A homozygous nonsense mutation (c.214C>A) in biliverdin reductase alpha gene (BLVRA) results in accumulation of biliverdin during episodes of cholestasis

Nikolaj S. Nytofte, Maria A. Serrano, Maria J. Monte, Zeynep Tumer, Karin Ladefoged, Jose J.G. Marin

A homozygous nonsense mutation (c.214C>A) in biliverdin reductase alpha gene (*BLVRA)* results in accumulation of biliverdin during episodes of cholestasis

Nikolaj S. Nytofte[1], Maria A. Serrano[2], Maria J. Monte[2], Ester Gonzalez-Sanchez[2], Zeynep Tumer[3], Karin Ladefoged[1], Oscar Briz[4], Jose J. G. Marin[2]

[1)] Queen Ingrid's Hospital, DK-3900 Nuuk, Greenland, Denmark.
[2)] Laboratory of Experimental Hepatology and Drug Targeting, CIBERehd, University of Salamanca, Salamanca, Spain.
[3)] Wilhelm Johannsen Centret, The Panum Institute, Copenhagen, Denmark.
[4)] Research Unit, University Hospital, CIBERehd, Salamanca, Spain.

Short Title: Green Jaundice

Key Words: Bilirubin, Biliverdin, Cholestasis, Cholelithiasis, Heme, Liver

Contact Information:

Jose J. G. Marin
Department of Physiology and Pharmacology
Campus Miguel de Unamuno E.I.D. S-09
37007-Salamanca, Spain
Telephone: 34-923-294674
Fax: 34-923-294669
E-mail: jjgmarin@usal.es

Abbreviations:

BLVRA, Biliverdin reductase alpha gene symbol; BVRα, Biliverdin reductase alpha; ICP, intrahepatic cholestasis of pregnancy; SNP, single nucleotide polymorphism.

Financial Support:
This study was supported in part by the Instituto de Salud Carlos III, FIS (Grants PI070517 and PI080151); the Junta de Castilla y Leon (Grants GR75/08, SA033A08, SA03508, and SA036A08), Spain; the Ministerio de Ciencia y Tecnología, Plan Nacional de Investigación Científica, Desarrollo e Innovación Tecnológica (Grant BFU2006-12577 and and SAF2009-08493), Spain and the Fundacion Investigacion Medica, Mutua Madrileña (Convocatoria 2009), Spain. The group is member of the Network for Cooperative Research on Membrane Transport Proteins (REIT), co-funded by the Ministerio de Educación y Ciencia, Spain and the European Regional Development Fund (ERDF) (Grant BFU2007-30688-E/BFI) and belongs to the CIBERehd (Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas) Instituto de Salud Carlos III, Spain.

ABSTRACT

Green jaundice is a rare finding usually associated with end-stage liver diseases. We investigated two unrelated Inuit women from different geographic areas in Greenland suffering from episodes of green jaundice associated with biliary obstruction. The crises were accompanied by elevation of the biochemical markers of cholestasis, together with absent or moderate hyperbilirubinemia. In contrast, using HPLC-MS/MS hypercholanemia and high levels of biliverdin IXα in serum, urine, bile and milk were found. Hyperbiliverdinemia disappeared after surgical correction of the cholestasis. Analysis of the coding sequence of the biliverdin reductase alpha (BVRα) gene (*BLVRA*) detected three single nucleotide polymorphisms: c.90G>A, c.214C>A and c.743A>C, which result in p.Ala3Thr, p.Ser44X and p.Gly220Gly, respectively. Using TaqMan probes, homozigosity for c.214C>A was found in both patients. Both parents of one of these patients were heterozygous for the inactivating mutation. Her brother was homozygous for normal alleles. Although her sister was also homozygous for the c.214C>A mutation, she had suffered from neither hyperbiliverdinemia nor cholestasis. Using human liver RNA, the BVRα coding sequence was cloned and the variant containing c.214C>A was generated by site-directed mutagenesis. Both proteins were expressed in human hepatoma liver cells and *Xenopus laevis* oocytes. Immunobloting, immunofluorescence and functional assays of BVRα activity revealed that the mutated sequence generates a truncated protein with no catalytic activity. The present is the first report of a homozygous *BLVRA* inactivating mutation indicating that the complete absence of BVRα activity is a non-lethal condition, whose most evident phenotypic characteristic is the appearance of green jaundice accompanying cholestasis episodes.

INTRODUCTION

Biliverdin is generated, together with Fe and CO, in heme catabolism, in a reaction catalyzed by heme oxygenase [1]. The main product is biliverdin IXα, although, depending on the protoporphyrin IX bridge cleaved, smaller amounts of the other three isomers (IXβ, IXγ and IXδ) are also generated in humans and other mammals [2]. Biliverdin IXα is converted into Bilirubin IXα by biliverdin reductase alpha (BVRα), which is expressed in many organs, mainly liver, brain, lung, pancreas, kidney, spleen and placenta [3, 4, 5]. The overall reduction of biliverdin to bilirubin is very efficient, and under physiological circumstances the level of serum biliverdin is low [6]. Subsequently, bilirubin is taken up by hepatocytes and conjugated with glucuronic acid by bilirubin uridine diphosphate-glucuronosyl transferase-1A1 [7] prior to its secretion into bile [6]. Thus, cholestasis normally results in hyperbilirubinemia.

Although for many years it has simply been considered a mere mechanism for the elimination of waste products, recently the physiological advantages of the transformation of heme into biliverdin and this into bilirubin have been recognized. Thus, generation of endogenous CO, a potent vasodilator, may play a role in the control of local vascular tone [8]. Fetal biliverdin can be taken up by the placenta, where it is converted into bilirubin by BVRα prior to its transfer to the maternal compartment [5]. Moreover, bilirubin has beneficial antioxidant properties, although they are limited to low concentrations of this potentially toxic pigment [9]. An explanation for the antioxidant function of bilirubin is a redox cycle in which bilirubin would be oxidized to biliverdin and then recycled back to bilirubin by BVRα [10]. However, recent in vitro studies argue against the BVRα-mediated redox cycle playing a general or important role as a cellular antioxidant defense mechanism [11].

Under physiological circumstances the levels of biliverdin are much lower than those of bilirubin [6]. However, in rare cases impairment in the biliverdin/bilirubin pathway has been reported to result in green jaundice and a green discoloration of body fluids [12, 13, 14, 15]. This has been ascribed to biliverdin accumulation, but identification of the pigment responsible has been hampered by the use of non-specific biliverdin assays [12]. Clinically, the appearance of green jaundice has been considered to be a sign of a poor prognosis [13].

In the present study we describe two cases of green discoloration of the skin and biliverdin accumulation in plasma and urine in two unrelated female Inuit patients from different geographic areas of Greenland who had suffered from episodes of obstructive cholestasis. The biliverdin concentrations in their body fluids were completely restored to normal levels after surgical correction of the cholestasis. The genetic bases for this alteration have been elucidated.

MATERIALS AND METHODS

Patients

The research protocol, which conformed to the ethical guidelines of the 1975 Declaration of Helsinki, was reviewed and approved by the Human Ethics Committees of the University of Salamanca (Spain) and the Queen Ingrid's Hospital (Nuuk, Greenland, Denmark). Written consent was obtained from the patients in all cases.

Case #1, Patient PJ

First episode: A 22-years-old pregnant woman from southern Greenland contacted the local Health Centre with complaints of back pain, nausea, vomiting and headache. The patient had previous been in good health. The local doctor noted that the urine was green. Urine microscopy revealed that the patient had a urinary infection. The patient developed contractions and was referred to the Queen Ingrid's Hospital where she was admitted to the Gynaecological Department on April 20th 2007. The contractions and the back pain had remitted, but the patient still complained of nausea and bilious vomiting. Apart from penicillin, the patient did not receive any medication before admission.

The patient was at gestation stage of 35(+1) weeks on the day of admission. The patient had a body mass index of 39.5 kg/m^2. Blood pressure was 143/84; pulse 100/min, and temperature 37.0ºC. An abdominal examination revealed a soft and initially non-tender abdomen. The skin of the patient presented a greenish hue. Both urine and plasma were green, the urine somewhat darker than the latter (Figure 1). Laboratory tests from the two days prior to the surgical intervention afforded normal values, except for serum alkaline phosphatase (282 U/l) and transaminases (ALAT-GPT: 58 U/l; ALAT-GOT: 54 U/l). Total bilirubin was only moderately elevated (18 µM). On April 22nd the patient complained of abdominal pain and her nausea and vomiting had worsened. Abdominal ultrasound imaging revealed a living foetus, signs of obstructive cholestasis and an enlarged, thin-walled gallbladder with multiple gallstones. Her blood pressure had risen (154/100 mmHg), a discrete oedema of the lower extremities was noticed, and the patient had mild proteinuria. On April 23rd a healthy-looking baby was delivered by Caesarean section. Neither the skin of the baby nor the amniotic fluid was green. A cholecystolithotomy was performed and very green bile was drained from the thin-walled gallbladder, after which multiple gallstones were removed. During the operation, a cholangiography revealed a very distended biliary tree without drainage to the duodenum. One week after the operation, a second cholangiography disclosed normal drainage to the duodenum. Laboratory tests afforded normal values (data

not shown). Moreover, all symptoms, as well as the green discoloration of the skin, urine and plasma (Figure 1), quickly regressed and the patient was discharged in good health.

Second episode: On October 15th 2008 the patient noticed that her urine was dark green again. On the following day, she developed abdominal pain in the epigastric region accompanied by bilious vomiting. No discoloration of the skin was observed. On October 17th the patient was admitted to the Queen Ingrid's Hospital. The patient was not febrile. The abdomen was soft but tender in the epigastric region. No skin discoloration was noted, but the urine and serum were green. Laboratory tests from October 16th to the day of surgical intervention revealed normal values except for alkaline phosphatase (370 U/l) and transaminases (ALAT-GPT: 324 U/l; ALAT-GOT: 140 U/l). Bilirubin levels were within the normal range (10 µM). On October 21st 2008 a cholocystectomy was performed. The patient was well after the operation and discharged in good health during the following week.

Case #2 Patient EA

A 33-years-old non-pregnant woman in previous good health contacted a Settlement Health Station in western Greenland complaining of epigastric pain, diarrhoea, nausea and vomiting on April 3rd 2008. The patient was not febrile and the abdomen was soft but tender. On April 18th 2008 the patient developed a green colour of the skin. She also noted that her urine had turned dark green. On April 21st 2008 the patient was transported to the Health Centre owing to a marked reduction (-8 kg) in body weight and she still complained of vomiting and diarrhoea, both described as greenish. The skin was visibly greyish green. The urine and serum were green. Laboratory tests afforded normal values except for serum bilirubin (56 µM), alkaline phosphatase (422 U/l) and transaminases (ALAT-GPT: 227 U/l; ALAT-GOT: 135 U/l). Leucocytosis was also observed (12.0 $x10^9$/l). The following day an ultrasound examination of the abdomen revealed that the gallbladder had increased in wall thickness and contained multiple gallstones. On May 9th, the patient was admitted to the Queen Ingrid's Hospital. The urine was still green and the skin had a greenish hue. Abdominal ultrasound showed cholelithiasis and cholestasis. On May 20th, a cholecystectomy was performed, gallstones were removed, and a drain was inserted. Postoperatively, the discoloration of the skin and body fluids normalized and a cholangiography now showed normal drainage to the duodenum. The patient was discharged in good health during the following week.

Analytical Methods

Adaptations of previously described methods for biliverdin [16], bilirubin [17] and bile acid [18] measurements by HPLC-MS/MS were used in a 6410 Triple Quad LC/MS (Agilent Technologies, Santa Clara, CA), using MS2Scan to select the precursor ions. For biliverdin

IXα and bilirubin IXα we used 583.3 and 585.3 m/z as the precursor ions, respectively, whereas for bile acids the selection was based on the molecular ion in each case. Positive (biliverdin IXα and bilirubin IXα) or negative (bile acid) electrospray ionization (ESI) was carried out. Precursor ions were then filtered and further fragmented in multiple-reaction monitoring (MRM) mode for using the product ions (297.2 m/z for biliverdin IXα, 299.2 m/z for bilirubin IXα, 80.2 m/z for taurine-conjugated bile acids, 74.0 m/z for glycine-conjugated bile acids and their own precursor ions for unconjugated bile acids) to measure the abundance of the indicated compounds.

HPLC was carried out using a Zorbax C18 column (30 mm x 2.1 mm, 3.5 µm) or Zorbax Eclipse XDB-C18 column (150 mm x 4.6 mm, 5 µm) for biliverdin/bilirubin and bile acids, respectively. The chromatographic conditions for biliverdin/bilirubin analyses were 85:15 methanol/water, both containing 5 mM ammonium acetate and 0.1% formic acid, pH 3.2. Flow rate was 0.3 ml/min at 35ºC. For bile acids, solvents were also methanol and water, but containing 5 mM ammonium acetate and 0.01% formic acid, pH 4.6, flow rate was 0.5 ml/min, the chromatography was started at 80:20 methanol/water, and the proportion of methanol was increased linearly up to 97% over 9 min. Standard biliverdin IXα, bilirubin IXα and bile acids (purity higher than 95%) were purchased from Porphyrin Products, Inc. (Logan, Utah, US) and Sigma-Aldrich (Madrid, Spain), respectively. All other reagents were of analytical grade.

Genetic Analyses

Total RNA-free DNA from blood cells was obtained using a commercial kit (QIAamp DNA Mini, Spin Protocol, Qiagen) after treatment of blood samples with proteinase K and RNAse A. DNA from oral epithelial cells was obtained using sterile buccal swabs (Omni Swabs, Whatman). The coding region of the BVRα gene was amplified by PCR using primers whose nucleotide sequence was located in the intronic regions of the gene. The amplicons contained both the exons and the exon-intron boundaries. The PCR products from at least three reactions of PCR per exon were purified by agarose gel electrophoresis followed by extraction of DNA from the gel bands using the QIAquick Gel Extraction kit (Qiagen). The amplicons were sequenced in both directions using forward and reverse primers. Thus, each exon was sequenced at least six times. To develop a PCR multiplex test to identify the c. 214C>A mutation found in exon 3, the two variants of this exon were cloned in pGEM-T vector and amplified in transfected *Escherichia coli* DH5α. TaqMan probes and multiplex PCR were used for allelic discrimination (forward primer: CCTTCCTCAGCGTTCCTGAA;

reverse primer: TCCATGAATTATGAAGCACAAAGA; wild-type probe: 6FAM-CTTCGTGTCGAGGTG-MGB; C214A probe: VIC-GCTTCGTGTAGAGGT-MGB).

Cloning and site-directed mutagenesis of BVRα

Using total RNA from human liver (Invitrogen) cDNA was synthesized by reverse transcription with random hexamers using the Cloned AMV First-Strand cDNA Synthesis kit (Invitrogen). The coding sequence (CDS) of BVRα including a Kozak sequence was then amplified by PCR, using high-fidelity AccuPrime Pfx DNA polymerase (Invitrogen) and two oligonucleotide primers specific to the sequence of GenBank accession number NM_000712 (forward: 5'-CCAAGATGAATGCAGAGCCCGA-3', reverse: 5'-CTTCCTTGAACAGCAATATTTCTGGATTTCTTCT-3') to which *att*B1 and *att*B2 sites were added, respectively, to obtain cDNA useful for Gateway cloning. PCR products were recombined with the *att*P-containing pDONR221 vector (Invitrogen) to generate Entry plasmids, which were further recombined with the pcDNA6.2/V5 destination vector (Invitrogen), containing the V5-tag downstream the insert, to generate an expression vector with the V5 epitope located at C-terminal end of BVRα. Complete sequencing of cloned BVRα CDS revealed the presence of the same silent polymorphism c.743C>A found in the patients. This construct (pcDNA6.2-BVRα-WT) was used to carry out homemade site-directed mutagenesis of the whole plasmid [19] and obtain pcDNA6.2-BVRα-C214A containing the mutated BVRα CDS. The presence of the mutation c.214C>A was confirmed by complete sequencing of the BVRα CDS in this new plasmid.

Expression of BVRα in cells

Synthesis of cRNAs for injection into oocytes was performed using the plasmids containing the CDS cDNA of both wild-type and mutated BVRα. These were linearized with the restriction enzyme *Pme*I (Promega). Capped and poly(A)-tailed cRNAs were synthesized using the T7 mMessage mMachine Ultra kit (Ambion; Applied Biosystems).

Mature female frogs (*Xenopus laevis*), purchased from Regine Olig (Hamburg, Germany), were used. The animals received humane care as outlined in the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and were treated in accordance with the indications of current Spanish (RD 223/1988) and European Union (86/609/CEE) laws. Experimental protocols were approved and supervised by the Ethical Committee for Laboratory Animals of the University of Salamanca.

The harvesting and preparation of oocytes were carried out as described elsewhere [20]. Oocytes were injected with 9 ng of cRNA and were subsequently cultured at 18°C for 2 days. The PLC/PRF/5 hepatoma cell line (Alexander, ATCC number CCL 8024) was purchased from the ATCC (LGC Standards, Barcelona). Cells growing on glass cover slips were transiently transfected with lipofectamine LTX (Invitrogen) and cultured for 3 days. V5-tagged BVRα was detected by immunostaining by using an anti-V5 monoclonal antibody (Invitrogen) and an anti-mouse FITC-conjugated secondary antibody (Invitrogen). DAPI (diamidino-2-phenylindole) was used to counterstain nuclei. Fluorescence photographs were obtained using a Nikon Eclipse TE 2000-S microscope.

Cell lysates (25 μg protein as measured determined using a modification of the Lowry method [21]) were used for immunoblotting analyses. Blots were probed with primary monoclonal antibodies against V5 epitope (Invitrogen). The appropriate horseradish peroxidase-linked secondary antibody was from Invitrogen. An enhanced chemiluminescence detection system (Hybond ECL; GE Healthcare) was used to visualize the bands.

Functional study of BVRα activity

BVRa activity was assessed by studying conversion of biliverdin IXα into bilirubin IXα by oocytes expressing the enzyme. Oocytes were washed with substrate-free medium (100 mM sodium chloride, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.5) and microinjected with 50 nl of similar medium but containing 300 pmol biliverdin IXα and then incubated at 25°C for 1 h. The oocytes were washed with 4 ml ice-cold medium before being placed individually in vials. Production of bilirubin IXα was measured by HPLC-MS/MS as described above after lysing the oocytes with 90% methanol.

RESULTS

Analysis of serum samples collected during green jaundice episodes in both patients revealed profiles of bile acid levels typically associated to biliary obstruction, i.e., high levels of conjugated bile acids (mainly taurocholic, glycocholic, taurochenodeoxycholic, glycocholic and tauro-sulfo-lithocholic acids) and low levels of unconjugated bile acids and secondary bile acids (data not shown).

To elucidate whether the greenish tinge of the skin, serum, urine and milk (Figure 1) during the episodes of cholestasis in both patients was actually due to biliverdin, HPLC-MS/MS was carried out. This permitted us to identify and measure the levels of biliverdin and to distinguish between the two major isomers: biliverdin IXα and biliverdin IXβ (Figure 2A). By filtering and following the specific ions (Figure 2B and 2C), it was possible to identify the major form of biliverdin in body fluids, which in both patients was biliverdin IXα, and to determine that this was markedly accumulated (Table 1). The time course of serum biliverdin IXα concentrations indicated that a marked reduction had occurred after surgical removal of the biliary obstruction (Table 1). An accumulation of biliverdin was also seen in urine and milk (Table 1). Interestingly, the bile of patient PJ collected after biliary drainage had been restored contained high amounts of biliverdin IXα (Table 1).

Since our findins suggested that patient PJ might have a deficient transformation of biliverdin IXα into bilirubin IXα we screened the coding region of the BVRα gene (*BLVRA*) for sequence variations. We detected three changes: c.90G>A (p.Ala3Thr, exon 2), c.214C>A (p.Ser44X, exon 3), and c.743A>C (p.Gly220Gly, exon 8). No changes in the intron-exon boundaries were observed (data not shown). Accordingly, the p.Ser44X mutation identified in this study, is predicted to generate a truncated protein containing only the 43 N-terminal amino acids: MNTEPERKFGVVVVGVGRAGSVRMRDLRNPHPSSAFLNLI GFV.

Using TaqMan probes in PCR multiplex analyses, both patients PJ and EA were found to be homozygous for the c.214C>A mutation. Material (oral epithelial cells) was available from only members of PJ's family. A pedigree analysis of c.214C>A in this family was carried out. This revealed that both PJ's parents were heterozygous for this mutation, whereas her brother was homozygous for the normal allele. In contrast, her sister was homozygous for the c.214C>A mutation. Although she had never suffered any episode of green jaundice, ultrasound investigation revealed a solitary stone in her gallbladder but no signs of biliary obstruction. Values of bile acid concentrations in the serum of PJ patient's sister were within

the normal range (data not shown) and no biochemical signs of cholestasis were found (data not shown). Values of biliverdin concentrations in the serum of PJ patient's sister were low and similar to that found in her sister in absence of cholestatic crisis but about two-fold higher than that found in control women (Table 1).

Expression in both human hepatoma cells and *Xenopus laevis* oocytes of the wild-type BVRα tagged with the V5 epitope at the COOH end of the protein resulted in a detectable band of the expected size in Western blot (Figure 3) and clear labelling of the cytoplasm of the cells with green florescence in immunostaining assays (Figures 4A, 4C and 4E). In contrast, when the construct containing the CDS of the mutated (c.214C>A) BVRα variant was used in the transfection of hepatoma cells or to obtain the mRNA that was injected in *Xenopus laevis* oocytes the result was a lack of detection of the V5 epitope both in Western blot (Figure 3) and immunofluorescence (Figures 4B, 4D and 4F).

HPLC-MS/MS was used to analyze in *Xenopus laevis* oocytes the biotransformation of biliverdin into bilirubin by chromatographically separating them and following the disappearance and appearance, respectively, of characteristic product ions (Figure 5A). The ability to generate bilirubin from endogenous biliverdin was significantly higher in oocytes expressing BVRα (Figures 5B and 5D). This enhanced ability was more clearly seen when the oocytes were microinjected with a bolus of exogenous biliverdin (Figures 5C and 5E). In this case, detection of a marked decrease in biliverdin content was accompanied by a similar increase in the amount of bilirubin in the cells 1 h after microinjection of biliverdin. Catalytic activity of BVRα either on endogenous or microinjected biliverdin could not be observed in oocytes expressing the mutated (c.214C>A) BVRα variant (Figures 5B-5E).

DISCUSSION

Serum biochemical markers, and low levels of unconjugated bile acids and secondary bile acids were consistent with obstructive cholestasis, i.e., a reduced transit of bile acids through the intestine, and poor biotransformation of them by intestinal bacteria [22]. Obstructive cholestasis was confirmed by additional analyses and subsequent surgery. When the drainage of bile to the duodenum was re-established, the values of laboratory tests and serum bile acids returned to within the normal range (data not shown). These findings support the hypothesis that biliary obstruction was the primary event and green jaundice appeared as a consequence. The marked accumulation of biliverdin, together with a moderate or absent hyperbilirubinemia, was the most interesting sign during crises of cholestasis in these patients. Accumulation of biliverdin has been already described in rare cases associated with biliary obstruction [12, 13], but also with malnutrition [14], and several liver diseases [23], in which the appearance of green jaundice has been considered as a sign of poor prognosis [13]. This is because the biliverdin-induced greenish colour of the skin, serum and urine probably masks the also enhanced levels of bilirubin, which might be accounted for by profound impairment of liver function. Indeed, in previously described cases of patients with enhanced biliverdinemia at levels similar to those found in patients studied here (30-40 μM) the levels of serum bilirubin were 10-fold higher (300-400 μM) [24]. In the patients included in the present study, pure hyperbiliverdinemia was found in one of the cases (PJ), and a moderate degree of hyperbilirubinemia accompanying hyperbiliverdinemia was detected in the other (EA).

Using a candidate gene approach to investigate green jaundice episodes, three single nucleotide polymorphisms were found, which result in: i) p.Ala3Thr, a variant that has already been described in this gene both in human [4] and rat [25] BVRα. The functional repercussions of the change in this residue are not known. ii) p.Gly220Gly, i.e., a silent mutation. iii) p.Ser44X, which is expected to account for the synthesis of a truncated protein.

The normal BVRα protein consists of 296 amino acids and contains two conserved sites: i) the Rossmann-fold NAD(P)H/NAD(P)(+) binding (NADB) domain at amino acid position 18-101, which is needed for the interaction with the cofactor and ii) the catalytic active site at amino acid position 133-246, which is involved in the interaction with the substrate (Figure 3). Both sites are necessary for the ability of BVRα to convert biliverdin to bilirubin. When the mutated sequence, to which a V5 epitope tag was linked at the COOH end, was transfected in human hepatoma cells and *Xenopus laevis* oocytes the complete protein was not synthesized. Moreover, the peptide synthesized before translation reaches the mutation-

generated stop codon, would lack most of the NADPH-binding site as well as the whole of the active site [26, 27]. This was consistent with the lack of catalytic activity observed in functional assays with human hepatoma cells and *Xenopus laevis* oocytes transfected with the mutated construct.

Recently in a Swedish male patient with end-stage liver cirrhosis a heterozygous nonsense mutation in exon 3 (Arg18X) of *BLVRA* has been described [15]. It is interesting that this heterozygous mutation resulted in biliverdin accumulation in spite of the presence of one normal allele. The cause of hyperbiliverdinemia in this patient was suggested to be the profound impairment in liver function due to advanced cirrhosis. The authors underline that human cases with a total deficiency of BVRα activity had not been described so far. The present study fills this gap and affords interesting information on the relevance of the complete lack of BVRα activity in humans.

In spite of the patients studied here having a complete lack of BVRα, they had no apparent signs of an abnormal phenotype in the absence of cholestasis. However, it cannot be ruled out that the complete absence of BVRα activity has any deleterious effect. For instance, it is not known whether a marked biliary secretion of this pigment might be indirectly involved in favouring the formation of gallstones that were present in both patients and in PJ's sister. Moreover, one of the subjects investigated here (PJ) was pregnant and gave birth during the first episode of green jaundice and biliverdin accumulation. The baby died a few months later of unknown causes. Thus, it cannot be ruled out that an accumulation of biliverdin during pregnancy may be harmful to the foetus.

In these patients, biochemical analyses of serum collected during green jaundice episodes revealed the presence of certain amount of total bilirubin (<20 µM and <60 µM in PJ and EA, respectively). As automated methods to determine serum bilirubin levels are based on the formation and colorimetric measurement of diazopigments and this method does not discriminate among bilirubin isomers [28], these finding were probably due to the generation in these patients of biliverdin/bilirubin isomers other than IXα.

It is interesting to note that even during cholestasis serum bilirubin levels were not markedly elevated. This, together with the presence of a large amount of biliverdin IXα in the bile of this patient, suggests that the main route for elimination of heme metabolites in individuals with complete BVRα deficiency could be the secretion into bile of biliverdin IXα. This maintains serum levels low as long as biliary function is not impaired. This may explain the

interesting observation that PJ's sister, despite being homozygous mutation for the inactivating in *BLVRA*, did not have any episodes of green jaundice. The findings of heterozygous mutation in both PJ's parents who did not suffer any liver disease and had not had signs of green jaundice along their lives were in agreement with the concept that green jaundice occurs in heterozygous patients only when liver function is severely deteriorated [15].

In conclusion, the present is the first report of a homozygous *BLVRA* inactivating mutation indicating that the complete absence of BVRα activity is a non-lethal condition, whose most evident phenotypic characteristic is the appearance of green jaundice accompanying cholestasis episodes.

Acknowledgements

The authors thank the laboratory-managing work by E. Keck, secretarial help by M.I. Hernandez, technical help by A.I. Barrios-Fernandez. Revision of the English spelling, grammar and style of the manuscript by N. Skinner are also gratefully acknowledged.

REFERENCES

1 Ryter SW, Tyrrell RM. The heme synthesis and degradation pathways: role in oxidant sensitivity. Heme oxygenase has both pro- and antioxidant properties. *Free Radic Biol Med* 2000;**28**(2):289-309.

2 Yamaguchi T, Nakajima H. Changes in the composition of bilirubin-IX isomers during human prenatal development. *Eur J Biochem* 1995;**233**(2):467-72.

3 McCoubrey WK, Jr., Cooklis MA, Maines MD. The structure, organization and differential expression of the rat gene encoding biliverdin reductase. *Gene* 1995;**160**(2):235-40.

4 Komuro A, Tobe T, Nakano Y, Yamaguchi T, Tomita M. Cloning and characterization of the cDNA encoding human biliverdin-IX alpha reductase. *Biochim Biophys Acta* 1996;**1309**(1-2):89-99.

5 Briz O, Macias RI, Perez MJ, Serrano MA, Marin JJ. Excretion of fetal biliverdin by the rat placenta-maternal liver tandem. *Am J Physiol Regul Integr Comp Physiol* 2006;**290**(3):R749-56.

6 Fevery J. Bilirubin in clinical practice: a review. *Liver Int* 2008;**28**(5):592-605.

7 Bosma PJ, Seppen J, Goldhoorn B, Bakker C, Oude Elferink RP, Chowdhury JR, Chowdhury NR, Jansen PL. Bilirubin UDP-glucuronosyltransferase 1 is the only relevant bilirubin glucuronidating isoform in man. *J Biol Chem* 1994;**269**(27):17960-4.

8 McLean M, Bowman M, Clifton V, Smith R, Grossman AB. Expression of the heme oxygenase-carbon monoxide signalling system in human placenta. *J Clin Endocrinol Metab* 2000;**85**(6):2345-9.

9 Gourley GR. Bilirubin metabolism and kernicterus. *Adv Pediatr* 1997;**44**:173-229.

10 Baranano DE, Rao M, Ferris CD, Snyder SH. Biliverdin reductase: a major physiologic cytoprotectant. *Proc Natl Acad Sci U S A* 2002;**99**(25):16093-8.

11 Maghzal GJ, Leck MC, Collinson E, Li C, Stocker R. Limited role for the bilirubin-biliverdin redox amplification cycle in the cellular antioxidant protection by biliverdin reductase. *J Biol Chem* 2009;**284**(43):29251-9.

12 Greenberg AJ, Bossenmaier I, Schwartz S. Green jaundice. A study of serum biliverdin, mesobiliverdin and other green pigments. *Am J Dig Dis* 1971;**16**(10):873-80.

13 Larson EA, Evans GT, Watson CG. A study of the serum biliverdin concentration in various types of jaundice. *J Lab Clin Med* 1947;**32**:481-8.

14 Prichard JS. Biliverdin appearing in a case of malnutrition. *Br J Clin Pract* 1972;**26**(10):481-2.

15 Gafvels M, Holmstrom P, Somell A, Sjovall F, Svensson JO, Stahle L, Broome U, Stal P. A novel mutation in the biliverdin reductase-A gene combined with liver cirrhosis results in hyperbiliverdinaemia (green jaundice). *Liver Int* 2009;**29**(7):1116-24.

16 Niittynen M, Tuomisto JT, Auriola S, Pohjanvirta R, Syrjala P, Simanainen U, Viluksela M, Tuomisto J. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced accumulation of biliverdin and hepatic peliosis in rats. *Toxicol Sci* 2003;**71**(1):112-23.

17 De Matteis F, Lord GA, Kee Lim C, Pons N. Bilirubin degradation by uncoupled cytochrome P450. Comparison with a chemical oxidation system and characterization of the products by high-performance liquid chromatography/electrospray ionization mass spectrometry. *Rapid Commun Mass Spectrom* 2006;**20**(8):1209-17.

18 Ye L, Liu S, Wang M, Shao Y, Ding M. High-performance liquid chromatography-tandem mass spectrometry for the analysis of bile acid profiles in serum of women with intrahepatic cholestasis of pregnancy. *J Chromatogr B Analyt Technol Biomed Life Sci* 2007;**860**(1):10-7.

19 Laible M, Boonrod K. Homemade site directed mutagenesis of whole plasmids. *J Vis Exp* 2009;(27), pii: 1135; doi: 10.3791/1135.

20 Briz O, Serrano MA, Rebollo N, Hagenbuch B, Meier PJ, Koepsell H, Marin JJ. Carriers involved in targeting the cytostatic bile acid-cisplatin derivatives cis-

diammine-chloro-cholylglycinate-platinum(II) and cis-diammine-bisursodeoxycholate-platinum(II) toward liver cells. *Mol Pharmacol* 2002;**61**(4):853-60.

21 Markwell MA, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* 1978;**87**(1):206-10.

22 Ridlon JM, Kang DJ, Hylemon PB. Bile salt biotransformations by human intestinal bacteria. *J Lipid Res* 2006;**47**(2):241-59.

23 Fenech FF, Bannister WH, Grech JL. Hepatitis with biliverdinaemia in association with indomethacin therapy. *Br Med J* 1967;**3**(5558):155-6.

24 Tickner TR, Gutteridge JM. A simple colorimetric method for the estimation of plasma biliverdin. *Clin Chim Acta* 1978;**85**(2):125-9.

25 Kikuchi A, Park SY, Miyatake H, Sun D, Sato M, Yoshida T, Shiro Y. Crystal structure of rat biliverdin reductase. *Nat Struct Biol* 2001;**8**(3):221-5.

26 Whitby FG, Phillips JD, Hill CP, McCoubrey W, Maines MD. Crystal structure of a biliverdin IXalpha reductase enzyme-cofactor complex. *J Mol Biol* 2002;**319**(5):1199-210.

27 Maines MD. New insights into biliverdin reductase functions: linking heme metabolism to cell signaling. *Physiology (Bethesda)* 2005;**20**:382-9.

28 Blumenthal SG, Stucker T, Rasmussen RD, Ikeda RM, Ruebner BH, Bergstrom DE, Hanson FW. Changes in bilirubins in human prenatal development. *Biochem J* 1980;**186**(3):693-700.

FIGURE LEGENDS

Figure 1. Representative images of samples collected from patient PJ during the first episode of cholestasis and after surgical removal of her biliary obstruction. The milk sample is compared with milk collected from a healthy volunteer (Control).

Figure 2. A. Schematic representation of the molecular structure of biliverdin IXα and biliverdin IXβ and the major fragments generated from these molecules in MS. B. Representative mass spectra of biliverdin IXα obtained in MS2-scan mode by HPLC-MS/MS using electrospray positive ionization to select the precursor ion (583.3 m/z), indicated with an asterisk. **C.** Mass spectra obtained in product ion mode by filtering the precursor ion at the first quadrupole showing the major product ion (297.25 m/z) generated in the second quadrupole, which was then used in MRM mode for quantitative analyses.

Figure 3. Representative Western blots of wild-type and mutated (c.214C>A; p.Ser44X) V5-tagged biliverdin reductase-IXα (BVRα) carried out with lysates of Alexander cells (10 µg protein) and *Xenopus laevis* oocytes (0.5 µg protein). Alexander cells expressing V5-tagged chloramphenicol acetyltransferase (CAT-V5) were used as a positive control. Detection was performed using a primary monoclonal antibody against the V5 epitope.

Figure 4. Representative phase-contrast (B,C) and fluorescence (D-G) microscopy photographs of human hepatoma Alexander cells expressing wild-type and mutated (c.214C>A; p.Ser44X) V5-tagged biliverdin reductase-IXα (BVRα). Nuclei were counterstained with DAPI (blue). Detection of BVRα (green) was performed using a primary monoclonal antibody against the V5 epitope and a FITC-conjugated secondary antibody.

Figure 5. A. Representative total ion chromatograms (TIC) showing peaks corresponding to biliverdin IXα (product ion 297.2 m/z) and bilirubin IXα (product ion 299.2 m/z) obtained by injection in HPLC-MS/MS of methanol lysates from *Xenopus laevis* oocytes control (Non-injected with cRNA) or expressing wild type (WT-BVRα) or mutated (c.214C>A; p.Ser44X) human biliverdin reductase alpha (Mutated-BVRα). All oocytes were microinjected with exogenous biliverdin IXα (300 pmol/oocyte) 60 min before analysis. B-E. Functional activity of human biliverdin reductase alpha (BVRα) in *Xenopus laevis* oocytes. Oocyte contents of biliverdin IXα (B,C) and bilirubin IXα (D,E) were measured by HPLC-MS/MS in oocytes control and expressing wild type (WT-BVRα) or mutated

(c.214C>A; p.Ser44X) BVRα (Mut-BVRα) without (B,D) or with (C,E) exogenous administration of 300 pmols of biliverdin IXα 60 min before collection for analysis. Results are means±SEM from at least 20 determinations per data point using oocytes from three different frogs. *, $p<0.05$ as compared with Control cells by the Bonferroni method of multiple-range testing.

Table 1. Biliverdin IXα concentrations in body fluids

Sample	Individuals	Day	Biliverdin (μM)
Serum	Control		0.12±0.04
	PJ's Sister		**0.36±0.03**
	Patients		
	EA	-1	**51.5**
	PJ	-2	**13.1**
		-1	**25.7**
		0	**32.4**
		+5	**0.33**
Urine	Control		<0.05
	Patients		
	EA	-1	**14.6**
	PJ	+5	**3.13**
Milk	Control		0.10±0.01
	Patients		
	PJ	+4	**1.11**
Bile	Patients		
	PJ	0	**124.7**

Surgical removal of biliary obstruction was carried out on day 0. Biliverdin IXα was determined in triplicate by HPLC-MS/MS. Control values were means±SD from samples collected from 7 volunteer women of ages similar to those of the probands. Four serum samples collected from the patient PJ's sister were also analyzed. Values are means±SD. Results above normal values are in bold case.

Licence Statement:

Jose J. G. Marin, the Corresponding Author of the article
Ref: JMG/2009/074567 -
"A homozygous nonsense mutation (c.214C>A) in biliverdin reductase alpha gene (BLVRA) results in accumulation of biliverdin during episodes of cholestasis",

has the right to grant on behalf of all authors and does grant on behalf of all authors, a licence to the BMJ Publishing Group Ltd and its licensees, to permit this Contribution (if accepted) to be published in Journal of Medical Genetics (JMG) and any other BMJ Group products and to exploit all subsidiary rights, as set out in our licence set out at: (http://jmg.bmj.com/site/about/licence.pdf)

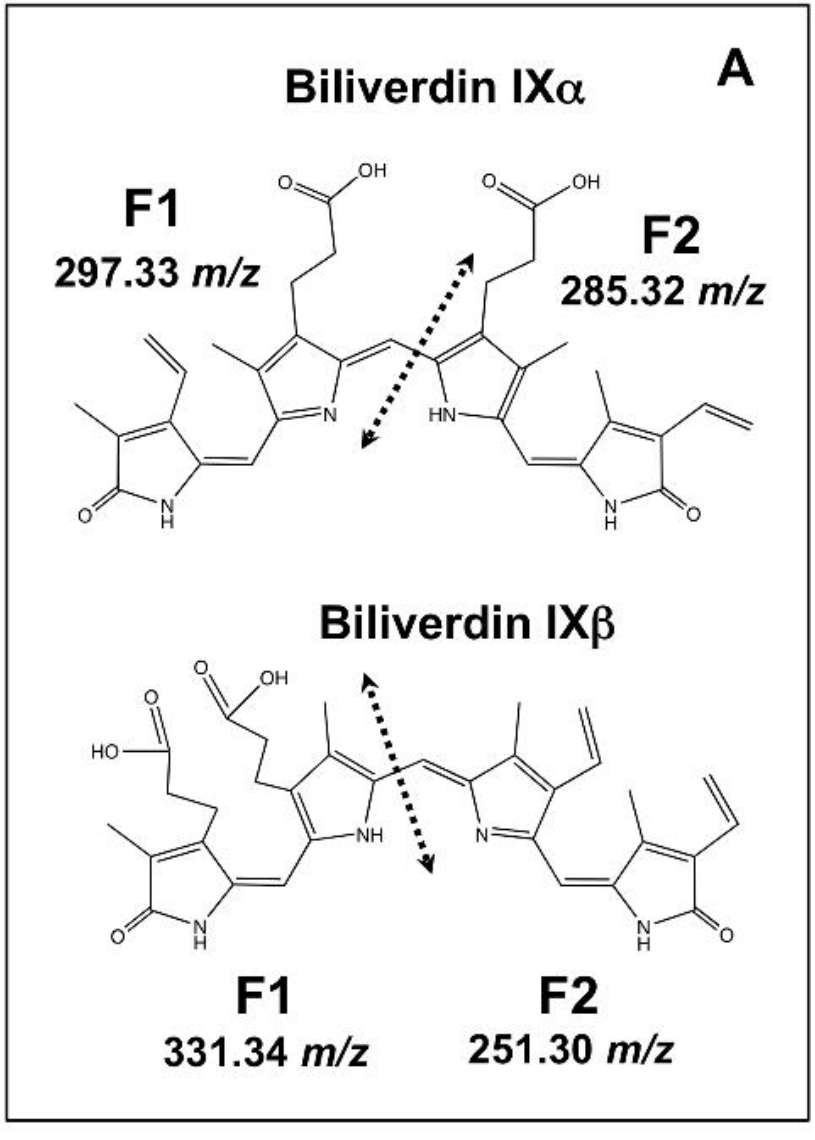
I am one author signing on behalf of all co-owners of the Contribution.

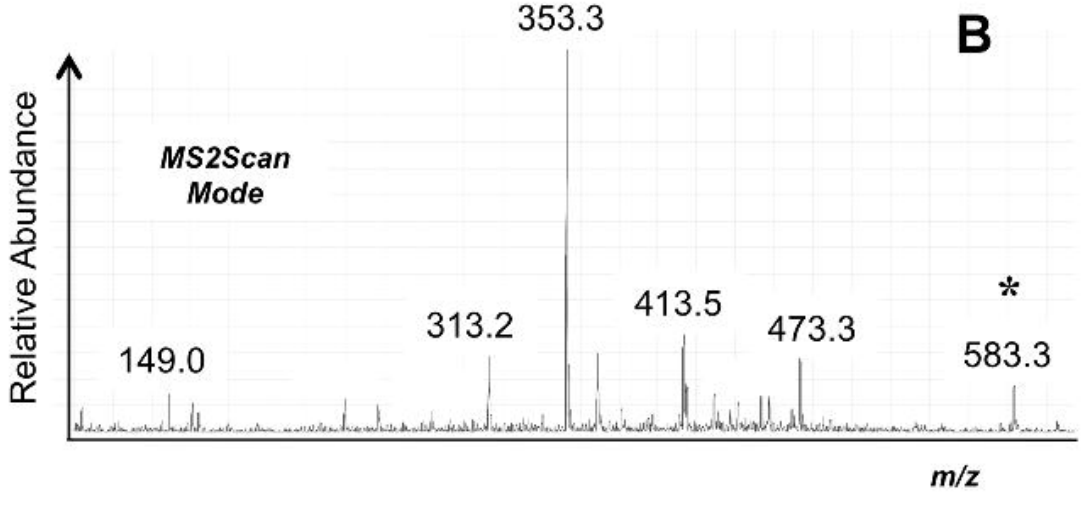
Competing Interest: None declared.

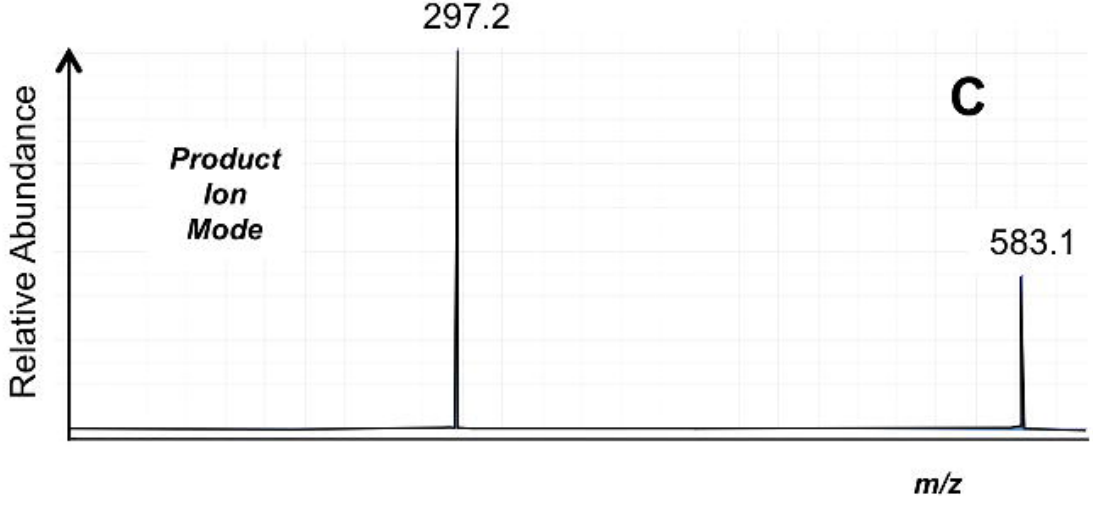
Patient PJ (time referred to surgical des-obstruction)	Control

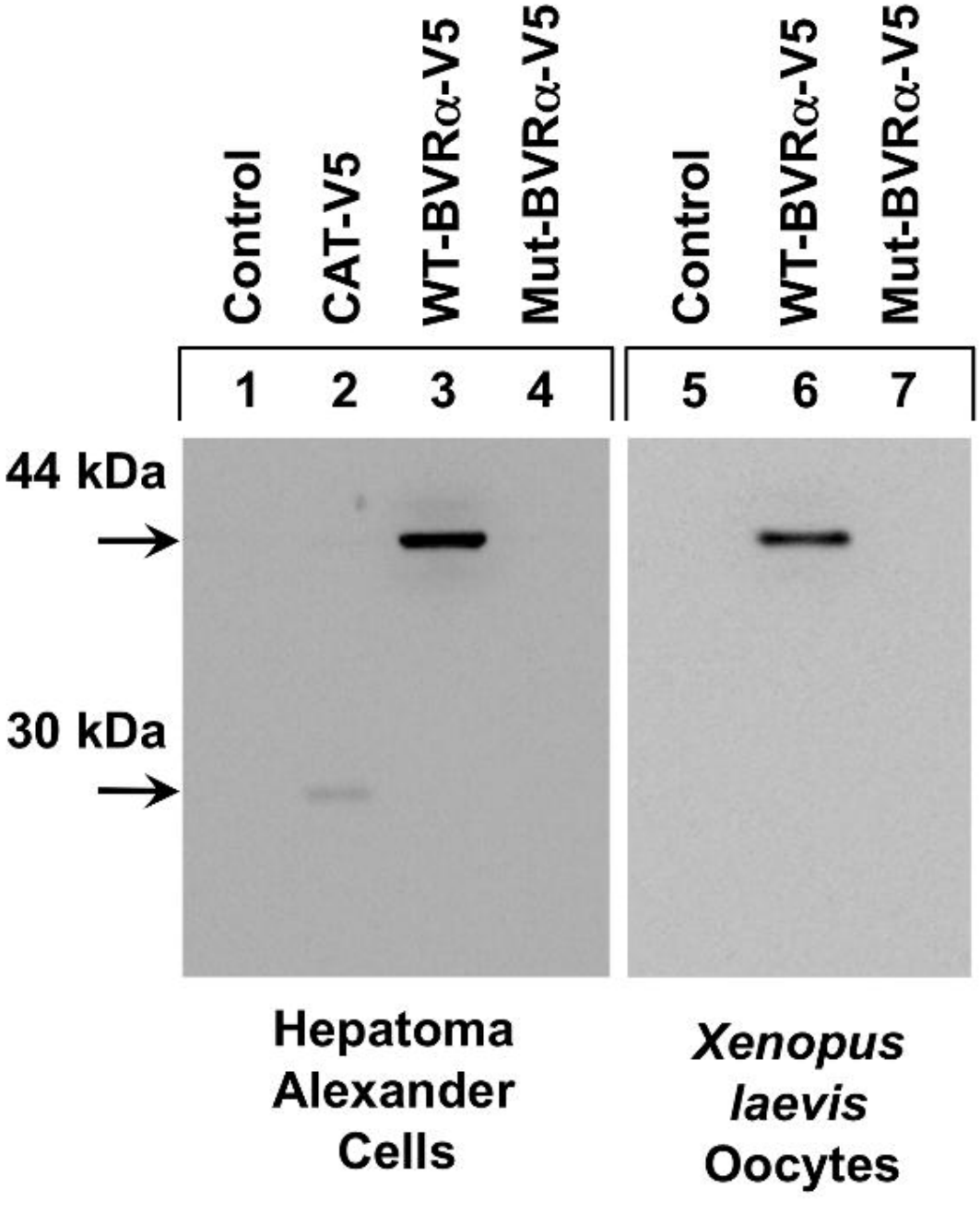
Days 0 -1 0 +5 +4

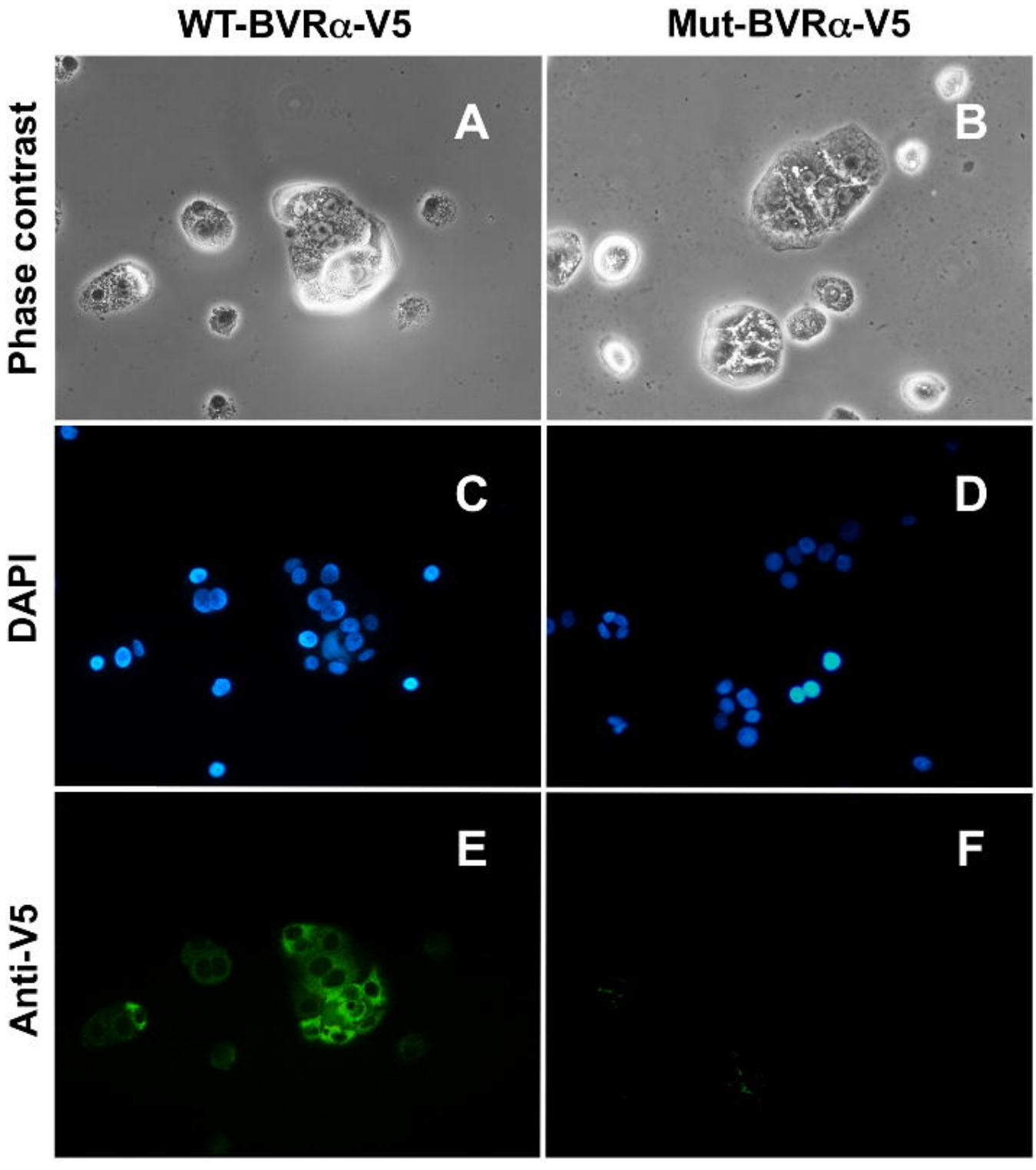
Bile	Urine	Serum	Milk

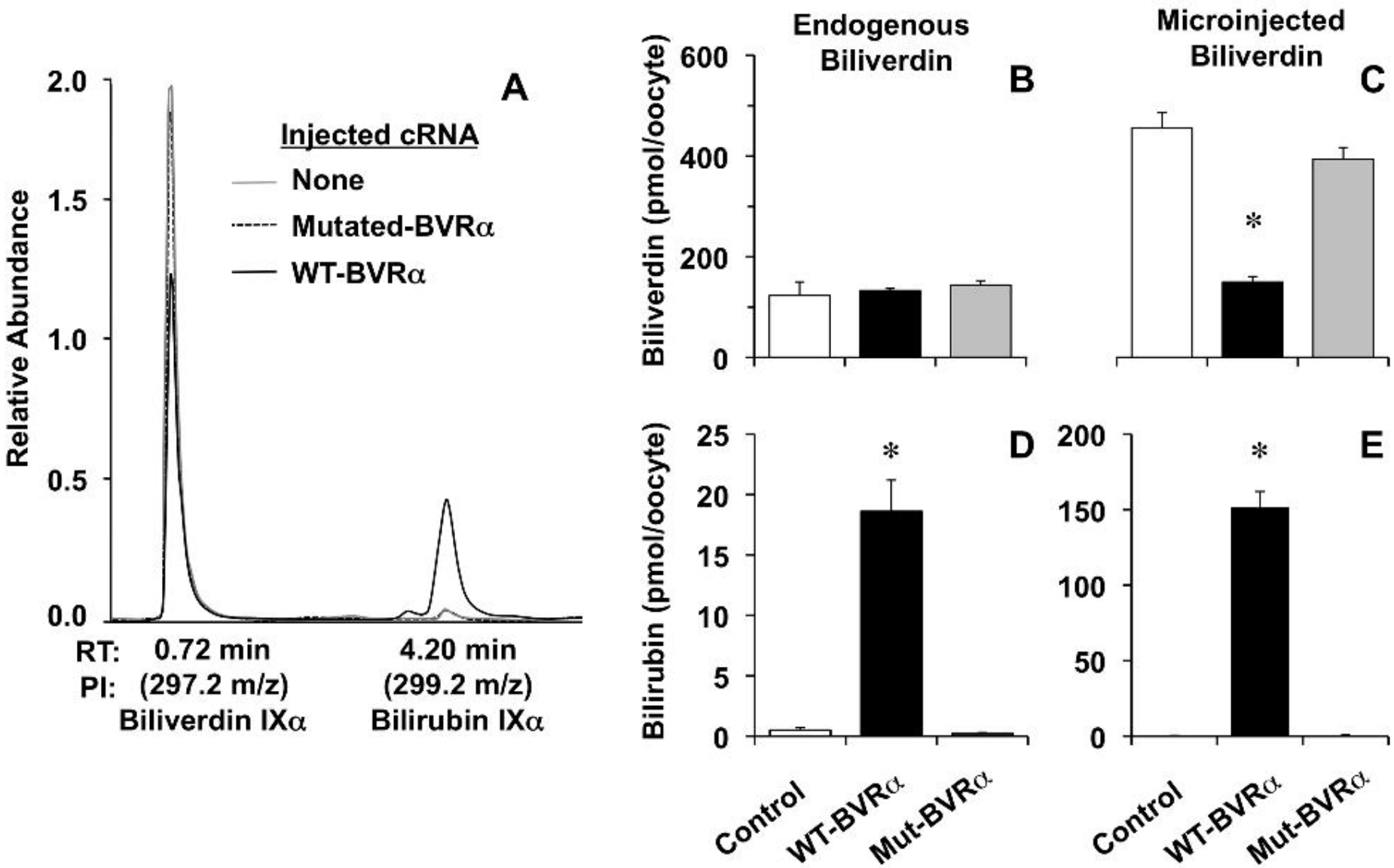

A
Biliverdin IXα
F1
297.33 m/z
F2
285.32 m/z
Biliverdin IXβ
F1
331.34 m/z
F2
251.30 m/z


B
353.3
MS2Scan Mode
Relative Abundance
313.2
413.5
473.3
*
583.3
149.0
m/z


C
297.2
Product Ion Mode
Relative Abundance
583.1
m/z


Control
CAT-V5
WT-BVRα-V5
Mut-BVRα-V5
Control
WT-BVRα-V5
Mut-BVRα-V5
1
2
3
4
5
6
7
44 kDa
30 kDa
Hepatoma Alexander Cells
Xenopus laevis Oocytes


WT-BVRα-V5
Mut-BVRα-V5
Phase contrast
DAPI
Anti-V5
A
B
C
D
E
F


A
Injected cRNA
None
Mutated-BVRα
WT-BVRα
Relative Abundance
2.0
1.5
1.0
0.5
0.0
RT: 0.72 min
PI: (297.2 m/z)
Biliverdin IXα
4.20 min
(299.2 m/z)
Bilirubin IXα
Endogenous Biliverdin
B
Microinjected Biliverdin
C
Biliverdin (pmol/oocyte)
600
400
200
0
*
Bilirubin (pmol/oocyte)
D
E
25
20
15
10
5
0
200
150
100
50
Control
WT-BVRα
Mut-BVRα