-14 mg/kg riboflavin (AM-II, Hope Farms, Woerden, The
Netherlands) and acidified water *ad libitum*.

**Chemicals.** Riboflavin, FMN and FAD were from Sigma Chemical Co. (St. Louis, MO). Methoxyflurane (Methofane) was obtained from Medical Developments Australia Pty. Ltd. (Springvale, Victoria, Australia).

**Foster experiments.** Newborn pups from wild-type dams were fostered with *Abcg2*\(^{−/−}\) dams and vice versa at the first day after birth. Subsequently, at day 5 or 12 after birth, pups were sacrificed by decapitation and blood was collected in heparinized Microvette® CB 300 LH capillary tubes (Sarstedt, Nümbrecht, Germany). At 21 days of age, pups were sacrificed by cardiac puncture under methoxyflurane anesthesia, followed by cervical dislocation. Stomach, small intestine, cecum and colon of the pups were also collected (all tissues including contents). All samples were protected from light and stored at -80°C until extraction.

**HPLC analysis of riboflavin, FMN and FAD.** Samples (gastrointestinal tissues including contents) were homogenized in 3 volumes of PBS. To 200 µl of each homogenate 600 µl methanol was added to precipitate proteins. To plasma samples four times the sample volume of methanol was added. Riboflavin, FMN and FAD were determined by HPLC as described previously (9), with modifications as described (4). All samples were protected from light.

**Statistical analysis.** All values are given as means ± standard deviations (SD). One-way ANOVA followed by Tukey’s multiple comparison test was performed to assess the significance of differences between data sets. Differences were considered to be statistically significant when the P-value was < 0.05.

**RESULTS**

**Genotype of the foster mother determines riboflavin contents in the stomach of suckling pups.**

To separate the effects of Abcg2 expression in mammary gland of the lactating mother and Abcg2 expression in the intestine of suckling pups, *Abcg2*\(^{−/−}\) pups were fostered with wild-type mothers and wild-type pups with *Abcg2*\(^{−/−}\) mothers at the day of birth (day 1). We subsequently used pups from these two groups, as well as control groups (wild-type pups with wild-type mothers and *Abcg2*\(^{−/−}\) pups with *Abcg2*\(^{−/−}\) mothers) at day 5 (when the pups solely ingest mother milk), day 12 (when the pups are about to start eating solid food, in addition to drinking milk) and day 21 (when the pups are weaned and are exclusively eating solid food) after birth to collect plasma, stomach, small intestine and cecum and colon. The levels of riboflavin, FMN and FAD in the different matrices were analyzed. In all cases gastrointestinal tissues including contents were measured.
The results for the stomach levels of the pups at different ages are shown in Figure 1A. This clearly shows that the amount of riboflavin ingested by suckling pups is completely dependent on Abcg2 expression in the mammary gland of the foster mother. Pups with an Abcg2<sup>-/-</sup> foster mother had 6-15-fold less riboflavin in the stomach than pups of the same genotype with a wild-type foster mother, both at 5 and 12 days of age, consistent with the drastically reduced riboflavin levels in milk of Abcg2<sup>-/-</sup> dams (4). When the pups were no longer drinking mother milk, but solely eating (riboflavin-rich) solid food (21 days), the difference between pups nursed by Abcg2<sup>2/-</sup> and wild-type dams disappeared (Figure 1A). At this age (day 21) stomach riboflavin levels in all groups were comparable to stomach riboflavin levels of 5-day old pups nursed by wild-type dams. This shows that the amount of riboflavin ingested on this standard diet is comparable to the amount ingested by pups drinking wild-type milk. The results show that riboflavin levels in the stomach of suckling pups are solely dependent on Abcg2 expression in the mammary gland of the mother and not on Abcg2 expression in the pups. Nevertheless, also pups nurtured by Abcg2<sup>2/-</sup> mice did have some riboflavin in the stomach, suggesting an alternative (but far less effective) mechanism for riboflavin excretion into milk, as shown before (4).

Levels of FMN in the stomach of pups from all groups were relatively low (<0.25 µg/g organ) in all groups and there were no consistent differences between the groups (not shown). Levels of FAD in the stomach of all groups were substantial and comparable to riboflavin levels in pups with wild-type foster mothers (2.0-4.4 µg/g organ). There were no consistent differences between the four groups analyzed (not shown).

**Genotype of the foster mother is the main determinant for riboflavin levels in the small intestine of pups.**

The riboflavin levels in the small intestines of the pups showed similar patterns as in the stomach, but the differences were much less pronounced (Figure 1B). This suggests that the genotype of the foster mother is still a determinant for riboflavin levels in the small intestine of pups. However, it seems that Abcg2 expression in the pup may lead to somewhat higher intestinal riboflavin levels, suggesting that Abcg2 in the pup can actively pump riboflavin into the small intestinal lumen (Figure 1B). At 21 days of age there were no differences in small intestinal levels between the different groups anymore, except for a minor difference between Abcg2<sup>2/-</sup> pups with either wild-type or Abcg2<sup>2/-</sup> foster mothers, which is (based on the data for the stomach) likely coincidental.

Levels of FMN in small intestine of the pups were relatively low (<0.45 µg/g) and not significantly different between the groups analyzed (not shown). Levels of FAD were comparable to riboflavin levels in the small intestine of the pups with wild-type foster mothers (8-13 µg/g), but not different between the four groups analyzed (not shown).
Figure 1. Endogenous levels of riboflavin in 5, 12 or 21 days old wild-type (WT) and \textit{Abcg2}^{−/−} pups nurtured by wild-type or \textit{Abcg2}^{−/−} foster mothers. A, riboflavin in stomach (tissue + contents) of the different groups. B, riboflavin in the small intestine (SI, tissue + contents) of the different groups. C, riboflavin in cecum and colon (cec + col, tissue + contents) of the different groups. Data are presented as means ± SD (n = 4-13, ns, not significant, *, P < 0.05, **, P < 0.01, ***, P < 0.001, ANOVA). Note the difference in axis scales between the different graphs.
Genotype of foster mother and pup together determine riboflavin levels in cecum and colon of pups.

In the cecum and colon of most 5- and 12-day old pups, the levels of riboflavin were much higher than in the small intestine (Figure 1C). The levels of riboflavin in this part of the intestinal tract were clearly determined both by Abcg2 expression in the mammary gland of the foster mother, and by Abcg2 in the intestine and/or liver of the pups. Pups with wild-type foster mothers had high concentrations of riboflavin in the cecum and colon (wild-type pups with wild-type foster mothers: 24 ± 6 µg/g, Abcg2<sup>+/−</sup> pups with wild-type foster mothers: 12 ± 3 µg/g, both at age 12 days) (Figure 1C), suggesting that high levels of riboflavin in the stomach also leads to high levels in cecum and colon. Nevertheless, wild-type pups that were fed by Abcg2<sup>+/−</sup> foster mothers, and therefore ingested relatively low levels of riboflavin (Figure 1A), still had quite high riboflavin levels in cecum and colon (32 ± 6 µg/g at age 12 days), whereas Abcg2<sup>−/−</sup> pups with Abcg2<sup>−/−</sup> foster mothers did not (1.1 ± 0.6 µg/g at age 12 days) (Figure 1C). Furthermore, as Abcg2<sup>−/−</sup> pups with wild-type foster mothers at 12 days of age had 2-fold lower levels of riboflavin in cecum and colon than their wild-type counterparts (see above, Figure 1C), this suggests that Abcg2 in the pups concentrates riboflavin in the intestinal lumen.

Riboflavin concentrations in cecum and colon of 21 days old pups of all groups were, despite comparable stomach levels (Figure 1A), much lower than in the younger wild-type pups and comparable to levels in suckling Abcg2<sup>+/−</sup> pups with Abcg2<sup>+/−</sup> foster mothers (Figure 1C). Furthermore, we found no significant differences between the four groups at this age. Levels of FMN in cecum and colon of all pups were quite low in all groups (<1.4 µg/g tissue) and showed similar patterns to the riboflavin levels in cecum and colon (although the differences between the groups were not in all cases significant (not shown)). This suggests that FMN levels in cecum and colon of pups of all ages were directly related to the levels of riboflavin in these pups. This is likely due to the fact that riboflavin can be converted to FMN in the intestine (5). FAD levels in cecum and colon of the pups were relatively low as well (<4.5 µg/g tissue) and no consistent differences between the groups were found (not shown).

Riboflavin plasma concentrations are mainly influenced by the genotype of the pup.

The plasma levels of riboflavin in all groups analyzed are shown in Figure 2. This shows that in suckling pups (5 and 12 days old) plasma levels were mostly dependent on the genotype of the pups. Abcg2<sup>+/−</sup> pups (also the ones with Abcg2<sup>+/−</sup> foster mothers and therefore low riboflavin intake (Figure 1A)) had significantly (1.3-5.5 fold) higher riboflavin plasma concentrations than the wild-type pups. This shows that Abcg2 expression in pups already early after birth reduces plasma levels of riboflavin. Also an effect of riboflavin intake (and therefore genotype of the foster
mother) was found: suckling $Abcg2^{-/-}$ pups with wild-type foster mothers (and therefore higher riboflavin intake) had 2-fold higher plasma levels than $Abcg2^{-/-}$ pups with $Abcg2^{-/-}$ foster mothers. However, this was only seen in Abcg2 deficient pups. In wild-type pups the genotype of the foster mother did not affect plasma levels of the pups (Figure 2). Collectively, these data suggest that Abcg2 expression in newborn pups strongly reduces the plasma levels of riboflavin, especially when riboflavin intake is high. On the other hand, Abcg2 in the mammary gland of the mother only has a modest effect on the riboflavin plasma levels of pups. In 21 days old pups, in contrast to what was previously found in adult $Abcg2^{-/-}$ males (4), we did not find significant differences between the groups analyzed (Figure 2).

FMN levels in plasma of all groups analyzed were relatively low (< 0.04 µg/ml) and appeared to correlate with riboflavin levels in plasma of the pups, although the differences between groups were less pronounced and not always significant (not shown). As shown before for adult mice (4), FAD levels in plasma of wild-type and $Abcg2^{-/-}$ pups were also low (0.01-0.02 µg/ml) and not significantly different between genotypes (not shown).

Figure 2. Endogenous levels of riboflavin in plasma of 5, 12 or 21 days old wild-type (WT) and $Abcg2^{-/-}$ pups nurtured by wild-type or $Abcg2^{-/-}$ mothers. Data are presented as means ± SD (n = 4-5, ns, not significant, *, P < 0.05, **, P < 0.01, ***, P < 0.001, ANOVA).
DISCUSSION

By fostering $Abcg2^{-/-}$ pups with wild-type mothers and vice versa we were in this study able to separate the effects of Abcg2 in the mammary gland of the mother, pumping riboflavin into milk, and Abcg2 in intestine and liver of the suckling pups, potentially pumping riboflavin into the intestinal lumen of the pup, thereby reducing the riboflavin plasma levels. We found that the levels of riboflavin in stomach and small intestine of suckling pups are primarily determined by Abcg2 expression in the mammary gland of the mother. On the other hand, riboflavin levels in cecum and colon of pups were dependent both on Abcg2 in the mammary gland of the mother and Abcg2 in liver and/or intestine of the pup itself. Furthermore, riboflavin plasma levels (and thereby the systemic exposure of suckling pups to riboflavin) were primarily determined by Abcg2 expression in the pups. This shows that, although Abcg2 actively pumps riboflavin into milk, this mainly increases riboflavin levels in the intestine of pups, but not in the circulation. However, Abcg2 in the pups appears to actively extrude riboflavin from the circulation into the intestine, thereby efficiently reducing riboflavin exposure of suckling pups. This observation is most evident in the wild-type and $Abcg2^{-/-}$ pups with $Abcg2^{-/-}$ foster mothers: in spite of a similarly low intake of riboflavin through milk, the riboflavin levels are far lower in cecum and colon of the $Abcg2^{-/-}$ pups (Figure 1C), whereas the plasma riboflavin levels are higher (Figure 2). This is to our knowledge the first demonstration of potential Abcg2 activity in newborn pups.

The finding that Abcg2 was expressed in the lactating mammary gland where it could actively pump its substrates, of which many are potential toxins, into the milk, led to the question what purpose this would serve (1-3). The subsequent finding that Abcg2 could transport the vitamin riboflavin seemed to partly explain this paradox (4). However, it was still not clear why a multispecific transporter is used for pumping such an important vitamin into the milk, at the expense of exposing vulnerable pups to all kinds of other potential toxins. Furthermore, it was unclear why $Abcg2^{-/-}$ pups did not suffer from riboflavin deficiency (4). The fact that the co-factor FAD is still abundantly present in the milk of $Abcg2^{-/-}$ mothers may explain this (4). Our results additionally suggest another explanation, namely that Abcg2 in the wild-type pups already early after birth plays an important role in pumping its substrates (including riboflavin) out of the body. Actually, as we show here, $Abcg2^{-/-}$ pups, even though they receive very low amounts of riboflavin via the milk (4), have relatively high riboflavin plasma levels due to absence of Abcg2 (Figure 2). In wild-type pups that receive high amounts of riboflavin, it appears that a large fraction of this riboflavin is effectively pumped back into the intestinal lumen by Abcg2 (Figure 1C). This suggests that Abcg2 in the mammary gland of lactating mothers is not very effective in increasing the systemic level of riboflavin in wild-type pups. The fact that Abcg2 actively pumps riboflavin into the milk, while at the same time Abcg2 in the pups actively pumps this vitamin into the
intestinal lumen (either directly or via the liver), leads to the hypothesis that high concentrations of riboflavin may be important in the lumen of the intestine of suckling and developing pups.

That riboflavin in the intestinal lumen may be important for the development of pups was previously also suggested by Yates et al. (2003), who showed that specific absence of riboflavin in the intestinal lumen (but not the systemic levels) of weanling rats led to disruption of normal gastrointestinal development (10). The mechanism behind this remains unclear. Although Yates et al. showed that riboflavin in the lumen is especially important for the development of the duodenum of pups, whereas our results suggest an effect of Abcg2 in riboflavin concentration especially in the cecum and colon, these findings may be related. In other words, it could well be that the biological function of Abcg2 in the mammary gland is to deliver high amounts of riboflavin to the pup, which subsequently remain high in the intestinal lumen of the pup by active (back-)transport via Abcg2 in intestine and liver. Whether this is indeed the case should be investigated in the future.

It is interesting to note that these high levels of riboflavin in small intestine, cecum and colon that we found here in wild-type pups (especially the ones fed by wild-type foster mothers, but also when fed by Abcg2<sup>−/−</sup> foster mothers) were only seen in pups up to 12 days old (Figure 1B and C). In 21 days old pups the riboflavin levels in plasma were quite comparable to the younger pups, but in the intestinal tract they were much lower than in the younger pups (Figures 1 and 2), suggesting that the effect of Abcg2 in retaining high intestinal riboflavin levels, is especially important in pups early after birth. Interestingly, whereas in adult mice the liver levels of Abcg2 are much lower in females than in males, in 10 days old pups Abcg2 expression in liver is similar between male and female pups (11). This suggests that early after birth, Abcg2 may (also in female pups) play an important role in pumping its substrates, like riboflavin, into bile. Merino et al. (2005) further showed that at 5 weeks of age the expression of Abcg2 in liver of female mice was already much lower than in males (11), suggesting that at this age the effect of Abcg2 in liver may already be less important. However, many other explanations for the differences between the 21 days old pups and the suckling pups can be suggested, as it is likely that when pups start dealing with solid food many physiological and anatomical changes occur in the intestines of pups.

The finding that Abcg2 in the newborn pups, as we show here, is already very active and important for the pharmacokinetics of riboflavin further explains why it might not be so problematic to have Abcg2 expression in the mammary gland of lactating females, pumping potential toxins into the milk. As Abcg2 in the pup is quite active already early after birth, the pups are, like adults, probably quite well protected from toxins in milk that are Abcg2 substrates, and will only be exposed to low systemic concentrations of these compounds. The majority of the toxins that are
ingested via the milk is likely efficiently excreted from the body of the pups by Abcg2.

Our results suggest that at least one physiological effect of Abcg2 functioning is to maintain high levels of riboflavin in the intestinal lumen of developing pups. However, although this mouse strain has been carefully studied in the past years (1;2), no severe problems with the intestinal development of Abcg2−/− pups have been reported. It could be that in the protective environment of laboratory mouse facilities, on a standard diet with high riboflavin content, an effect of Abcg2 may be missed. Possibly, under more natural conditions, with lower amounts of riboflavin in the diet, it may be much more important to have Abcg2 expression in lactating mammary gland as well as in the intestine and liver of suckling pups. It would be interesting to investigate this possibility in more detail in future studies.

ABCG2 is also expressed in the mammary gland of lactating humans (3), and many ABCG2 polymorphisms in humans are known, in some cases leading to altered function of the protein (12). Furthermore, many compounds (either drugs or dietary components) have been identified that can inhibit the function of ABCG2 (12). As we show here that Abcg2 appears important in suckling pups (and therefore likely also in suckling infants) for reducing the systemic exposure to ABCG2/Abcg2 substrates, it might be important to investigate whether polymorphisms in ABCG2 of infants can lead to increased systemic exposure to toxins in the mother milk. Furthermore, it would be interesting to investigate whether reduced activity in ABCG2 of mother and/or suckling infant may lead to problems with gastrointestinal development of children due to low riboflavin levels in the intestinal lumen.

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