The aetiology of cholestasis in pregnancy

Catherine Williamson

BSc MB ChB MRCP (UK)

A thesis submitted to the University of Manchester for the degree of MD in the Faculty of Medicine

August 2002

TABLE OF CONTENTS

List of figures and tables

Figures

ABSTRACT

Obstetric cholestasis is a liver disease of pregnancy characterised by maternal pruritus and raised serum bile acids. The fetal complications include spontaneous prematurity, fetal distress and unexplained third trimester intrauterine death. There are currently no established ways to predict which pregnancies will have fetal complications. The aetiology of the condition, and that of the fetal complications is currently not known. The aims of the studies reported in this thesis were to establish the clinical features, inheritance and aetiology of the condition in the UK.

The clinical studies confirmed that 90% of intrauterine deaths occur after 37 weeks gestation, but it was not possible to identify a reliable way to predict 'at-risk' pregnancies. Spontaneous prematurity was shown to be commoner in pregnancies where the pruritus started at an earlier gestation. Pedigree studies revealed that 11% of parous sisters develop the condition, suggesting a genetic component to the aetiology. Gallstones are commonly coinherited with obstetric cholestasis in affected pedigrees.

Genetic studies of the *MDR3* gene demonstrated a mutation (A546D) in one woman from a subgroup of 8 cases with a raised gamma-glutamyl transpeptidase. Functional studies in P-glycoprotein (P-gp1) demonstrated that this mutation does not alter the function of the protein, but that it causes a reduced amount of protein at the cell surface, suggesting that it is a trafficking mutant.

Primary cultures of rat cardiomyocytes were used as an *in vitro* model of the fetal heart. The primary bile acid taurocholate was demonstrated to cause an abnormal rate and rhythm of contraction and to influence cellular calcium dynamics that are associated with contraction. The therapeutic agents dexamethasone, and to a lesser

extent ursodeoxycholic acid, protected cardiomyocytes from taurocholate-induced dysrhythmias.

These results help explain the aetiology of obstetric cholestasis, and give insights into the mechanisms of the fetal complications. The results from the clinical and inheritance studies reported in this thesis will be of value for counselling of women with the condition and their relatives. Subsequent studies to the *MDR3* experiments will reveal what additional genetic abnormalities confer susceptibility to the condition, and the ways they influence the development of the fetal complications. The results of the cardiomyocyte studies suggest that the therapeutic agents ursodeoxycholic acid and dexamethasone could protect against the fetal complications of obstetric cholestasis as well as treating maternal symptoms.

DECLARATION:

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institution of learning, with the exception of the clinical data presented in Chapter 2. Some of these data were collected by a medical student, Laura Hems, and presented as a BSc project (B Clin Sci, Imperial College, 2001) that was supervised by Dr Williamson.

COPYRIGHT AND THE OWNERSHIP OF INTELLECTUAL PROPERTY RIGHTS:

This work is dedicated to Richard

and Alexander

Acknowledgements

I would like to acknowledge and profoundly thank Professor Michael de Swiet and Professor Des Johnston for their supervision, insight and guidance whilst I performed this work.

I would also like to thank the Professor Chris Higgins, Clinical Sciences Centre Faculty of Medicine, Imperial College of Science, Technology and Medicine for the provision of facilities and support whilst this work was performed.

I would like to thank the scientists and colleagues with whom I have worked and collaborated, including Jenny Chambers, Peter Dixon, Elwyn Elias, Vicki Froome, Julia Gorelik, Dimitri Goulis, Laura Hems, Yuri Korchev, Kenneth Linton, Cath Nelson-Piercy, Poorvi Patel, Ian Walker, Judith Weaver and Natasha Weerasekera.

ABBREVIATIONS

ABC transporter	ATP-binding cassette
ATP	adenosine triphosphate
BA	bile acid
BRIC	benign recurrent intrahepatic cholestasis
BSEP	bile salt export pump
CA	cholic acid
CDCA	chenodeoxycholic acid
CESDI	confidential enquiry into stillbirths and deaths in utero
CYP7A1	cholesterol 7α-hydroxylase
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribosenucleic acid
ER	endoplasmic reticulum
FACS	fluorescent activated cell sorting
FIC1	familial intrahepatic cholestasis type 1
FXR	farnesoid X receptor
GGT	gamma-glutamyl transpeptidase
HEK293T	human epithelial kidney
ICP	intrahepatic cholestasis of pregnancy
IUD	intrauterine death
LRH	liver-related hormone 1
LXR	liver X receptor
MDR	multidrug resistance
MRP	multiresistance associated transport proteins
NBD	nucleotide binding domain
NICU	neonatal intensive care unit
NTCP	Na^+-taurocholate co-transporting polypeptide
OATP	organic anion transporting peptide
OC	obstetric cholestasis
OCPO	obstetric cholestasis patient organisation
OD	optic density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC	phosphatidyl choline
PCR	polymerase chain reaction
PFIC	progressive familial intrahepatic cholestasis
P-gp1	P-glycoprotein
RXR	retinoid X receptor
SHP	short heterodimer protein
SICM	scanning ion conductance microscopy
SPGP	sister of P-glycoprotein
SNP	single nucleotide polymorphism
UDCA	ursodeoxycholic acid
UV	ultraviolet

1. INTRODUCTION

1.1 Pathogenesis of cholestasis

1.1.1 Physiology of bile formation

Bile formation is a complex, multi-step process, which requires distributed liver perfusion with an intact lobular gradient, normal hepatocyte uptake and excretion mechanisms, a rapid transcellular transport system, sealed tight junctions to retain the cellular products and a patent biliary tree.

Canalicular bile is the major secretion product of hepatocytes. It is an aqueous solution of organic and inorganic compounds with an osmolarity close to that of plasma. A normal human adult produces approximately 600 ml of bile per day. Above a critical concentration, bile acids form macromolecular complexes (micelles) due to their amphipathic character. The concentrations of cholesterol and phospholipids can far exceed their water solubility due to the formation of mixed micelles consisting of bile acids, phospholipids and cholesterol.

The functions of bile formation include the excretion of endobiotics such as cholesterol, porphyrins, bilirubin and aged proteins, the excretion of xenobiotics such as drugs, heavy metals and environmental toxins, and the facilitation of digestion by providing bicarbonate-rich fluid and bile acids. In addition it performs the immunological function of IgA transport to the intestinal mucosa. Bile is the major route of excretion of lipid-soluble organic compounds with a molecular weight of 300-500 Da, after these have been converted into more hydrophilic metabolites by hepatic biotransformation.

Bile acids and other organic anions are actively transported from the blood into the hepatocyte and the bile canaliculus, where osmotic gradients are formed that stimulate the flow of bile. The bile canaliculus is formed between adjacent hepatocytes and is

sealed by 'tight junctions' that form a diffusion barrier between the extracellular environment and the bile canalicular lumen. Tight junctions also divide the hepatocyte plasma membrane into canalicular (apical) and sinusoidal (basolateral) domains that define the cells polarity. These membrane domains differ with respect to lipid composition and ion transport proteins. Thus hepatocytes are highly polarised.

1.1.2 Bile acid synthesis

Bile acids are synthesised from cholesterol in the liver. The first, and rate-limiting enzyme is cholesterol 7α-hydroxylase (CYP7A1), a microsomal cytochrome P450 that converts cholesterol to 7α-hydroxycholesterol. Bile acid synthesis is one of the major mechanisms for cholesterol elimination from the body, with approximately 50% of daily cholesterol disposal occurring by this route. This process is believed to be tightly regulated, and several recent studies have shed light on the molecular regulation of this process. This will be discussed in section 1.1.4.

There is another 'alternative' pathway of bile acid synthesis. This is thought to be a back-up pathway for when CYP7A1 is defective, and it preferentially synthesises chenodeoxycholic acid from cholesterol. The rate limiting step in this pathway is catalysed by a mitochondrial enzyme, sterol 27-hydroxylase (CYP27).

After biosynthesis from cholesterol and before excretion from the hepatocyte, bile acids are conjugated with either glycine or taurine, and this converts a weak acid to a strong acid. As a result, conjugated bile acids are fully ionised at the range of pH values seen in the intestine, and thus exist as charged bile *salts*, to which membranes are impervious.

When bile acids are described, the side chain (i.e. whether conjugated with glycine or taurine, or unconjugated) is usually described separately from the nature of the steroid

nucleus. In humans, the majority of bile acids are conjugated with glycine. Figure 1 shows the structure of the major human bile acids, including the structure of the steroid nucleus in the main primary and secondary bile acids, and that of ursodeoxycholic acid, a tertiary bile acid in humans.

Primary bile acids

Two *primary* bile acids are synthesised from cholesterol in the human liver: cholic acid (CA) predominantly, and chenodeoxycholic acid (CDCA). Chenodeoxycholic acid, a dihydroxy bile acid with α-hydroxy groups at the 3rd and 7th carbon atoms (C-3 and C-7), can be considered the building block of other bile acids, because every bile acid must initially have a hydroxy group at C-3 and C-7 of the steroid nucleus. A C-3 is present in cholesterol and a C-7 is always present because hydroxylation at this site occurs in the biosynthetic pathway of all bile acids. In humans, cholic acid is formed by the addition of a hydroxy group at C-12.

Secondary and tertiary bile acids

The *secondary* bile acids are derived from bacterial modification in the terminal ileum and colon, mostly de-conjugation and 7α-dehydroxylation. Removal of the C-7 hydroxy group by bacterial dehydratases forms the 7-deoxy bile acids (the term *deoxy* means that an oxygen-containing group has been lost). By this process cholic acid (hydroxy groups at C-3, C-7 and C-12) is converted to deoxycholic acid (C-3 and C-12), and chenodeoxycholic acid (C-3 and C-7) is converted to a monohydroxy-bile acid, lithocholic acid, with a hydroxy group only at C-3.

Ursodeoxycholic acid is thought to be formed by bacterial epimerisation of the hydroxy group at C-7 in the distal small intestine, or large intestine, followed by

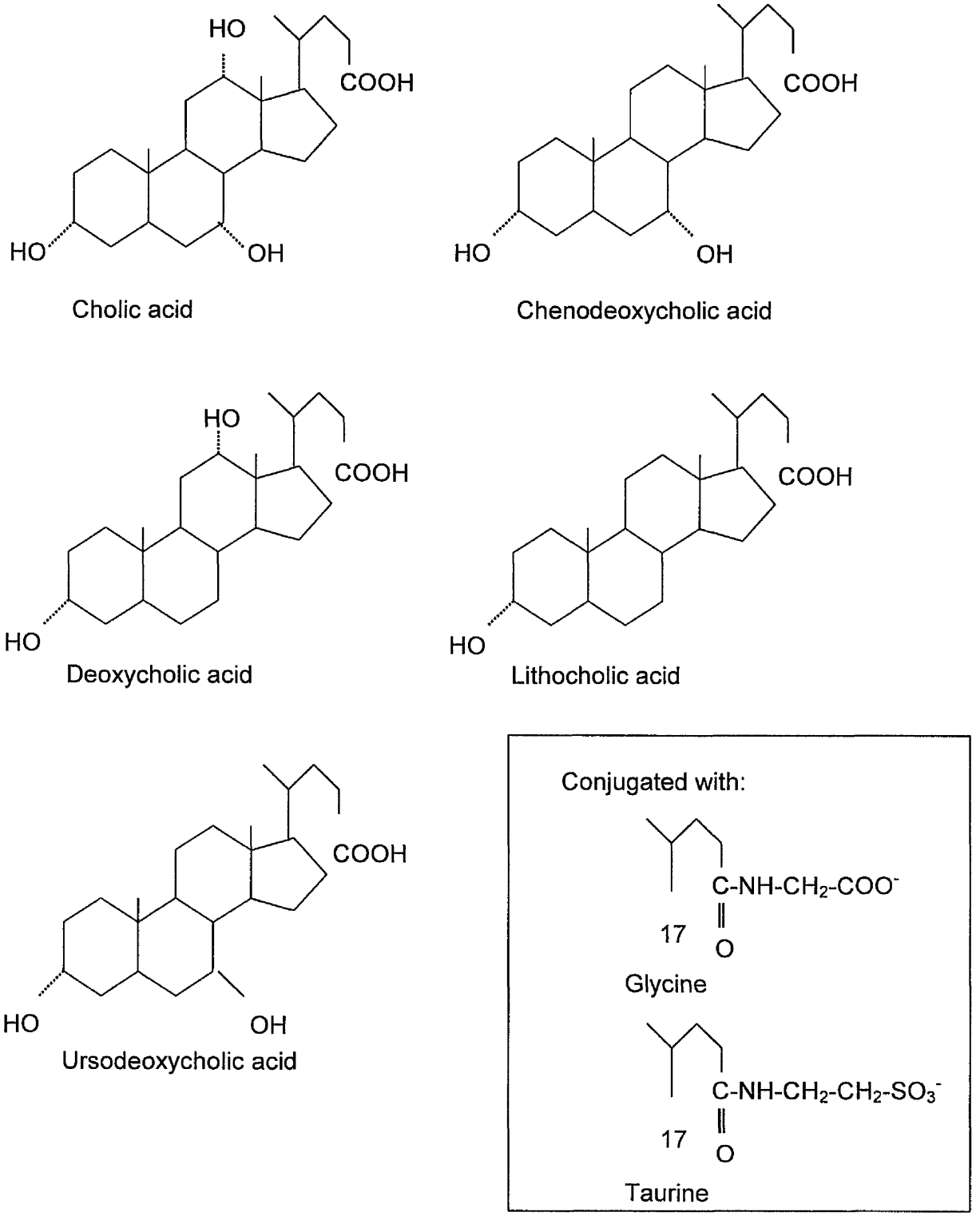


Figure 1. Structural formulae of bile acids.

hepatic metabolism (MacDonald et al. 1981), and is therefore sometimes referred to as a *tertiary* bile acid. It is detectable only in trace amounts in humans.

1.1.3 Mechanisms of bile flow.

There are two major mechanisms of bile flow; bile salt-dependent and bile salt-independent flow. Bile salt-dependent flow depends on hepatocyte uptake, translocation and secretion of bile acids. Bile acid secretion is the major driving force for bile flow, and is regulated by the co-ordinated action of transporter proteins in the sinusoidal and canalicular membranes of hepatocytes. The proteins in the sinusoidal membrane transport organic anions, cations and bile acids. The canalicular proteins transport endogenous and exogenous metabolites into the bile against a concentration gradient.

1.1.3.1. Sinusoidal membrane transporters

Na^+-taurocholate co-transporting polypeptide (NTCP)

Human NTCP consists of 349 amino acids and belongs to the Na^+-bile acid symporter family in the superfamily of Na^+-solute symporters (Reizer et al. 1994). This family also contains the human ileal-localised NTCP (iNTCP) which shares 35% identity with the human NTCP. NTCP is dependent on the transmembrane sodium gradient, and is relatively specific for conjugated and unconjugated bile salts, and some other conjugated sterols (Kouzuki et al. 2000). It is responsible for about 95% of sinusoidal bile acid transport. Its expression is regulated by bile acid concentrations (Jansen et al. 2000).

Polyspecific organic anion transport proteins

Members of the family of organic anion transport proteins (OATPs) are sodium-independent and have a much broader specificity, transporting bile salts, conjugated steroids such as 17β-oestradiol-glucuronide, organic anions (including conjugated bilirubin) and thyroid hormones. OATP proteins that have been characterised to date include OATP-A to E and OATP-8. Not all members of this family have been fully characterised, but it is apparent that they have different tissue-specific expression patterns and substrate specifities. OATP-B, OATP-C and OATP-8 are predominantly expressed at the sinusoidal (basolateral) membrane of hepatocytes, and are the members of the OATP family that account for the majority of sodium-independent bile acid transport across this membrane of the liver (Kullak-Ublick et al. 2001).

1.1.3.2. Canalicular membrane transporters

Most of these proteins belong to the ATP-binding cassette (ABC) superfamily (Higgins et al. 1986, 1992), and their transport function directly depends on the hydrolysis of Mg^{2+}/ ATP. ABC transporters consist of four domains: two homologous nucleotide binding domains (NBDs) which bind and hydrolyse ATP and whose sequence is highly conserved throughout the ABC transporter family, and two hydrophobic transmembrane domains which span the membrane multiple times. The NBDs contain an ATP-binding site with characteristic Walker motifs (A and B) and the S signature of ABC transporters. The ABC transporters that are most important with respect to bile acid secretion are those that transport organic solutes (i.e. the bile salt export pump (BSEP), the multiresistance associated transport proteins (MRP2-4) and phospholipids (i.e. multidrug resistance 3 protein (MDR3)). The other canalicular

transporter that plays a role in phospholipid and bile transport is the familial intrahepatic cholestasis 1 (FIC1) protein, which is a P-type ATPase.

Bile salt export pump (BSEP)

The *BSEP* gene (also known as *ABCB11*) maps to chromosome 2q24 (Strautnieks et al. 1996, 1997), and codes for the major canalicular bile salt export pump in man (Strautnieks et al. 1998). *BSEP* is the human orthologue of the rat gene *Spgp* that encodes the ABC transporter sister of P-glycoprotein (SPGP) (Gerloff et al. 1998). There is 82% amino acid identity between the human and rat predicted sequences (Strautnieks et al. 1998).

Mutations in *BSEP* have been demonstrated in patients with the childhood liver disease progressive familial intrahepatic cholestasis type 2 (PFIC2) (Strautnieks et al. 1998) (section 1.1.6.1).

Multidrug resistance 3 protein (MDR3)

The *MDR3* gene (also known as *ABCB4* and *MDR2*), the homologue of murine *mdr2* (Linke et al. 1991, Gros 1986) maps to chromosome 7q21, and encodes the MDR3 protein. The MDR3 protein has been shown to be localised to the bile canilicular membrane and is a phosphatidyl choline (PC) flippase (Ruetz and Gros 1994, Smith et al. 1994, van Helvoort et al 1996).

Mice homozygous for disruption of the *mdr2* gene lack the Mdr2 P-glycoprotein and develop a liver disease that is caused by the complete inability to secrete phospholipid into bile (Smit et al. 1993). Transgenic mice that express human *MDR3* were generated using the *mdr2* -/- strain. In these mice, the MDR3 protein is exclusively located in the canalicular membrane of hepatocytes, and phospholipid secretion into bile is restored (Smith et al. 1998). The clinical, histological and biochemical features of the *mdr2* -/- mice are very similar to those of progressive familial intrahepatic

cholestasis type 3 (PFIC3), and it was demonstrated that a patient with PFIC3 had no *MDR3* messenger RNA (mRNA) present in the liver (Deleuze et al. 1996). Some heterozygote mothers of children with PFIC3 have symptoms consistent with a diagnosis of obstetric cholestasis (see section 1.1.6.1).

The *MDR3* gene is located immediately downstream from the *MDR1* gene which codes for the ABC transporter, P-glycoprotein (P-gp1) (Lincke et al. 1991). Although P-gp1 may not influence biliary bile acid transport directly, it will be briefly discussed in this section, because its similarities to the MDR3 protein were utilised for some of the functional experiments described in this thesis. Figure 2 gives a schematic representation of the interaction between MDR3 and BSEP at the canalicular membrane in the transport of PC and bile acids and their incorporation into mixed micelles.

P-glycoprotein (P-gp1)

P-gp1 is expressed in many tissues, in particular in the epithelia of excretory organs, and plays a role in the elimination of toxic metabolites from the body. P-gp1 shares 77% identity with the MDR3 protein and it has been shown that substitution of as few as three adjacent MDR3 protein residues in the first transmembrane domain by the equivalent residues from P-gp1 is sufficient to allow the chimeric molecule to transport substrates specific to P-gp1 (Zhou et al. 1999). The level of sequence identity between P-gp1 and the MDR3 protein increases to 87% in the NBDs. In addition, the NBDs of P-gp1 and the MDR3 protein, have been shown to be interchangeable (Buschman et al. 1991).

Familial intrahepatic cholestasis 1 (FIC1)

The familial intrahepatic cholestasis 1 (*FIC1*) gene maps to chromosome 18q21, and encodes a P-type ATPase that has been localised to the canalicular membrane of

hepatocytes (Eppens et al. 2001). It has been shown to also be expressed in a wide variety of tissues using Northern blots. The role of FIC1 has not been clearly

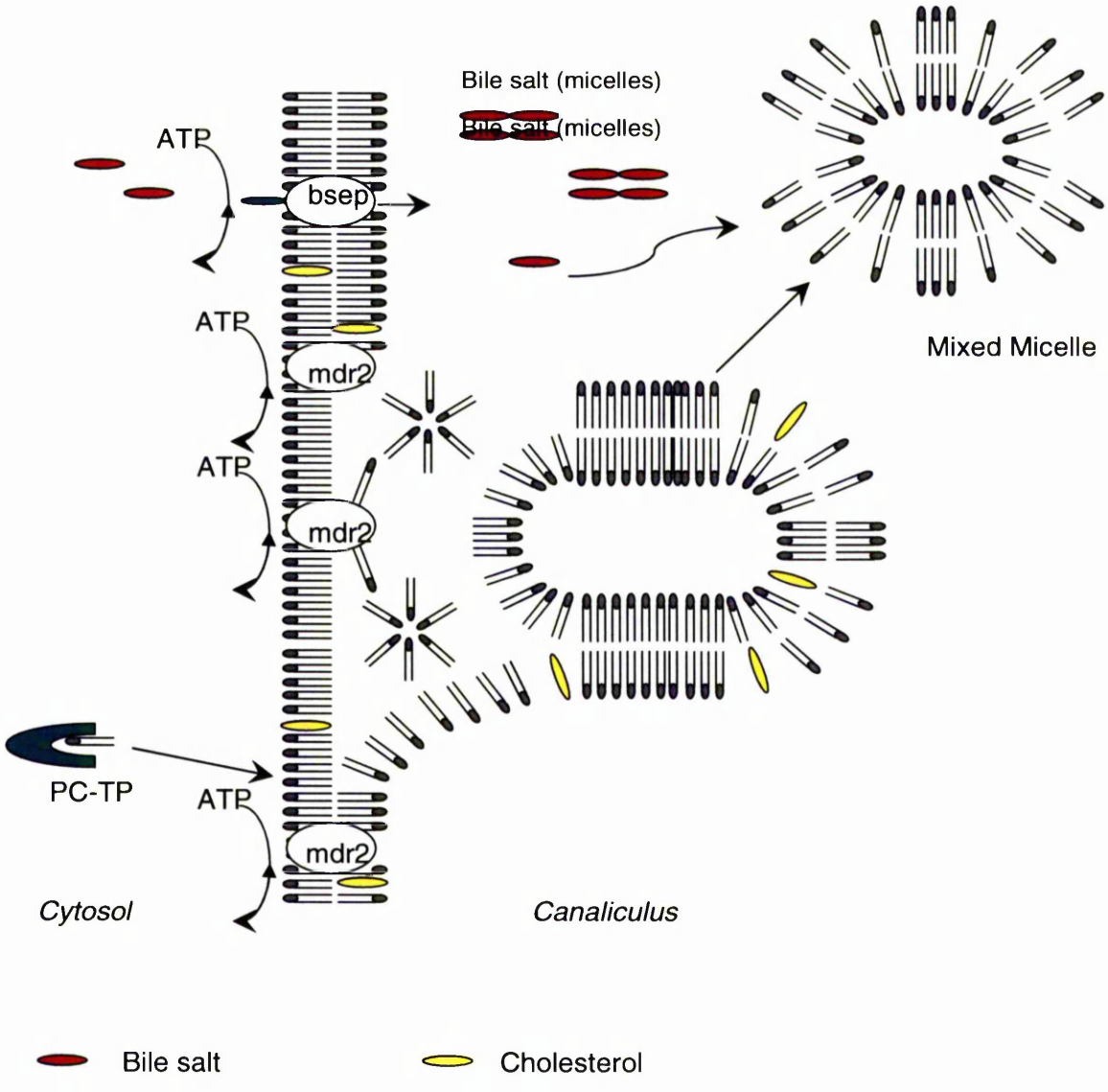


Figure 2. Events at the canalicular membrane. Bile acids are transported to the canalicular lumen by the ATP-stimulated bile salt export pump (BSEP). Phosphatidyl choline (PC) molecules are transported to the canalicular membrane by the PC transport protein and across the canalicular membrane by the PC 'flippase' (mdr2, the murine equivalent of MDR3). When PC molecules achieve sufficient enrichment at the luminal face of the canalicular membrane, they bud out forming bilayer vesicles that adsorb bile acid molecules. When the proportion of bile acid molecules is sufficiently high, a mixed micelle is formed (modified from Hoffman 1999).

established. As it is most highly expressed in liver, intestine, cholangiocytes and colon, it has been proposed that it plays a role in the enterohepatic circulation of bile acids (Bull et al. 1998). It has also been suggested that it plays a role in the secretion of highly hydrophobic bile acids, as *FIC1* mutations have been reported in progressive familial intrahepatic cholestasis type 1 (PFIC1) (Bull et al. 1998), a condition in which there is a marked reduction of hydrophobic bile acids in bile (Oude Elferink et al. 1998) (see section 1.1.6.1).

Multiresistance associated transport proteins

The human MRP family currently has 6 members (MRP1-6). The length of the proteins and their membrane topology distinguishes the MRPs from the MDR P-glycoproteins. In addition to the typical six plus six membrane topology described for the MDR family, most of the MRP family have an additional aminoterminal membrane-spanning domain. MRP2, the canalicular multispecific organic anion transporter (cMOAT) protein transports a broad range of amphipathic anionic conjugates as substrates (Ishikawa et al. 1990, Jedlitschky et al. 1996). The human *MRP2* gene is mainly expressed in liver and kidney and maps to chromosome 10q24 (Taniguchi et al. 1996). MRP2 is mutated in patients with Dubin-Johnson syndrome, a condition characterised by mild jaundice, raised conjugated bilirubin and accumulation of black pigment in the liver due to the retention of the oxidation products of tryptophan (Kartenbeck et al. 1996, Toh et al. 1999). Mrp2 transport of estradiol-17-beta-glucuronide is necessary for estrogen-induced cholestasis secondary to inhibition of Bsep (Steiger et al. 2000; Huang et al. 2000) (section 1.1.7).

Unlike human and rat MRP1 and MRP2, rat MRP3 is able to transport bile acids (Hirohashi et al. 2000). MRP3 mediates the transport of anionic conjugates across the basolateral hepatocyte membrane into sinusoidal blood. Its precise role has not been

elucidated, but it is thought to play a compensatory role in the hepatic secretion of anionic conjugates during impaired transport into bile (Keppler and Konig 2000).

1.1.3.3 Translocation of bile acids across hepatocytes

Little is known about the translocation of bile acids across the hepatocyte. Either diffusion or cytosolic bile acid-binding proteins may be involved. There is some evidence for vesicular bile acid transport through the hepatocyte, since intracellular bile acid-containing vesicles are detectable by electron microscopy (Crawford et al. 1994). Once bile acids enter the hepatocyte, they bind to cytosolic binding proteins. There is evidence that protein-bound bile salts are delivered to the canalicular membrane primarily by diffusion (Crawford 1996).

A familial form of cholestasis has been described in North American Indian children. This is characterised by progressive hepatic damage, cholestasis, hepatomegaly, portal hypertension with variceal bleeding and facial telangiectasia (Weber at al. 1979, 1981). Physical development is otherwise normal. Hepatocyte changes are chiefly characterised by a widening of the pericanalicular microfilamentous zone, resembling the changes caused by phalloidine poisoning (Gabbiani et al. 1975). It has therefore been suggested that this condition may represent a model of microfilament dysfunction-induced cholestasis in humans (Weber et al. 1981).

1.1.3.4 Bile salt-independent flow

Approximately one third of canalicular bile flow is formed independently of bile acid secretion. This is partly dependent on the presence of bicarbonate (Hardison 1978) and may involve chloride/ bicarbonate exchange on the canalicular membrane (Meier 1985). Bicarbonate secretion may explain the 'hypercholeresis' caused by some bile

acids, such as ursodeoxycholic acid, although the source of the bicarbonate may be the bile ductules rather than the hepatocyte (Yoon 1986). Other mechanisms for bile salt-independent flow are biliary excretion of glutathione and its metabolites, secretin-stimulated bile secretion from the bile ducts, and exocytosis.

A lobular gradient exists for bile secretion within the liver. The majority of bile acid transport is performed by periportal hepatocytes (zone I). Perivenular (zone III) hepatocytes are located downstream in the lobule, and because of efficient clearance of bile acids in zone I, and the countercurrent flow of bile in the opposite direction, hepatocytes in zone III are usually exposed only to low concentrations of bile acids. Bile secretion in this region usually occurs by bile salt-independent mechanisms. This may explain why, in cholestasis, bile staining is most marked in zone III; i.e. when canalicular bile secretion is impaired, a large proportion of bile acid bypasses zone I and is then transported by zone III hepatocytes at the 'blind-end' of the canaliculus.

1.1.4 The bile acid receptor and related transcription factors

The bile acid receptor was first identified as an orphan nuclear receptor, the farnesoid X receptor (FXR). FXR functions as a heterodimer with the retinoid X receptor (RXR). It was subsequently shown that bile acids, particularly the primary bile acid chenodeoxycholic acid, were physiological ligands for FXR:RXR (Makashima et al. 1999; Parks et al. 1999; Wang et al. 1999). The rate-limiting enzyme for bile acid synthesis from cholesterol is CYP7A1, and both bile acids and cholesterol are the main regulators of bile acid synthesis by acting as inducers or repressors of CYP7A1. Animals fed a high cholesterol diet show upregulation of CYP7A1 activity, while bile acid feeding down-regulates CYP7A1. The molecular mechanisms underlying these regulatory changes are mediated by FXR, LXR (liver X receptor) and related

transcription factors. This process is summarised in Figure 3. LXRs function as RXR heterodimers, and their ligands are a group of oxysterols that are derived from tissue

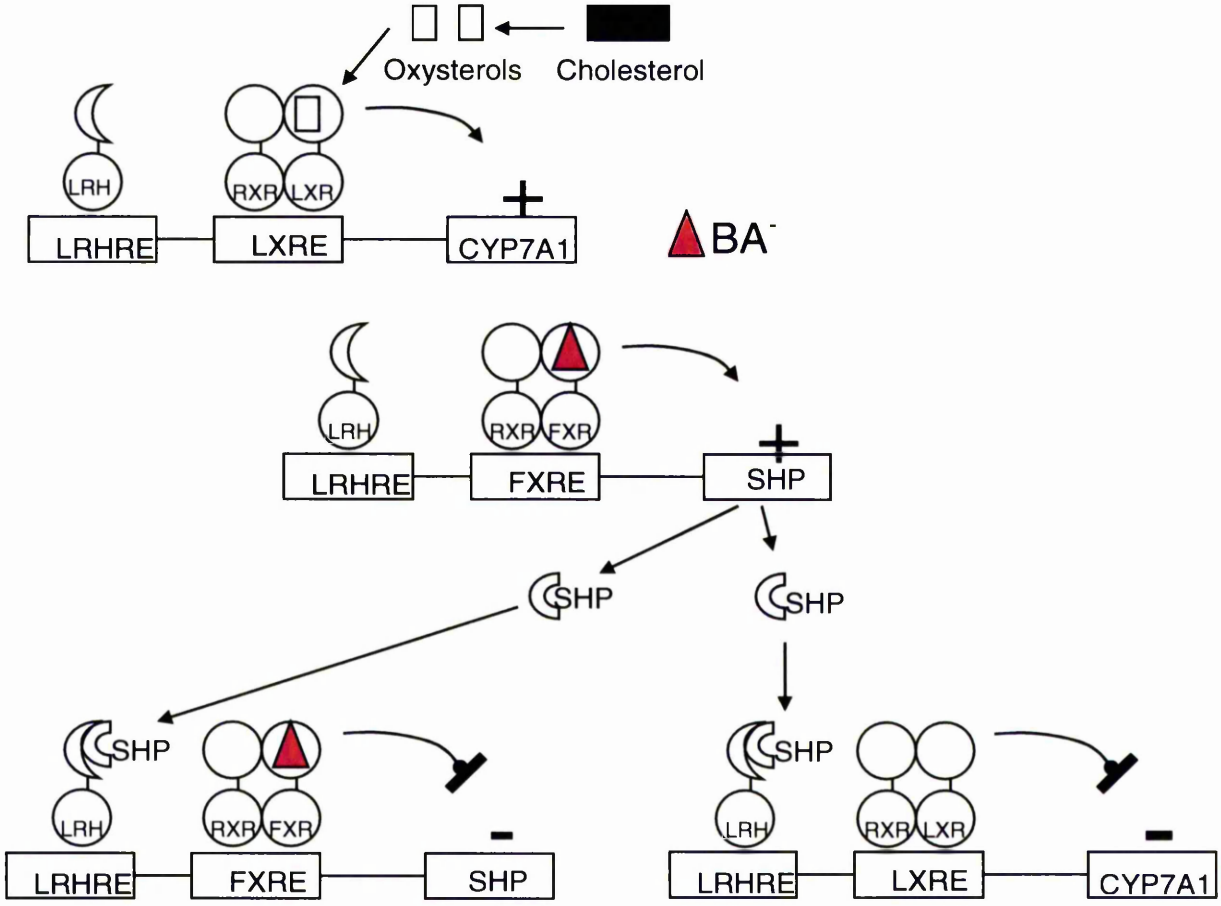


Figure 3. Schematic representation of the interaction between farnesoid X receptor (FXR), liver X receptor (LXR), liver-related hormone 1 (LRH) and short heterodimer protein (SHP) in the control of bile acid synthesis from cholesterol.

specific metabolism of cholesterol in the liver, brain and gonads (Janowsk et al., 1996). LXRs have been shown to be the key sensor of dietary cholesterol that up-regulate CYP7A1 mRNA and subsequently eliminate excess cholesterol via bile acid synthesis and excretion (Peet et al. 1998a). The murine *Cyp7a1* promotor has been shown to contain a potent LXR response element (LXRE) that directly binds RXR:LXR heterodimers and activates transcription in a ligand-dependent manner (Peet et al., 1998b).

In the opposing regulatory pathway, FXR:RXR heterodimers inhibit *CYP7A1* transcription when bound to bile acids (Makishima et al. 1999). However, the *CYP7A1* promotor does not have a FXR binding site. The repression of bile acid synthesis by CYP7A1 is mediated indirectly by bile acid-activated FXR, which is acting via two additional nuclear receptors, LRH-1 (liver-related hormone 1) and SHP (short heterodimer protein). LRH-1 is a monomeric orphan nuclear receptor that acts as a tissue-specific transcription factor that is required for the basal expression of *CYP7A1* and SHP is an atypical nuclear receptor that does not bind DNA but can dimerise with and repress a subset of nuclear receptors.

LRH-1 works as a competence factor for *CYP7A1* promotor ectivity and is required for LXR-mediated upregulation of the gene (Lu et al. 2000). The same group also demonstrated that the SHP promotor contains a FXR:RXR binding site, and that bile acids when bound to FXR activate *SHP* gene transcription (Lu et al. 2000). The increased level of SHP results in complete repression of *CYP7A1*, and subsequent suppression of the *SHP* promotor itself. SHP achieves repression of both promoters through direct interaction with LRH-1 (Lu et al. 2000). A separate study demonstrated that a FXR-specific agonist (GW4064) downregulates *CYP7A1* more strongly than bile acids, and that it strongly induced *SHP* (Goodwin et al. 2000). Thus it is

becoming apparent that at least five different nuclear receptors govern bile acid metabolism (i.e. FXR, RXR, LXR, SHP and LRH-1), and therefore also play an important role in the regulation of cholesterol homeostasis (Figure 3).

Studies in rats have shown that experimentally induced cholestasis, following bile duct ligation, is associated with downregulation of *ntcp* (Gartung et al. 1996) and enhancement of *bsep* (Lee et al. 2000). The downregulation of *ntcp* is the result of *fxr*-mediated enhancement of *shp* expression (Denson et al. 2001). Separate studies have shown that the FXR:RXR heterodimer activates the human BSEP promotor (Ananthanarayanan et al. 2001).

The rate limiting enzyme in the 'alternative' pathway of bile acid synthesis (CYP27) is also regulated by hydrophobic bile acids, but to a lesser extent than CYP7A1 (Twisk et al. 1995).

1.1.5 Mechanisms of cholestasis

Cholestasis results from a generalised impairment in the secretion of bile. Intrahepatic cholestasis refers to all cholestatic disorders that impair bile secretion within the liver. The term 'cholestasis' refers to the morphological appearance of bile plugs within the bile canaliculus of the hepatocyte. Ultrastructural findings usually demonstrate dilation of the canalicular lumen, loss of microvilli, and pericanalicular accumulation of microfilaments and small vesicles.

The causes of human intrahepatic cholestasis are summarised in Table 1. The mechanisms that are of relevance to intrahepatic cholestasis of pregnancy are shown in red font, and will be considered in more detail.

Table 1. Cellular mechanisms of cholestasis.

Mechanism	Animal model	Possible clinical correlates
Genetic disorders • *FIC1*		PFIC1
• *BSEP*		PFIC2
• *MDR3*	*Mdr2 -/- mouse*	PFIC3
Altered plasma membrane lipid composition	Oestrogens	Pregnancy, oral contraceptives
	Chlorpromazine	Phenothiazines
	Monohydroxy BA	Total parenteral nutrition
	Hypothyroidism	Hypothyroidism
↓		
Decreased membrane fluidity		
↓		
Decreased • Na^+/ K^+ ATPase	Endotoxin	Sepsis
	Protoporphyria	Porphyria
	Hypoadrenalism	Adrenal/ pituitary insufficiency
• Na^+/ H^+ exchange		
• Bile acid uptake		
• Bile acid excretion		
Microtubular dysfunction	Colchicine	
↓		
Impaired transcellular transport (vesicles)	Chlorpromazine	Phenothiazines
	Phorbol esters	
Microfilament dysfunction	Phalloidin	N. American Indian cholestasis
	Cytochalasin B	
↓	Chlorpromazine	Phenothiazines
	Norethandrolone	Androgens
Increased biliary permeability (tight junction or canalicular membrane)	Oestrogens	Pregnancy, oral contraceptives
	Monohydroxy BA	Total parenteral nutrition
↑	Bile duct ligation	Biliary obstruction
Biliary obstruction	Bile duct ligation	Diseases of bile ducts
↑		
Precipitation in bile ductules	Monohydroxy BA	Total parenteral nutrition
	Protoporphyria	Porphyria
	Chlorpromazine	Phenothiazines
Portal vasoconstriction	Chlorpromazine	Phenothiazines
	Phorbol esters	Shock, cardiac failure

PFIC=progressive familial intrahepatic cholestasis, BA=bile acids, FIC1=familial intrahepatic cholestasis 1, BSEP= bile salt export pump, MDR3=multidrug resistance 3.

1.1.6 Genetic disorders of cholestasis

Several human autosomal recessive disorders are recognised to cause abnormal biliary bile transport. Mutations have recently been identified in several hepatocyte canalicular transporters and have been shown to cause two forms of hereditary cholestasis, progressive familial intrahepatic cholestasis and benign recurrent intrahepatic cholestasis.

1.1.6.1 Progressive familial intrahepatic cholestasis

Progressive familial intrahepatic cholestasis (PFIC) is characterised by the onset of cholestasis in early childhood which can progress to cirrhosis and liver failure before adulthood (Bull et al. 1997), and can be classified into three subtypes (PFIC1-3). PFIC1 and 2 have low concentrations of biliary bile acids and low to normal gamma-glutamyl transpeptidase (GGT) in the serum. PFIC1 (also called Byler disease) is clinically characterised by features of hepatic failure including jaundice, steatorrhoea and reduced growth, and is caused by mutations in the *FIC1* gene (Bull et al. 1998).

The clinical features of PFIC2 are similar to those of patients with PFIC1, although the appearance of the bile by electron microscopy is different (Bull et al. 1997), and the clinical outcome following orthotopic liver transplantation is better than for some PFIC1 patients (Bull et al. 1997, Strautnieks et al. 1998). PFIC2 is caused by mutations in the *BSEP* gene (Strautnieks et al. 1998). Both nonsense and missense mutations have been reported (Strautnieks et al. 1998).

PFIC3 patients are distinguished by high serum levels of GGT and bile which lacks phospholipid but has a normal biliary bile acid concentration (de Vree et al. 1998), together with distinctive liver histology that shows portal duct inflammation and ductular proliferation (Maggiore et al. 1991). The raised serum GGT is a result of the

detergent effect of the relatively high level of bile acids compared with phospholipid in the bile causing release of GGT from the biliary epithelium.

Several heterozygote mothers of children with PFIC3 have symptoms consistent with OC (de Vree et al. 1998, Jacquemin et al. 1999, Jacquemin et al. 2001). In large consanguineous pedigree with co-existing PFIC3 and OC, three of the six mothers with OC had pregnancies complicated by unexplained intrauterine death (Jacquemin et al. 1999). Four of the six women were investigated and shown to be heterozygotes for the *MDR3* mutation for which the proband was a homozygote (Jacquemin et al. 1999). In a recent study of 31 patients with PFIC3, *MDR3* mutations were found in 17 cases, and three heterozygote mothers were either diagnosed or suspected of having OC (Jacquemin et al. 2001).

1.1.6.2 Benign recurrent intrahepatic cholestasis

Benign recurrent intrahepatic cholestasis (BRIC) is a rare disorder characterised by episodes of cholestasis without permanent liver damage. The following diagnostic criteria have been proposed for BRIC: (1) several episodes of pronounced jaundice with severe pruritus and biochemical evidence of cholestasis; (2) bile plugs on liver biopsy; (3) normal intra- and extrahepatic bile ducts on direct cholangiography; (4) absence of a factor known to produce cholestasis; (5) symptom-free intervals of several months or years (Tygstrup and Jensen 1969). The disease is benign in nature without progression to chronic liver dysfunction (Putterman et al. 1987). Both sporadic and familial cases have been described, and the published pedigrees support autosomal dominant inheritance (de Koning et al. 1995, de Pagter et al. 1976). In one pedigree, a subgroup of female members only had symptoms consistent with

intrahepatic cholestasis of pregnancy, and no other features of BRIC (de Pagter et al. 1976). Mutations in the *FIC1* gene have been reported in BRIC (Bull et al. 1998).

1.1.7 Endocrine causes of cholestasis

Oestrogens are thought to cause cholestasis during pregnancy and after oral contraceptive use in humans. Oestrogens impair bile flow and excretion of bile acids and bilirubin in rats, but do not cause the ultrastructural changes in the canalicular membrane (dilatation and loss of microvilli) associated with most cholestatic agents (Schreiber et al. 1983). Bile salt-independent flow is uniformly reduced by oestrogen administration (Gumucio et al 1971, 1973). The mechanisms by which oestrogens are thought to cause cholestasis include altered plasma membrane composition and associated impairment of plasma membrane ion transport, the metabolism of oestrogens to cholestatic metabolites, and inhibition of hepatocyte bile acid transporters.

The fluidity of rat plasma membranes can be changed by altering the membrane lipid composition (Davis et al. 1978a). Oestrogen treatment results in increased cholesterol uptake by the liver, and resultant increased cholesterol content of liver homogenate and plasma membranes (Simon et al 1980, Davis et al. 1978b). Fluidity of these membranes is reduced as measured by electron spin resonance probes and fluorescence polarization, and it has been proposed that the reduced membrane fluidity is responsible for the decrease in bile flow, sinusoidal membrane Na^+/K^+ ATPase, and the transport maximum for taurocholate (Simon et al 1980). This postulate is based on the demonstration that the nonionic detergent WR-1339 can reverse all these oestrogen effects, without having a significant effect in control rats (Simon et al 1980).

Oestrogens also inhibit the sinusoidal and canalicular bile acid transporters. While the reduction of basal bile flow induced by oestrogen has a half time ($t_{1/2}$) of 12.5 hours, initial taurocholate uptake was not significantly reduced until 3 days to 59% of control, and 5 and 7 days to 13% and 10% of control, respectively ($t_{1/2}$ 4.3 days) (Simon et al. 1996). These physiological changes were correlated with measurement of protein mass and steady-state mRNA for rat Na^+/K^+ ATPase, Ntcp, Oatp and membrane lipid fluidity (Simon et al. 1996). Neither Na^+/K^+ ATPase α-subunit, nor β-subunit mass was altered by ethinylestradiol administration. In contrast, the protein content of Ntcp was reduced to 21% of control at 5 days. Oatp protein mass was similarly reduced to 21% of control at 5 days. Ethinyl estradiol also reduced the mRNA levels of Ntcp and Oatp to 50% and 15% of control levels at 5 days, respectively (Simon et al. 1996). These results are consistent with a primary effect of oestrogens on both membrane fluidity and on sinusiodal bile acid transporters contributing to the aetiology of cholestasis.

Chronic ethinyl estradiol administration has also been shown to decrease ATP-dependent bile acid and multiorganic anion transporters in bile canalicular vesicles (Bossard et al. 1993). Murine *mBsep* expression is decreased by 95% following administration of ethinylestradiol (Green et al. 2000). The expression of *mNtcp* was also diminished, but to a lesser degree (Green et al. 2000). Studies of vesicles isolated from Bsep-, Mrp2- and Bsep/Mrp2-expressing Sf9 cells have shown that the oestrogen metabolite estradiol-17-beta-glucuronide inhibits ATP-dependent taurocholate transport only in vesicles that coexpress rat Bsep and Mrp2, indicating that it can only inhibit Bsep after its secretion into bile canaliculi by Mrp2 (Steiger et al. 2000).

The effect of oestrogens on the expression and function of *MDR3* and *FIC1* have not been investigated to date. However, oestrogens and progestogens can inhibit murine mdr1-catalysed drug efflux and interact with P-gp (Metherall et al. 1996). As the MDR3 protein shares 77% identity with P-gp1, it is possible that a similar effect would be seen if these experiments were repeated using mdr2 or the MDR3 protein.

It has been demonstrated that certain classes of oestrogen metabolites have a more profound effect on cholestasis. The intravenous administration of the D-ring glucuronide conjugates (i.e. the 16- and 17- glucuronides) of estradiol, estriol and ethinylestradiol to rats causes an immediate, profound, dose-dependent and reversible cholestasis (Vore et al. 1985, Meyers et al. 1981, Vore et al. 1983). In contrast, under identical experimental conditions, the A-ring glucuronides cause choleresis (Meyers et al. 1981). Hepatic biotransformation of estriol, the major oestrogen in pregnancy, includes sulfation and glucuronidation, and helps to reduce the cholestatic effect of oestrogens. However, the oestrogen conjugate that increases most in pregnancy is estriol-16α-D-glucuronide, one of the D-ring conjugates that has been shown to be cholestatic in rats.

1.2 Pathogenesis of obstetric cholestasis

Obstetric cholestasis (also called intrahepatic cholestasis of pregnancy) is the commonest disorder of liver function unique to pregnancy and can have severe consequences for the mother and fetus. It is associated with maternal morbidity and with fetal morbidity and mortality.

1.2.1 Epidemiology

The prevalence of OC varies in different populations, with reported rates varying from 0.2% in France (Perreau et al. 1961), 1% in Poland (Wojcicka-Jagodzinska et al.

1989) to 0.5-2% in Finland (Svanborg et al. 1959, Berg et al. 1986, Heinonen et al. 1999), and 12% in Chile (Reyes et al. 1978). It affects 0.6% of pregnancies in UK white Caucasians (Abedin et al. 1999) and double this proportion of Indian and Pakistani Asians (Abedin et al. 1999, Kenyon et al. 2002). Epidemiological studies in Latin America have indicated that descendants of the Araucanian Indians in Chile and the Quechua Indians in Bolivia have a higher prevalence of OC than individuals of more European/ mixed ancestry (Reyes et al. 1978, 1979). In individuals of overt Araucanean descent, the prevalence of OC has been quoted as high as 27.6% (Reyes et al. 1978). The Araucanean Indians were the predominant racial group occupying central Chile before the arrival of Spanish colonisers. The relatively low prevalence of obstetric cholestasis in Spain, and in Latin American countries with a non-Araucanean native population suggests that there may be a genetic predisposition to the condition in the Araucaneans.

There is a seasonal variation in the prevalence of OC, with a greater number of cases reported in the winter months (Berg et al. 1986, Reyes 1997). In addition, there has been a reduction in the number of reported cases in Chile and in Scandinavia in recent years (Reyes 1997). The reasons for these epidemiological fluctuations are not clear, but they indicate that environmental factors must play a role in the aetiology of the condition. One Finnish study has demonstrated that OC occurs more commonly in women of relatively advanced age (>35 years) (Heinonen et al. 1999). In addition, the condition is commoner in multiple pregnancy, and this is thought to be due to the higher levels of oestrogen in such pregnancies (Gonzalez et al. 1989).

1.2.2 Maternal clinical features

The classical maternal clinical feature of OC is generalised pruritus which commonly develops in the third trimester, becoming more severe with advancing gestation, and which disappears shortly after delivery in the majority of cases (Berg 1986). It is usually most marked on the trunk, soles and palms and is not associated with any skin rash apart from dermatitis artefacta secondary to scratching. The severity of the pruritus is often sufficient to prevent women from sleeping. Eighty percent will develop pruritus after 30 weeks gestation (Reyes 1992), but symptoms can occur as early as the sixth week of pregnancy (Berg 1986). Rarer symptoms include anorexia, malaise, abdominal discomfort, pale stools and dark urine (Williamson and Nelson-Piercy 1998). If jaundice does develop, it tends to follow the pruritus by two to four weeks and to plateau relatively quickly (Lunzer 1989). An increased risk of post-partum haemorrhage (PPH) has been reported (Reid et al. 1976). This may be a consequence of malabsorption with steatorrhoea (Reyes et al. 1987), and resultant vitamin K deficiency (Reid et al. 1976).

1.2.3 Fetal clinical features

While OC is associated with maternal morbidity, it causes perinatal morbidity and mortality. It causes fetal distress (defined as either meconium stained amniotic fluid or fetal heart rate abnormalities), spontaneous premature delivery and unexplained third trimester intrauterine death (Reid et al 1976, Reyes et al 1982, Fisk 1988a, Rioseco 1994). The major studies of the prevalence of fetal complications in OC are summarised in Table 2. The perinatal mortality rate has reduced from 10-15% in older studies (Reid et al 1976, Reyes et al 1982), to $\leq$ 3.5% in more recent series in which most women were delivered before 38 weeks gestation (Reyes et al 1982, Fisk 1988a,

Rioseco 1994). Davies et al. (1995) reported an uncontrolled series of 13 OC pregnancies that had been managed expectantly in the UK with resultant perinatal morbidity or mortality complicating 11 cases. This included 8 intrauterine deaths, two premature deliveries with fetal distress (one died in the perinatal period), and one emergency caesarean section for fetal distress. All but one of the pregnancies complicated by intrauterine death were delivered at ≥ 37 weeks gestation (Davies et al. 1995).

Table 2. Summary of the major studies of fetal outcome in OC.

Date of study	No. of cases	PMR or IUD*(%)	Meconium staining (%)	Pre-term labour (%)	Delivery <37-38/40	Reference
1964-69	87	9*	-	54	No	Reyes 1982
1965-79	56	11	27	36	No	Reid et al. 1976
post-1969	91	3*	-	-	Yes	Reyes 1982
1988	83	4	45	44	Yes	Fisk et al. 1988a
1994	320	2	25	12	Yes	Rioseco et al. 1994
1990-96	91	0	15	14.3	Yes	Heinonen et al. 1999

PMR = perinatal mortality rate as a percentage, IUD = intrauterine death rate as a percentage.

1.2.4 Biochemical features

Abnormal liver function tests (LFTs) are necessary to make the diagnosis of OC. The serum total bile acid concentration is increased, and this is largely due to primary bile acids (Sjovall et al. 1966, Laatikainen et al. 1977, Bacq et al, 1995).

In normal pregnancy serum bile acid levels do not change significantly during the course of the pregnancy (Sjovall 1966; Heikkinen et al. 1981) although one study has reported reduced levels in the first trimester (Laatikainen and Hesso 1975a). Several studies have demonstrated a slight rise in serum levels of specific bile acid levels in the third trimester (Sjovall 1966; Heikkinen et al. 1981; Lunzer et al. 1986), but the overall bile acid levels did not differ significantly from the normal range. Cholic acid is the primary bile acid in pregnant women, while chenodeoxycholic acid and deoxycholic acid predominate in non-pregnant women.

In patients with OC, cholic acid is the most sensitive bile acid for early diagnosis and follow-up, and can rise as much as 100-fold (Laatikainen et al. 1977). It is the first bile acid to rise and this has been shown to precede the onset of symptoms in up to 50% of cases (Laatikainen et al. 1977, Heikkinen et al. 1981). Chenodeoxycholic acid also increases, but this is less marked than the rise in cholic acid, and only occurs in approximately 74% of patients (Laatikainen et al. 1977).

A study of 13 patients that compared measurement of serum bile acid using either high-performance liquid chromatography or an enzymic procedure that measured total bile acid revealed that there was no difference in the total bile acid level measured by either procedure (Bacq et al. 1995). Therefore, although cholic and chenodeoxycholic acid are the main serum bile acids to rise in OC, many clinical laboratories use the simpler total bile acid assay, and this is equally valid for diagnostic purposes.

The liver transaminases are also usually moderately raised (commonly two to three fold), although more marked elevation has been reported (Laatikainen et al. 1977, Misra et al. 1980). Both the alanine (ALT) and aspartate (AST) transaminase may be raised, and they are thought to be released as a result of hepatocyte damage. Some studies have shown an increase that only occurs after a rise in primary bile acids and

this may reflect the severity of the disease with advancing hepatocyte damage (Heikkenen 1983, Shaw et al. 1982). ALT appears to rise first and is thought to be a more sensitive test (Laatikainen et al. 1977, Heikkinen 1983, Fisk et al. 1988b). When measuring the liver transaminases, it is important to use the reference ranges for pregnancy (Girling et al. 1997), i.e. the upper end of the normal range should be reduced by about 20% compared to the quoted normal range for a laboratory. Normal ranges for LFTs in pregnancy are summarised in Table 3.

Table 3. Normal ranges for liver function tests in pregnancy.
(from Girling et al. 1997, Fagan 1995)

Liver enzyme	Non-pregnant	1st trimester	2nd trimester	3rd trimester
Aspartate transaminase (u/L)	7-40	10-28	11-29	11-30
Alanine transaminase (u/L)	0-40	6-32	6-32	6-32
Bilirubin (μmol/L)	0-17	4-16	3-13	3-14
Gamma glutamyl transpeptidase (u/L)	11-50	5-37	5-43	3-41
Alkaline Phosphatase (u/L)	30-130	32-100	43-135	133-418

Bilirubin has been shown to correlate with raised primary bile acids (Heikkinen 1983), but has no value in the early diagnosis or follow-up of patients with OC as it is

usually normal (Lunzer et al. 1986). GGT is raised in 20-30% of cases (Milkiewicz et al. 2001).

In one study of 21 women with cholestasis of pregnancy, serum lipids (cholesterol, phospholipids, triglycerides and very low density lipoproteins) were increased, and high density lipoproteins decreased, when compared with pregnant controls (Johnson et al. 1975). One other study has found raised cholesterol levels in a cohort of pregnant women with raised glycocholic acid levels, compared to pregnant women with a normal glycocholic acid (Lunzer et al. 1986).

1.2.5 Management of obstetric cholestasis

OC is a diagnosis of exclusion and other causes of cholestasis, such as gallstones or extrahepatic obstruction, should be excluded by ultrasound examination. Investigations should also be performed to exclude infectious and autoimmune hepatitis. One large Italian study reported an increased prevalence of OC in women who are hepatitis C positive (Locatelli et al. 1999).

The management of OC aims to avoid an adverse fetal outcome and improve the maternal morbidity. The mother should be counselled with regard to the fetal risks and the need for close surveillance. LFTs, including prothrombin time, should be regularly checked, and fetal well being monitored at frequent intervals. There is accumulating evidence that the perinatal mortality is considerably reduced by induction of labour before 38 weeks gestation (Reyes et al 1982, Fisk 1988a, Rioseco 1994). There are currently no established forms of fetal surveillance that allow prediction of the at-risk fetus. Therefore, although women with OC are often seen several times per week for fetal assessment by CTG, they are usually told that this

will give reassurance that there is no current problem, but cannot exclude subsequent fetal complications.

Drug treatment

A variety of drug therapies have been used for the maternal pruritus. The two most commonly used drugs, ursodeoxycholic acid (UDCA) and dexamethasone, have been reported to improve both symptoms and LFTs. Other treatments that have been reported to be beneficial in some studies are S- adenosylmethionine, cholestyramine and guar gum. Vitamin K should be given to reduce the risk of postpartum haemorrhage.

Ursodeoxycholic acid (UDCA) is a hydrophilic bile acid which acts by altering the bile acid pool, and reducing the proportion of hydrophobic, and therefore hepatotoxic, bile acids. There have been several reports on the use of UDCA in OC in which patients are given up to 2000 mg/day for periods of seven days to ten weeks, mainly during the third trimester. When the results of nine studies with a total of 85 affected women who have been treated with UDCA are combined, 74 (87%) showed clinical or biochemical improvement, or both (Berkane et al. 2000; Brites et al. 1998a; Davies et al. 1995; Floreani et al. 1996; Mazzella et al. 1991, 2001; Nicastri et al. 1998; Palma et al. 1992, 1997). All babies born to mothers given UDCA were delivered safely and no problems attributable to treatment were reported. UDCA treatment has been shown to improve the serum bile acid levels measured in cord blood and amniotic fluid at the time of delivery (Mazzella et al. 2001). In addition, there is very little accumulation of UDCA in the amniotic fluid or in cord blood (Mazzella et al. 2001). These findings are encouraging as several authors have postulated that raised maternal and fetal bile acids are the cause of the adverse fetal consequences of OC (Bacq et al. 1995; Heikkinen et al. 1980, Laatikainen et al. 1975c, 1977). One study

has demonstrated that while bile acid levels are raised in the colostrum of women whose pregnancies were complicated by OC compared with controls, these are markedly reduced by UDCA treatment (Brites et al. 1998b).

Dexamethasone can also be an effective therapy. A Finnish study of ten women at between 28 to 37 weeks gestation reported reduced pruritus and lowered bile acids and transaminases following treatment with dexamethasone 12 mg per day for seven days, and gradually reduced over three days (Hirvioja et al, 1992). The serum oestriol and oestradiol levels in the patients reduced significantly after one day of treatment, and the authors postulated that this is the mechanism of action of dexamethasone in treating OC. Symptoms of cholestasis did not recur after cessation of therapy, even in one patient who delivered more than two months after treatment with dexamethasone. The results of this study are encouraging, but this is the only series of cases treated with dexamethasone in the literature to date. There has been one case report of worsening of maternal pruritus and alanine aminotransferase (ALT) following dexamethasone treatment (Kretowicz et al. 1994), but it is difficult to draw conclusions from a single case report.

S-adenosylmethionine (SAMe) reverses oestrogen-induced cholestasis in rats. It has been reported to improve symptoms and LFTs in women with OC in some studies (Nicastri et al. 1998; Frezza et al. 1990) and not in others (Ribalta et al. 1991; Floreani et al. 1996). In one Italian study thirty women were randomised to receive either 800 mg SAMe daily or placebo until delivery for a mean period of 18 days. Treated women had significantly lower levels of serum bile acids, bilirubin and aminotransferases compared with pre-treatment levels and with the placebo group (Frezza et al. 1990). In another study 32 women with OC were subdivided into four groups treated with either UDCA, SAMe, both drugs or placebo for 20 days.

Improvements in pruritus and serum bile acids were seen in all groups, but the serum glutamic pyruvic transaminase (SGPT) did not improve in the placebo group (Nicastri et al. 1998). The combination of UDCA and SAMe caused a greater reduction in pruritus, but not in serum bile acids or SGPT (Nicastri et al. 1998). A Chilean double-blind study in which 18 patients with OC were ramdomised to receive either 900 mg SAMe daily or placebo did not demonstrate any difference in pruritus, nor in the levels of serum bile salts, ALT, total bilirubin or alkaline phosphatase between the groups (Ribalta et al. 1991). An Italian study that compared the efficacy of SAMe and UDCA in the treatment of OC failed to show any significant changes in pruritus, serum bile acids, aspartate aminotransferase (AST), ALT, or alkaline phosphatase tests following SAMe treatment, while UDCA resulted in a significant lowering of serum bile acids and improvement of pruritus (Floreani et al. 1996).

Cholestyramine is an anion exchange resin that can bind bile acids in the intestine, thereby reducing their enterohepatic recirculation, and increasing their faecal elimination. It also binds cholesterol and is used as a cholesterol-lowering therapy. There have been two studies of the use of cholestyramine in OC; in one five of seven women that were treated with 18 g daily had improved pruritus (Heikkinen et al. 1982). However, the only biochemical change of note was a reduction in the serum levels of one bile acid, chenodeoxycholic acid (CDCA) in four patients, but not in the levels of cholic acid (CA), the main bile acid that is raised in OC (Heikkinen et al. 1982). There was no reduction in the serum transaminases, alkaline phosphatase nor gamma-glutamyl transpeptidase, and there was an increase in conjugated bilirubin (Heikkinen et al. 1982). Another study in which ten women were treated with 8-12 g daily reported a reduction in both CA and CDCA in five patients, but there was no response in those women who had higher serum bile acid levels when treatment was

commenced (Laatikainen et al. 1978). It is important to use cholestyramine treatment with caution as it may bind vitamin K, and therefore reduce its absorption. This can result in exacerbation of the maternal vitamin K deficiency that can occur in OC, and may increase the risk of postpartum haemorrhage. In addition, there has been a case report in which fetal intracranial haemorrhage complicated cholestyramine treatment in OC (Sadler et al. 1995). Cholestyramine therapy should not be given at the same time as UDCA treatment as it will prevent absorption of the UDCA.

Guar gum is a gel-forming dietary fibre that binds bile acids in the intestinal lumen and enhances their elimination. There have been two Finnish double-blind placebo controlled studies in which up to 15 g guar gum per day was used to treat women with OC (Gylling et al. 1998; Riikonen et al. 2000). Both studies demonstrated an improvement in pruritus and the serum bile acids did not rise following treatment. However, in both studies the serum total bile acid level did not fall, and remained at approximately 20 μmol/l following treatment (Gylling et al. 1998; Riikonen et al. 2000).

Vitamin K is essential for normal coagulation. The active vitamin K dependent clotting factors (II, VII, IX and X) are formed from precursors in the liver, and patients with liver disease are at risk of haemorrhage due to deficiency of these clotting factors. It is thought that the increased prevalence of post-partum haemorrhage in OC is caused by Vitamin K deficiency that develops as a result of impaired intestinal absorption secondary to steatorrhoea, but hepatic impairment may also contribute in some cases. There has been one report of a reduction in the prothrombin level to below 5% in a woman with OC (Herre et al. 1976), and the case report of fetal intracranial haemorrhage complicating cholestyramine treatment was

thought to be due to vitamin K deficiency (Sadler et al. 1995). Therefore vitamin K treatment is advised from approximately 32 weeks gestation in OC.

Postpartum course

Biochemical and clinical features of OC resolve following delivery, but recurrence commonly occurs in subsequent pregnancies. Some women may report pruritus associated with the use of oestrogen-containing oral contraceptives and these should either be avoided, or if taken the LFTs should be checked on a regular basis. In addition, gallstones are commoner in women with OC and their relatives.

1.2.6 The pathophysiology of obstetric cholestasis

The condition has a complex aetiology with hormonal, genetic and environmental factors playing a role. The hormonal factors that contribute to the pathogenesis of OC include raised serum oestrogen and progestogen levels. It has also been demonstrated that the administration of oestrogens to women with a previous history of OC causes symptoms and biochemical changes of OC (Kreek et al. 1967; Holzbach et al. 1983). In addition, a subgroup of women with pregnancies complicated by OC have been reported to develop symptoms after taking the combined oral contraceptive pill and in the second half of the menstrual cycle when not pregnant (Larsson-Cohn et al. 1965; de Pagter et al. 1976; Lunzer 1989). The cholestatic effect of oestrogens in *in-vitro* and animal models is discussed in section 1.1.6. Progestogens may also play a role as 34 (68%) of 50 women in a French prospective series of OC cases had been treated with oral micronized natural progesterone for risk of premature delivery (Bacq et al. 1997).

A genetic component to the aetiology of OC is supported by epidemiological studies, which suggest a founder mutation in certain populations, e.g. the Chilean Araucanean

Indian population (Reyes et al. 1978). There are also a small number of reported pedigrees that show autosomal dominant sex-limited inheritance (Holzbach et al. 1983, Reyes 1976, Hirvioja et al. 1993). We conducted a preliminary study of the genetic epidemiology of OC in 81 UK cases from Queen Charlotte's Hospital and the OC Patient Organisation prior to the commencement of the larger study reported later in this thesis. We demonstrated that 33% have a positive family history and a pedigree that demonstrates sex-limited dominant inheritance (Williamson et al. 1998). In the other pedigrees the only affected woman is the index case. This could be because the other women in the pedigree who potentially have the OC phenotype are non-parous, or the condition has been inherited from male relatives. In this study, the relative risk for parous siblings (λ_S) was 24 and for parous daughters (λ_O) was 30, assuming a population prevalence of 0.5% of pregnancies (Williamson et al. 1998).

The only gene that has been shown to be mutated in OC to date is *MDR3*, and this is almost entirely in a rare subgroup of women identified as having a relative with PFIC3 (see section 1.1.5.1). In one pedigree in which a large consanguineous family had co-existing PFIC3 and OC, three of the six heterozygote female relatives had pregnancies complicated by unexplained intrauterine death (Jacquemin 1999). There has also been a recent report of two additional *MDR3* mutations in three cases with coexistant OC and a peculiar form of cholelithiasis (i.e. at least one episode of biliary cholic, pancreatitis or cholangitis, chronic cholestasis, recurrence of symptoms following cholecystectomy and prevention of recurrence by UDCA therapy) (Rosmorduc et al. 2001).

The mothers of children with PFIC1 and 2 have not been reported to have symptoms of cholestasis in pregnancy. However, in one large, extended BRIC pedigree, the only complaint of eight female relatives was OC (de Pagter et al. 1976).

Other than these studies of the heterozygote mothers and relatives of two children with PFIC3, no other candidate genes for OC had been investigated prior to the commencement of this project, although one study found no common HLA haplotypes in five female relatives (Hirvioja et al. 1993).

Environmental factors that may play a role in the aetiology of OC include selenium and erucic acid. Plasma selenium levels decrease with advancing gestation, but are maintained at normal levels if dietary intake is normal (Butler et al. 1982). Selenium levels were significantly reduced in pregnant women with OC compared with controls in a Finnish study (Kauppila et al. 1987), and at approximately the same time a lower dietary intake of selenium was also demonstrated in Chile. A more recent Chilean study demonstrated that plasma selenium levels are lower in normal pregnant women near term, and that OC patients had significantly lower plasma selenium levels than normal pregnancies (Reyes et al. 2000). This study also demonstrated that in normal pregnancies, plasma selenium levels are higher in the summer. In the Finnish study, the activity of the selenoenzyme glutathione peroxidase had a significant positive correlation with selenium concentration, and it was also significantly lower in women with the disease (Kauppila et al. 1987). Oestrogens can cause oxidative damage but in normal pregnancy antioxidants prevent this. Glutathione peroxidase is an antioxidant that reduces the concentration of antioxidants, e.g. steroid hydroperoxides and other peroxides (Ursini et al. 1985). Thus it is possible that selenium deficiency in women with OC may contribute to oestrogen-induced oxidant damage to hepatocytes. The more recent Chilean study demonstrated that plasma selenium levels have increased in non-pregnant individuals since the 1980s, and the authors suggested that this may partly explain the reduction in prevalence of OC in Chile since this time (Reyes et al. 2000).

It has also been reported that changes in the availability of dietary rapeseed oil, which has a high content of erucic acid, mirrored reductions in the prevalence of OC in Chile and Sweden from the 1960s (Reyes et al. 1995). It was therefore hypothesised that dietary erucic acid was involved in the pathogenesis of OC. A study in which rats and hamsters were fed diets containing a high proportion of erucic acid did not demonstrate an increased incidence of cholestasis either during pregnancy or when not pregnant (Reyes et al. 1995). However, it is still possible that dietary erucic acid plays a role in the aetiology of the condition in genetically predisposed individuals.

1.2.7 The aetiology of intrauterine death and fetal distress in obstetric cholestasis

The pathophysiological mechanisms that cause intrauterine death in OC are currently not understood. The majority of stillborn infants are of appropriate weight with no evidence of utero-placental insufficiency (Fisk et al. 1988a, Davies et al. 1995). Thus, the evidence to date suggests that the intrauterine death is a sudden event. Two studies have demonstrated an abnormal fetal heart rate ($\leq$ 100 or $\geq$ 180 beats/ minute) in the children of women with OC (Laatikainen et al. 1984, Laatikainen 1975c). A severe fetal bradycardia (<100 beats/ minute) was noted in 16% of neonates in another study (Reid et al. 1976).

Two studies have reported an association between raised maternal bile acid levels and fetal distress (Laatikainen et al. 1977, Laatikainen et al. 1984), and several authors have postulated that raised bile acids are the cause of the adverse fetal consequences of OC (Laatikainen 1977, Bacq et al. 1995, Heikkinen et al. 1980, Laatikainen 1975c), although there has been no proven mechanism to date. Two studies have demonstrated increased bile acid levels in the fetus, which were uniformly lower than the maternal levels, suggesting that maternal bile acids cross the placenta and enter the fetal

circulation and are then excreted from the fetus to the mother via the placenta in OC (Laatikainen 1975c, Shaw et al. 1982). The fetal bile acid levels were raised and similar to maternal levels in 50% of these OC pregnancies (Laatikainen 1975c, Shaw et al. 1982), suggesting that abnormal placental bile acid transport may predispose these fetuses to raised serum bile acids, and may result in associated fetal distress and IUD. These results are summarised in Figure 4.

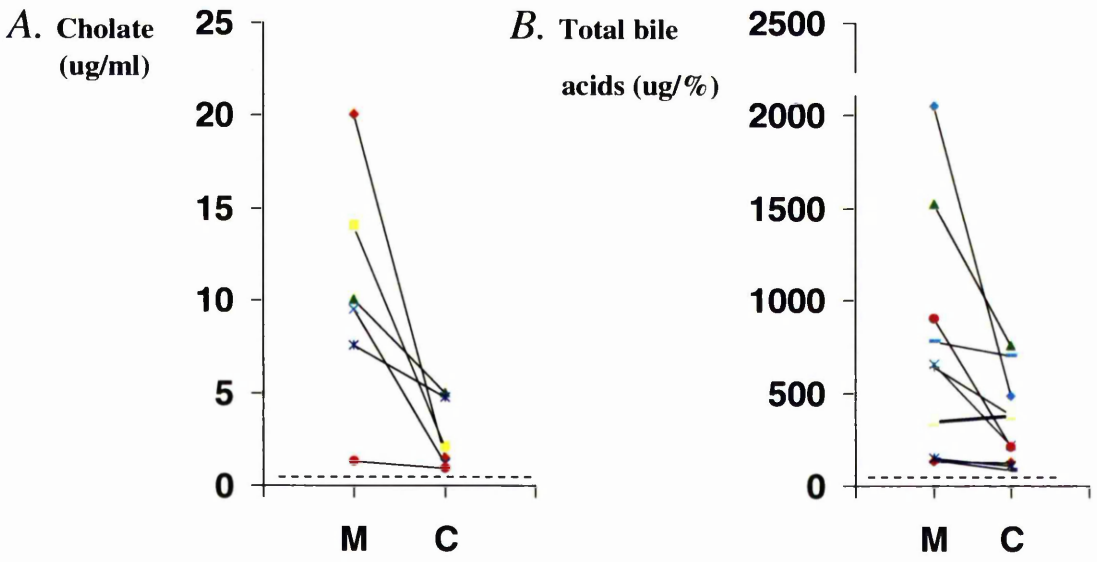


Figure 4. Comparison of serum cholate (ug/ml) and total bile acid (ug/%) levels from maternal and fetal cord blood specimens taken at term (A. from Laatikainen 1975c, and B from Shaw et al. 1982). Normal range is represented by the dashed line.

Histological examination of placentas from OC pregnancies revealed non-specific changes, including reduced syncitial sprout formation, maturation defects, villous oedema and trophoblastic swelling (Laatikainen et al. 1975b, Costalo 1975, Costoya 1980). Measurement of intervillous blood flow has demonstrated both no change and a reduction in flow in two different studies (Rauramo et al. 1988, Kaar et al. 1980).

One study demonstrated a dose-dependent vasoconstriction of the placental chorionic veins following administration of bile acids (Sepulveda 1991).

The cause of the increased incidence of spontaneous pre-term labour is not fully understood. Cholic acid has been shown to cause a dose-related increase in myometrial contractility in rat myometrium (Campos et al. 1988), and it increases the incidence of pre-term labour when given as an infusion to sheep (Campos et al. 1986). The reason for the increased frequency of pre-term labour is not clear, but it has been postulated that it may be a consequence of bile acid-induced release of prostaglandins, which in turn may initiate labour (Campos et al. 1986). There is also some evidence that in OC the myometrium is more responsive to oxytocin (Israel et al. 1986).

In the study in which bile acids were given as an infusion to sheep, all the bile acid-treated fetuses developed meconium stained amniotic fluid by the third day of infusion (Campos et al. 1986). This may be because the bile acid infusion caused fetal distress and subsequent passage of meconium into amniotic fluid. Alternatively, it may be due to bile acid-induced increases in bowel motility, as bile acids are implicated in diarrhoea in humans following extensive ileal resections (Hofmann 1967), and *in vitro* studies have demonstrated that deoxycholic acid activates luminal potassium and choloride secretion in rabbit distal colon (Mauricio et al. 2000).

1.3 Hypotheses

The hypotheses that are tested in this thesis are as follows:

1. Obstetric cholestasis has serious fetal complications, including spontaneous prematurity and intrauterine death in UK white Caucasians (Chapter 2).
2. The aetiology of obstetric cholestasis has a genetic component in the UK (Chapter 2).
3. Mutations in the *MDR3* gene cause obstetric cholestasis in a subgroup of women with raised GGT (Chapter 3).
4. The primary bile acid taurocholate will alter the rate, rhythm and calcium dynamics in an *in vitro* model of the neonatal heart at term (Chapter 4).
5. The therapeutic agents ursodeoxycholic acid and dexamethasone can reverse the taurocholate-induced abnormalities in cardiomyocytes (Chapter 4).

2. CLINICAL SURVEY OF 352 AFFECTED OBSTETRIC CHOLESTASIS PREGNANCIES IN 227 WOMEN

2.1 Background and aims

There have been no large studies of the clinical features of complicated OC pregnancies (e.g. by IUD or spontaneous prematurity). It is therefore difficult to know at what stage such pregnancies should be delivered, or the extent to which fetal surveillance should be performed. Also there have been no studies of these complications in UK Caucasians. The aims of the studies reported in this chapter were firstly to establish the clinical features of OC pregnancies in UK white Caucasians and to investigate in more detail a series of cases complicated by IUD and prematurity.

There have also been no studies of the inheritance of OC in UK Caucasians. We therefore also aimed to establish details of the family histories of affected women, and the prevalence of the condition in parous relatives.

2.2 Subjects and methods

2.2.1 Recruitment of cases

Women with OC were identified retrospectively and prospectively by the UK Obstetric Cholestasis Patient Organisation (OCPO) and were asked whether they would be interested in participating in a study of the genetic aetiology of OC. Initially, index cases were mailed a questionnaire to ascertain details of their symptoms, their pedigree and whether they were willing to participate. The questionnaire is shown in the Appendix. When the questionnaires had been completed and returned, they were reviewed to confirm that a definite diagnosis of OC had been made. Women met the diagnostic criteria for OC if they had pruritus and biochemical confirmation of the diagnosis by raised serum bile acids and/ or raised serum transaminases in at least one

pregnancy. As hospitals have different normal ranges for liver transaminase levels, the upper end of the normal range in pregnancy was assumed to be 80% of the level quoted outside pregnancy for each case, consistent with published studies (Girling et al. 1997), and any values above this were considered to be abnormal. For some women who fulfilled the diagnostic criteria, previous pregnancies had been complicated by pruritus, but biochemical confirmation of OC had not been obtained. These prior pregnancies were included as OC pregnancies providing the diagnosis had been confirmed in the subsequent pregnancy. Further details were obtained by telephoning the women to obtain information about their symptoms. Women were asked at what gestational week the different features of the condition occurred. In most cases this was given as total weeks. If a woman also quoted the number of days, e.g. 35 weeks and 4 days gestation, the figure was rounded down to the number of weeks. Women were also asked whether they had experienced pruritus with exogenous oestrogens and whether they had cyclical pruritus or a history of gallstones.

As almost all of the women who had contacted the OCPO were white Caucasians, it was decided to limit the study to this ethnic group. The clinical information and family history of each index case was entered on a phenotype data sheet (see Appendix) and stored in a Microsoft Excel® spreadsheet.

Caucasian control women with normal pregnancies were identified daily on the postnatal wards at Queen Charlotte's and Chelsea Hospital, London, between 8th March and 11th May, 2001, and the aim was to recruit all eligible women that delivered over this time period. Women with a diagnosis of obstetric cholestasis, or with documented abnormal liver function tests were excluded. The aim was to recruit all women that delivered over this time period. All women willing to participate in

the study were recruited and they were given the option of either completing the questionnaire with the interviewer, or in their own time. Clinical information was obtained from the controls in the same way as for the patients except that the information was obtained in person rather than by telephone. Local research ethics committee approval was granted for this study.

2.2.2 Data analysis

The incidence of pre-term labour and IUD were determined for OC cases and controls. Information was collected on the gestation at onset of pruritus. The gestation at delivery was recorded for all pregnancies that progressed past 24 weeks gestation. A delivery was classified as premature if it occurred before 37 weeks (i.e. less than or equal to 36 weeks and 6 days gestation). This group was subdivided into spontaneous and iatrogenic premature deliveries. Iatrogenic premature deliveries included those that were induced and those in which there was an elective caesarean section.

The gestational week at delivery and incidence of premature labour (spontaneous and iatrogenic) was compared in OC pregnancies and controls. The gestational week at which the IUDs occurred was recorded. The gender of the fetus in each IUD and premature delivery was also noted and the features of these pregnancies were compared in males and females. It was established whether a diagnosis of OC was made and what treatment was given in affected pregnancies. Data about pregnancies complicated by fetal distress defined as meconium stained amniotic fluid or cardiotocograph abnormalities were not obtained as accurate information could not be obtained without access to the hospital notes of all cases. However, information was

collected on whether the infant was admitted to the neonatal intensive care unit (NICU) as an indicator of the condition of the child at birth.

The number of twin pregnancies and the gender of the fetuses were recorded. In addition, the prevalence of cyclical and oral contraceptive pill-induced pruritus was compared in both women with OC and controls. The frequency of clinically apparent gallstones was also determined.

2.2.3 Assessment of the Inheritance pattern of OC

In order to assess the pattern of inheritance of OC, the following variables were analysed from the data obtained.

(i) The number of index cases with affected relatives.

(ii) The total number of parous female siblings and the proportion of these with OC, (i.e. the prevalence of OC in siblings).

(iii) The number of index cases with informative pedigrees and the pattern of inheritance in the pedigrees.

The number of first-degree relatives with a history of gallstones was also recorded.

A medical student assisted with collection of clinical data about cases and controls, and added a large proportion of the information to the excel datasheet, but Dr Williamson designed the study and supervised the data analysis.

2.2.4 Statistical analysis

Data were expressed as median (interquartile range). The Mann-Whitney U test was used to identify differences in the values between two groups whereas the Kruskal-Wallis H test was used for more than two groups. The Chi-square test 2 x 2 was used

to compare proportions and replaced by Fischer's exact test when there were small sample sizes. The odds ratios were calculated using the Gart's method for a 95% confidence interval (95% CI). Statistical analysis of the data was performed using the software SPPS for Windows, release 10.0, SPSS Inc, Chicago, Ill. Advice and assistance with statistical analysis were given by Dr Dimitri Goulis.

2.3 Results

2.3.1 Subject details

Details of the 227 women in the OC group and the 234 women in the control group are given in Figure 5.

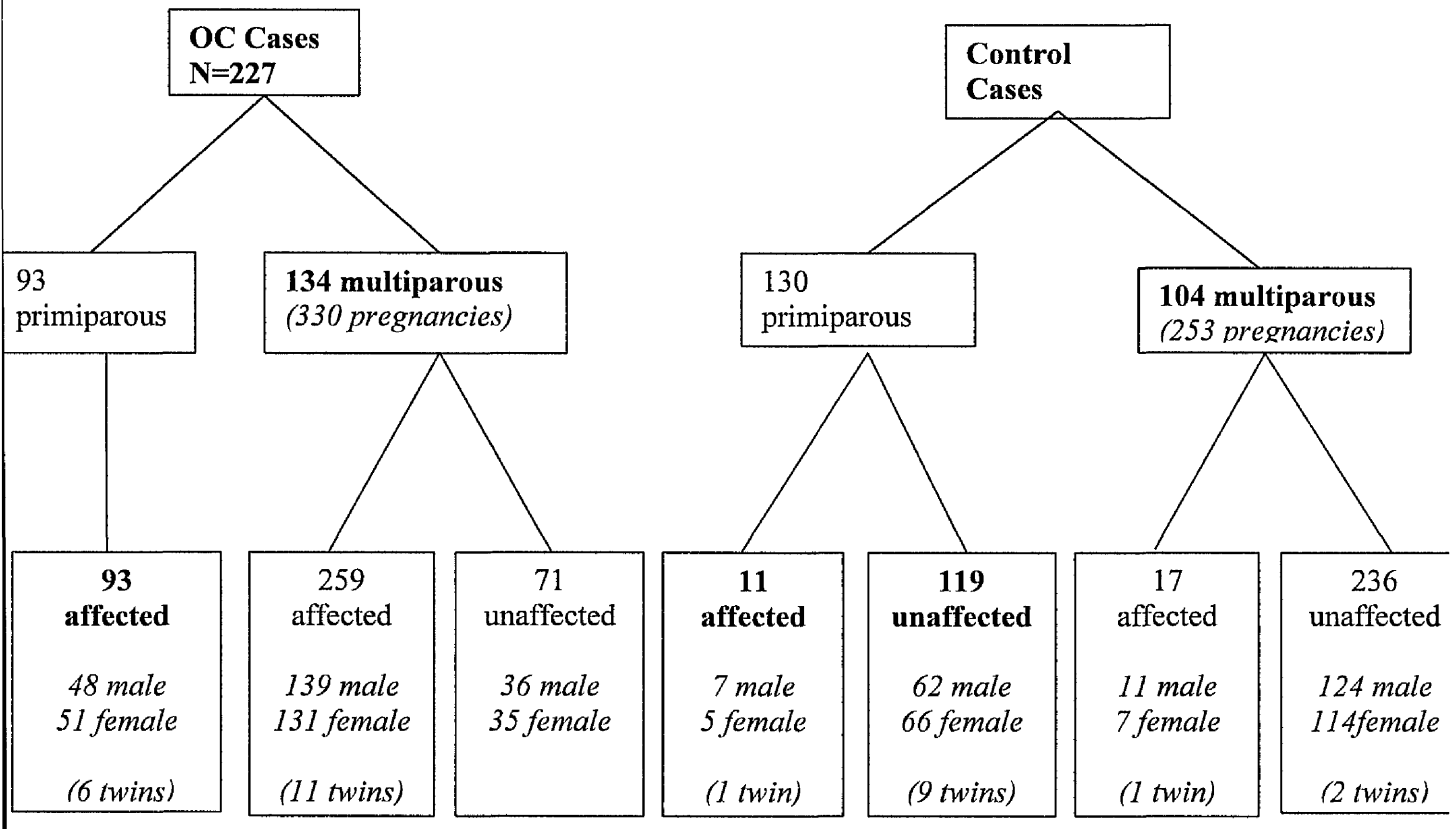


Figure 5. Subject details.

There were 423 pregnancies in the OC group and 383 pregnancies in the controls. In the OC group 352 (83%) pregnancies were complicated by pruritus (i.e. classified as affected pregnancies) and 71 (17%) were unaffected. In the OC group, 223 fetuses were male and 217 were female. In the control group there were 192 females and 204 males. There was no significant difference in the gender of the fetus in either group. 5% of women who were approached and asked to be in the control group declined to be interviewed. Table 4 shows a comparison of the prevalence of pruritus, spontaneous and iatrogenic premature delivery and IUD in OC and control pregnancies, and quotes the odds ratio for each feature in OC pregnancies.

Table 4. Indidence of pruritus, premature delivery and intrauterine death in OC and control pregnancies.

	OC pregnancies N=352	Control pregnancies N=383	**Odds Ratio (95% CI)**
Pruritus	**100%**	**7%**	-
Spontaneous prematurity	**16%**	**3%**	**6.4 (3.2-13.8)**
Iatrogenic prematurity	**22%**	**2%**	**13.3 (6.3-32.4)**
IUD	**7%**	**0.3%**	**23.5 (3.7-976.3)**

2.3.2 Intrauterine death

23 (7%) OC pregnancies were complicated by an IUD compared with 1 (0.26%) in the control group ($p<0.001$). 20 were in singleton and three were in twin pregnancies. In the singleton pregnancies the median gestation at which IUD occurred was 38 weeks (IQR 2.5) (Figure 6).

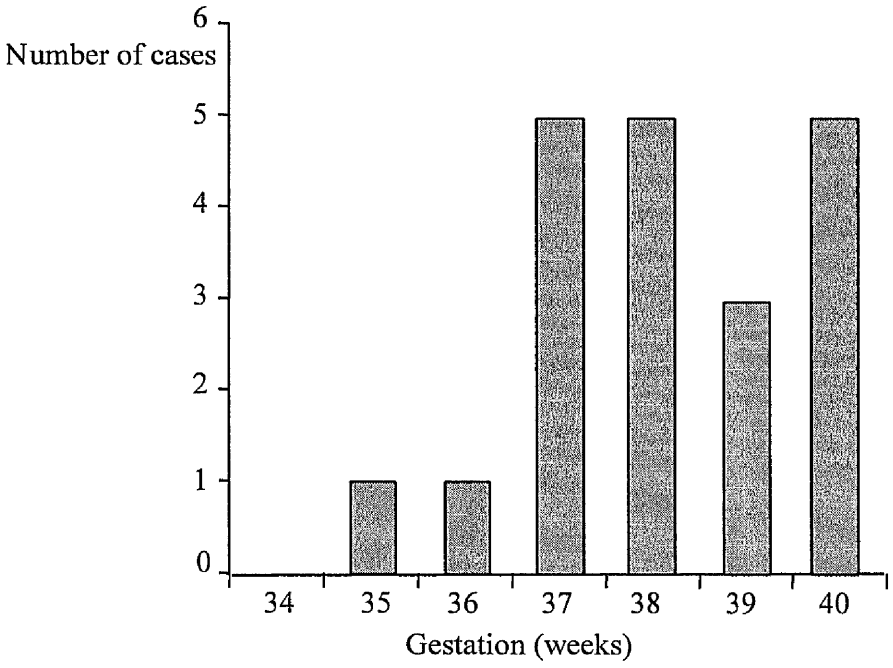


Figure 6. Gestational week at which IUD occurred in singleton OC pregnancies.

Of the IUDs in this group 13 of the fetuses were female and 7 were male. The control group IUD was female. There was an additional female IUD delivered at 26 weeks gestation in the OC group but this pregnancy was not complicated by pruritus (i.e. not defined as being affected by OC).

This gender difference in singleton pregnancies complicated by IUD (female preponderance) compared with the gender in other OC pregnancies (Chi-square,

p=0.16), or with that in control pregnancies (Chi-square, p =0.22) was not statistically significant. The median gestational week at which IUD occurred in singleton pregnancies was 38 for females and 39 for males.

Three of the IUDs occurred in twin pregnancies, i.e. 3 of the 17 (18%) twin pregnancies in women with OC. Two female IUDs occurred in a male plus female twin and a female plus female twin at 35 weeks and 31 weeks gestation respectively. One male IUD occurred in a male plus male twin pregnancy at 36 weeks gestation.

If all IUDs are considered, 22% occurred before 37 weeks gestation. However, only 2/20 (10%) of singleton IUDs occurred before 37 weeks gestation, and the fetus in both cases was female.

There were no significant differences in gestational week for the onset of pruritus in OC pregnancies complicated by an IUD when compared with other OC pregnancies.

A clinical diagnosis of OC had not been made in any of the pregnancies before they were complicated by IUD. As a consequence, none of the affected mothers had received treatment with either UDCA or dexamethasone.

2.3.3 Prematurity

133 (38%) of OC pregnancies were delivered prematurely, compared with 19 (5%) of controls (p<0.001).

2.3.3.1 Spontaneous prematurity

There were 56 (16%) spontaneous premature deliveries in women with OC and 11 (3%) spontaneous premature deliveries in the controls (p<0.001). Figure 7 shows the gestation at which spontaneous prematurity occurred in each group. The median gestation of the spontaneous premature deliveries in the OC group was 34 weeks (IQR

3.6) and in the control group was 36 weeks (IQR 4.4) (p=0.2). There were 33 (55%) female and 27 (45%) male fetuses in the OC spontaneous prematurity group.

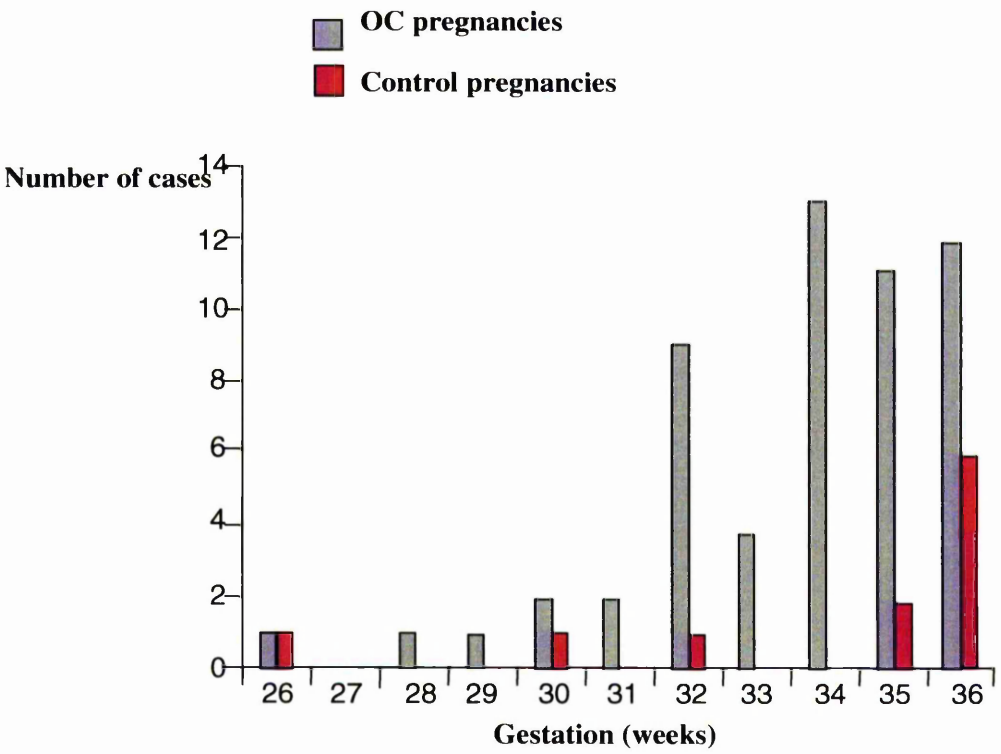


Figure 7. Gestational week at which spontaneous prematurity occurred in pregnancies complicated by OC and controls.

The pruritus started earlier in pregnancies complicated by spontaneous prematurity (median 28.0 weeks (IQR 6.8)) when compared with other OC pregnancies (30.0 (IQR 7.0)) weeks) (p<0.001) (Figure 8). Also, when all OC pregnancies were considered, those in which pruritus started earlier were more likely to be complicated by spontaneous prematurity (Chi-Square, p=0.04) (Table 5).

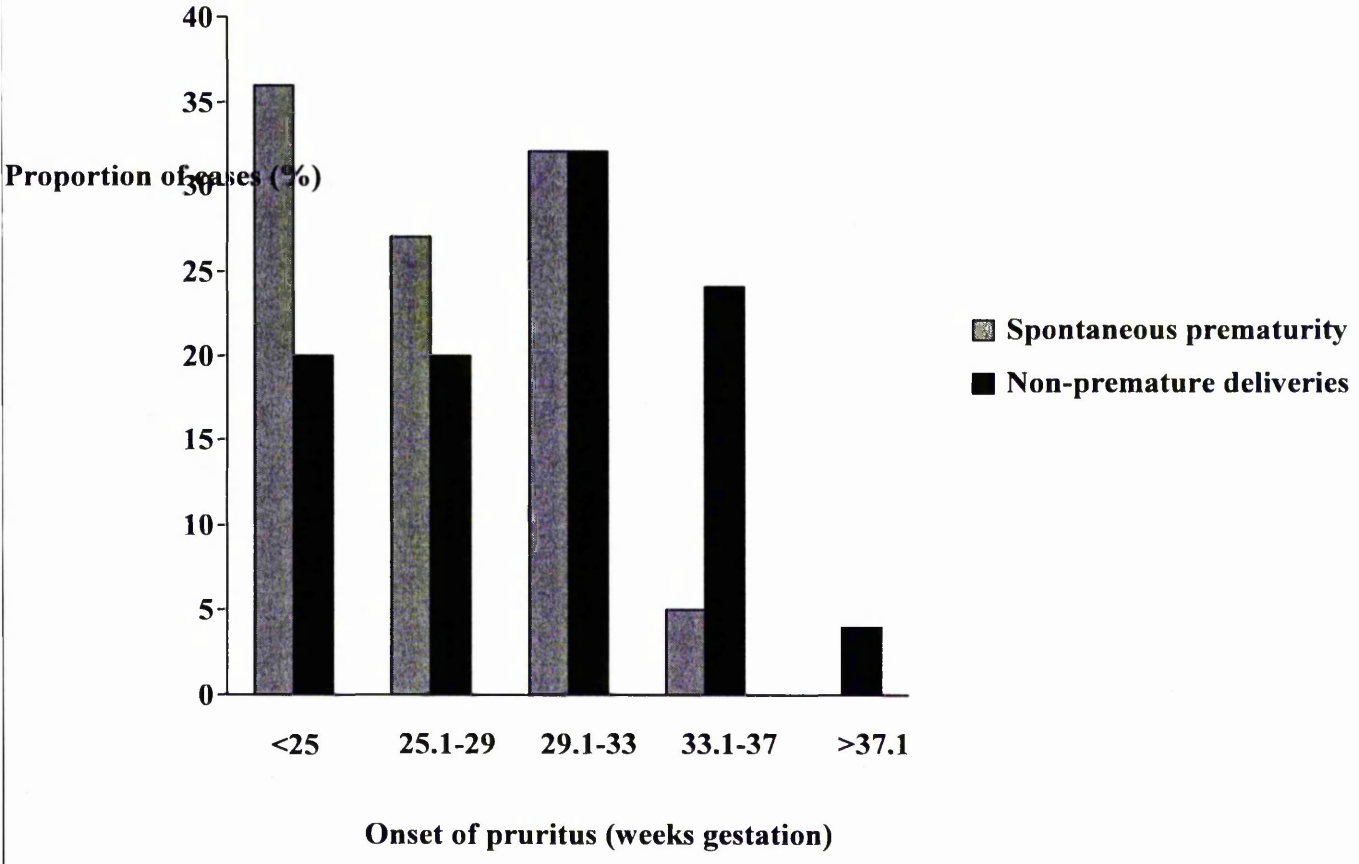


Figure 8. Gestational week at which pruritus started in OC pregnancies complicated by spontaneous prematurity and in those in which there was no prematurity.

OC had been diagnosed earlier in the pregnancy in 33/56 (59%) of those complicated by spontaneous prematurity. Sixteen were treated with UDCA and 2 with UDCA and dexamethasone. The median gestation at delivery in the treated group was 33.9 weeks compared with 34.8 weeks in the untreated group (p=NS).

Table 5. Relationship between gestational week at which pruritus started and the rate of spontaneous prematurity in OC pregnancies.

Gestation pruritus started	n	**Spontaneous prematurity (%)**
<25 weeks	**91**	**22**
25.1-29 weeks	**79**	**19**
29.1-33 weeks	**111**	**16**
33.1-37 weeks	**63**	**5**
> 37.1 weeks	**8**	**0**
Total	**352**	**56**

2.3.3.2 Iatrogenic prematurity

There were 77 (22%) iatrogenic premature deliveries in OC women (i.e. 46 induced, 25 elective caesarean sections and 6 pre-labour emergency caesarean sections), and 8 (2%) iatrogenic premature deliveries (2 induced, 5 elective caesarean sections and 1 pre-labour emergency caesarean section) in the controls ($p<0.001$). The rates of spontaneous and iatrogenic prematurity in the controls were 3% and 2% respectively.

2.3.4 Mode of delivery

In the OC group 48 women had a caesarean section before labour and 304 went into labour. The mode of delivery in the group that laboured is summarised in Table 6.

Table 6. Mode of delivery in affected pregnancies of women with OC who went into labour.

	Obstetric Cholestasis cases (304)				**Controls** (339)			
	Premature delivery (102)		**Delivery >37 weeks** (202)		**Premature delivery (13)**		**Delivery >37 weeks** (326)	
	Spont. Lab (56) (%)	**IndLab** (46) (%)	**Spont. Lab** (89) (%)	**Ind. Lab** (113) (%)	**Spont. Lab** (11) (%)	**Ind. Lab** (2) (%)	**Spont. Lab** (234) (%)	**Ind. Lab** (92) (%)
SVD	**48 (85.7)**	**28 (60.9)**	**76 (85.4)**	**81 (71.7)**	**9** (81.8)	**1** (50.0)	**176** (75.2)	**50** (54.3)
Instrum	**0**	**2** (4.3)	**6 (6.7)**	**11 (9.7)**	**0**	**0**	**35** (15.0)	**12** (13.0)
EMCS	**8 (14.3)**	**16** (34.8)	**7 (7.9)**	**21 (18.6)**	**2** (18.2)	**1** (50.0)	**23** (9.8)	**30** (32.6)

Spont. = Spontaneous Labour **NB. Elective Caesarean Sections not inclu**
Ind. = Induced Labour
Instru = Instrumental delivery
SV = Spontaneous Vaginal Delivery
EMC = Emergency Caesarean Section

Of the 304 women who laboured, there were 252 (83%) vaginal deliveries and 52 (17%) caesarean sections. The corresponding figures for the 339 women in the control group who laboured were 283 (83%) and 56 (17%) respectively. Of all 352 OC pregnancies, 159 (45%) were induced (46 before and 113 after 37 weeks gestation), compared with 94 of the 383 controls (27%) (X^2, $p<0.0001$). Of the 159 women with OC who were induced, 122 (77%) had a vaginal delivery (including instrumental deliveries) compared with 63 (67%) controls (X^2, p=NS). Of the women

with OC who laboured, 19 (6%) pregnancies were delivered by either forceps or vacuum extraction. The corresponding figures for controls was 47 (14%) (Chi-square, p=0.002) (Table 6).

Of the 48 women with OC who had a caesarean section before labour, 31 occurred before 37 weeks gestation (including 6 twin pregnancies), and a diagnosis of OC was made in all cases. It was beyond the scope of this study to establish whether the decision to perform a caesarean section was made for maternal or fetal reasons.

2.3.5 Admission to neonatal intensive care unit

When analysing the number of pregnancies in which the infant was admitted to the neonatal intensive care unit (NICU) for more than three days, the 23 pregnancies complicated by IUD were not considered. Therefore, of 329 affected OC pregnancies, 64 (20%) infants were admitted to the neonatal ICU, compared with 13/382 (3%) of control pregnancies (Chi-square, p<0.0001). When only premature deliveries (spontaneous and iatrogenic) were considered, 53/128 (41%) infants were admitted to the ICU, compared to 6/18 (33%) controls (Chi-square, p=NS). The proportion for spontaneous prematurity was 27/54 (50%) for OC pregnancies compared with 4/11 (36%) controls (Chi-square, p=NS), and for iatrogenic prematurity were 26/74 (35%) compared with 2/7 (29%) (Fisher's exact test, p=NS).

2.3.6 Recurrence

Of the 131 women, 80 (61%) had OC in all pregnancies and 57 (39%) did not. If a woman had OC in a previous pregnancy, her risk of having it in a subsequent pregnancy was 90%. The pruritus started at an earlier gestation in subsequent pregnancies (Kruskal Wallis, p=0.006) (Table 7).

Order of affected pregnancy	**Number of cases** *(total 352)*	**Gestational week** ***median (IQR)***
1st	182	30 (7)
2nd	113	28 (8)
3rd	43	28 (8)
4th	10	25 (6)
5th	4	21 (15)

Table 7. Median gestation at which pruritus started in successive OC pregnancies.

2.3.7 Other clinical features

Cyclical pruritus was present in 46 (20%) of women with OC and 3% of controls (p<0.001). Some women experienced pruritus at the time of ovulation (day 14),

others just prior to or during the menses, supporting the role of oestrogen and progesterone in the pathogenesis of OC.

Pruritus was experienced following the oral contraceptive pill in 30 (14%) of women with OC and in 1 (0.5%) of controls ($p<0.001$). Cyclical pruritus occurred in combination with oral contraceptive-induced pruritus in 15 women. Therefore 61 (27%) women with a history of OC had either cyclical or oral contraceptive-induced pruritus.

In addition more women with OC had a history of gallstones compared with controls, i.e. 30 (13%) versus 3 (1%) ($p<0.001$, odds ratio 11.7, 95% C.I. 3.5-60.7). 21 women with OC had symptomatic gallstones and 9 had asymptomatic gallstones previously documented following ultrasound examination in pregnancy.

2.3.8 Pattern of Inheritance

There were 76 parous sisters in the OC group and 82 parous sisters in the control group. The prevalence of OC in the parous sisters was 14% (11 cases), for the OC group and zero for the controls ($p<0.001$). There were 3 index cases whose sisters were also recruited to this study as a separate case. If one sister from each of these sibling pairs was removed from the study, the number of parous sisters was 73 and the prevalence of OC in sisters was 11% (8 cases). Twenty (9%) of women with OC and no controls had an affected mother ($p<0.001$), and 32 (14.1%) had an affected first-degree relative (mother or parous sister) ($p=0.001$). Ten (4.4%) additional cases had an affected relative who was not a first degree relative. Therefore, in total 42 (18.5%) women with OC had an informative pedigree.

2.3.8.1 Co-inheritance of OC and gallstones

A large proportion of women with OC had first degree relatives with symptomatic gallstones (26%), compared with controls (9%) ($p<0.001$). A diagnosis of gallstones was coinherited with OC in some pedigrees (see below).

2.3.8.2 Pedigrees

Pedigrees were drawn for all cases with a positive family history, even if the first degree relatives were unaffected. A typical pedigree is shown in Figure 9 to illustrate features of the inheritance of OC in this series. The remainder are shown in the Appendix.

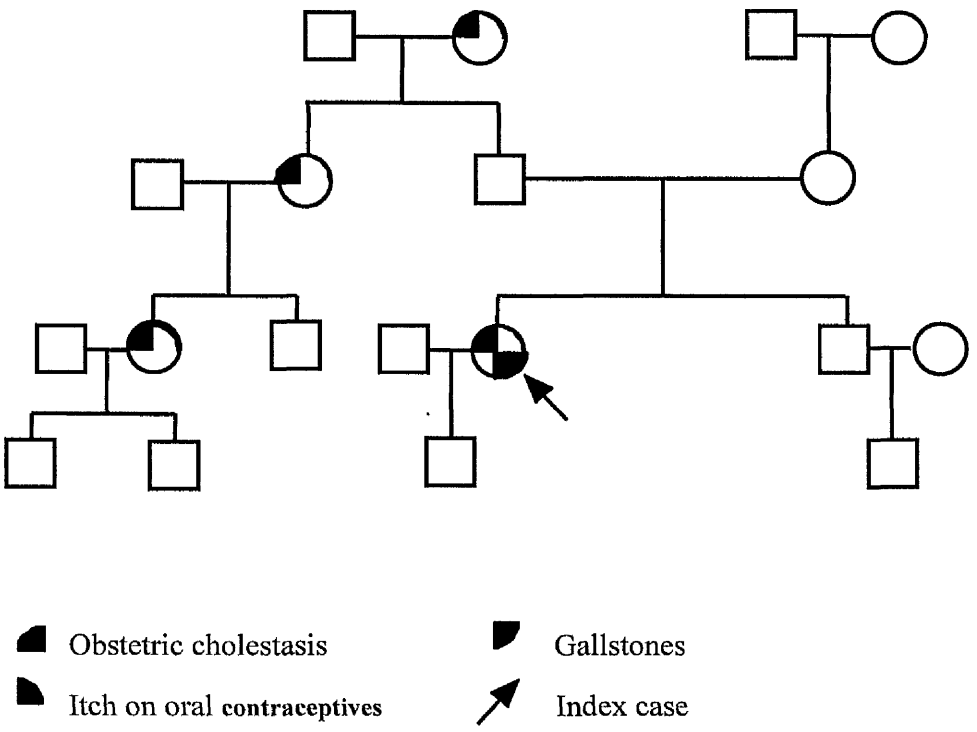


Figure 9. Pedigree from an OC index case demonstrating sex-limited autosomal dominant inheritance.

2.4 Discussion

2.4.1 Clinical features of OC in UK Caucasians

The cases in this study represent the largest collection of affected OC pregnancies in the world literature. Significant differences in the incidence of pruritus, prematurity and IUD were demonstrated in pregnancies complicated by OC compared with controls. In addition this study has confirmed that OC has serious consequences in UK white Caucasians. Specific features were demonstrated that will be of use to clinicians managing the condition; in particular the findings that in singleton pregnancies IUD mainly occurs from 37 weeks gestation (although the IUDs in multiple pregnancies occur earlier); also the finding that the earlier in gestation at which pruritus is first reported, the higher the incidence of spontaneous prematurity.

The high prevalence of complications in the OC group in this study may result from ascertainment bias, as the women had self-referred to the OC patient organisation. Therefore this study does not claim to predict the frequency of IUD or prematurity in the total OC population. It does however allow features of the pregnancy complications of the condition to be studied. In addition, the control group was recruited prospectively while the majority of the cases were recruited retrospectively. However as the control group contained approximately 95% of white Caucasian women who delivered at Queen Charlotte's Hospital over the study period, it is anticipated that it will be a fair representation of the obstetric population at that time. Indeed, the IUD rate in the control pregnancies (2.6 per 1000) was similar to the rate of IUD at term from a study of 171 527 births in the North East Thames region, i.e. 1.3 per 1000 (Cotzias et al. 1999). Similarly, the spontaneous and iatrogenic prematurity rates in the controls (3% and 2% respectively) were similar to those in a study of 625 646 liveborn singleton deliveries in Scotland (Magowan et al. 1998).

Although several case series have reported high rates of IUD in OC, the largest number of cases reported in any study to date is nine (Davies et al. 1995). Eight of the cases in this previous study occurred from 37 weeks gestation (Davies et al 1995), consistent with the current findings. The demonstration that the majority of the IUDs occur from 37 weeks gestation should be of clinical value. In singleton pregnancies there were only two (10%) IUDs before 37 weeks gestation, and there were no IUDs before 35 weeks gestation. Therefore elective delivery at 37 weeks gestation may be a reliable way to prevent IUD in affected singleton OC pregnancies, particularly given that there have been no reliable methods reported for the prediction of fetuses at risk to date.

Meconium staining of the amniotic fluid is reported in almost all cases of IUD that complicate OC in the current literature (Fisk et al. 1988a; Rioseco et al. 1994; Alsulyman et al. 1996) and amniocentesis for the presence of meconium has been proposed as the best way to predict the at-risk fetus (Fisk et al. 1988a). Given that there were no IUDs before 35 weeks gestation in the current study, a possible alternative strategy for the prevention of IUD could be weekly amniocentesis or amnioscopy for meconium from 34 weeks gestation, and if meconium is not present, delivery could be deferred until 37 weeks gestation. However, such an approach is likely to be considered too intrusive to be used routinely by most obstetricians.

When considering elective delivery at 37 weeks gestation to reduce the risk of IUD, it is important to also consider the fetal risks of early delivery. A study of neonatal respiratory morbidity following elective caesarean section reported a higher incidence of respiratory distress syndrome and transient tachypnoea of the newborn in those that were delivered during the week 37^{+0}-37^{+6} compared with 38^{+0}-38^{+6}, and a similar difference was seen when this was compared with the following week (Morrison et al.

1995). Another study demonstrated that a markedly higher proportion of babies required ventilation for respiratory distress syndrome when delivered at an earlier gestation; i.e. 1:73 (1.4%) at 35 weeks, compared with 1:557 (0.2%) at 37 weeks, and 1:1692 at 38 weeks (Madar et al. 1999). These risks are lower than the published rates of IUD in OC pregnancies that are not delivered by 38 weeks, i.e. 9-11% (Reid et al. 1976; Reyes 1982).

A diagnosis of OC had not been made in any of the pregnancies complicated by IUD in this study. If a diagnosis had been made, it is possible that treatment with ursodeoxycholic acid (UDCA) or dexamethasone, both of which improve symptoms and liver function tests including serum bile acid levels (see section 1.2.5), could have reduced the risk of IUD in these pregnancies. Animal studies have implicated bile acids in the pathophysiology of spontaneous prematurity (Campos et al. 1986), and the cardiomyocyte experiments reported later in this thesis implicate them in the aetiology of IUD. If future prospective studies support the suggestion that bile acids cause the IUD in OC, it may be justifiable to defer delivery to a later gestation than 37 weeks in women who have responded to treatment and whose serum bile acid levels have returned to normal.

Three of the five IUDs that occurred before 37 weeks gestation were in twin pregnancies, suggesting that twin pregnancies may be at risk of IUD at an earlier gestation than singleton pregnancies. There was a non-significant tendency towards female fetuses in pregnancies complicated by IUD. The data on IUDs in twin pregnancies and in female fetuses also did not reach statistical significance, and a much larger series would be required to assess a possible gender difference.

A recent initiative is the establishment of a web-based international registry of all OC-related IUDs (http://escuela.med.puc.cl/iris/welcome.html). This is called IRIS

(International Registry of Intrahepatic Cholestasis of Pregnancy related Stillbirth) and should permit prospective information from pregnancies complicated by IUD to be collected.

The rate of spontaneous prematurity in women with OC (16%) was significantly higher than that of controls. However, it was lower than in most other studies (36-44%) (Reid et al. 1976; Fisk et al. 1988), with the exception of one recent UK study (Kenyon et al. 2001). This may be because 77/352 (22%) of OC pregnancies in this present study had received treatment with either UDCA alone or in addition to dexamethasone. While it has not been proved that treatment reduces the rate of spontaneous prematurity, studies in animals (Campos et al. 1986) and using myometrium from women with OC (Israel et al. 1986) have implicated raised serum bile acids. However, 32% of women with pregnancies complicated by spontaneous prematurity in this study had been treated with UDCA alone, or together with dexamethasone, and this did not prevent premature delivery in these cases.

This study has demonstrated that the onset of pruritus occurs at a significantly lower gestation in OC pregnancies complicated by spontaneous premature delivery when compared with unaffected OC pregnancies or with controls. A Chilean study has also found a positive correlation between prematurity and earlier gestation at onset of pruritus during pregnancy (Reyes 1982). If bile acids are the cause of the spontaneous prematurity, it is possible that the duration of exposure to bile acids may influence myometrial contractility or other processes that can initiate labour.

The proportion of iatrogenic premature deliveries was significantly higher than the rate in controls and a diagnosis of OC had been made in all cases. However, the caesarean section rate was no higher in the women with OC than the controls, despite the large proportion of women with OC who were induced. Of the women who were

induced, a smaller proportion of those with OC required instrumental delivery, and there was a non-significant trend for them to require caesarean section less frequently than controls. This may be because women with OC respond better to induction of labour, possibly due to the same mechanisms that cause the increased incidence of spontaneous prematurity.

It was not possible to obtain accurate data about the rates of fetal distress in this study as most women did not know whether they had cardiotocograph abnormalities or fetal distress in their pregnancies. However, admission to the neonatal ICU was used as a surrogate marker of fetal distress, and the rates were significantly higher in OC pregnancies. When the results were analysed further, the risk appears to be related more to premature delivery than to OC. It was beyond the scope of this study to assess the subsequent morbidity of the infants that were admitted to the neonatal ICU. However, given that elective delivery at 37 weeks is currently advocated as the best strategy for the prevention of intrauterine death, a large prospective study of the outcome of such OC pregnancies would allow the consequences of both iatrogenic and spontaneous premature delivery to be evaluated.

The prevalence of oral contraceptive pill-induced pruritus was higher than in a smaller French study of 24 women diagnosed with OC during their pregnancies in which only one (4%) suffered pruritus and abnormal liver function tests after taking the oral contraceptive pill (Bacq et al. 1997). This may be because OC in French women has a different aetiology (i.e. genotype or environment), or because of the small number of cases in the study. These symptoms disappear once women stop taking the oral contraceptive pill. At present there is no evidence that prolonged use of oral contraceptives in women with OC can cause permanent liver damage. Given that 34%

of women with cyclical pruritus also developed pruritus if they took oral contraceptives, these women may have a stronger genetic predisposition to OC.

In summary, this study has confirmed that OC can have serious fetal consequences in UK white Caucasians. This study contains the largest series of pregnancies complicated by IUD to date, and has demonstrated that the majority of IUDs occur from 37 weeks gestation in singleton pregnancies, suggesting that delivery by this stage of pregnancy will prevent 90% of fetal deaths. IUDs complicating twin pregnancies occur at an earlier date. The gestation at which the pruritus started did not allow prediction of pregnancies complicated by IUD, but did allow prediction of risk of spontaneous prematurity.

2.4.2 Inheritance of OC in UK Caucasians

A family history of OC was demonstrated in 18.5% of cases in this study. 9% of the index cases had an affected mother and 14% had an affected first degree relative. 11% of parous sisters developed the condition. These figures will be of use for the counselling of relatives of affected women.

Although it was possible to ascertain 42 pedigrees in which at least two relatives had a history of OC, these pedigrees have a limited use for genetic studies because the diagnosis of OC in many older relatives was not confirmed by measurement of either serum bile acids or transaminases. OC is not the only cause of generalized pruritus in pregnancy. Therefore, the pedigrees in this study can not be reliably used for studies of genetic linkage.

It will be possible to use the pedigrees that were identified in this study if a test of phenotype is identified for males and non-parous females. Several authors have demonstrated that the adminstration of the oral contraceptive pill to women with a

history of OC causes recurrence of the symptoms and biochemical changes of the condition (Kreek et al. 1967; Holzbach et al. 1983). In the more recent study, the administration of orthonovum 10mg/day for 2 weeks caused changes in the liver transaminases in all the women who participated. One man who was presumed to carry the susceptibily locus for OC from pedigree studies did not respond. The authors concluded that this was a good test for women, but not for men (Holzbach et al. 1983).

We have shown that gallstones can be co-inherited with OC. It is possible that this is an additional phenotype for individuals with OC. However, the high prevalence of gallstones within the population may preclude its use due to the risk of a high false positive rate.

3. INVESTIGATION OF THE ROLE OF THE *MDR3* GENE IN THE MOLECULAR AETIOLOGY OF OBSTETRIC CHOLESTASIS

3.1 Background and aims

The studies in chapter 2 demonstrated that the aetiology of OC has a genetic component. Therefore the decision was made to collect a DNA resource, and while collecting this to investigate a candidate gene. There are several candidate genes for OC, including the genes that influence bile acid transport (see sections 1.1.3.1 and 1.1.3.2), the bile acid receptor and associated transcription factors (see section 1.1.4). At the time this study commenced, the *MDR3* gene was the only one in which mutations had been reported in women with cholestasis in pregnancy, but this was only in a small subgroup of 7 women from two PFIC3 pedigrees (de Vree et al. 1998; Jacquemin et al. 1999). However it was not known whether *MDR3* mutations cause OC in women without a family history of PFIC3. Children with PFIC3 are distinguished by having a raised GGT, and therefore a subgroup of women with OC and a raised GGT were identified in whom *MDR3* was investigated for sequence variants that predispose to cholestasis in pregnancy.

3.2 Subjects and methods

3.2.1 Subjects used for genetic studies of the *MDR3* gene

For mutational analysis of the *MDR3* gene, 8 women with OC and a raised GGT were analysed together with a normal control individual. Of these 8 individuals, 2 were recruited prospectively upon detection of raised GGT and the remaining 6 were added to the cohort following retrospective measurement of GGT levels from stored serum in 87 patients. The samples were obtained from Selly Oak Hospital, Birmingham, where the serum bile acid levels had been previously checked, and the serum had then

been stored at –20°C. Of the 87 patients with a confirmed diagnosis of OC and raised serum bile acids, 20 (23%) had a raised GGT. As different hospitals have different normal ranges for liver function tests, the upper end of the normal range in pregnancy was assumed to be 80% of the level quoted outside pregnancy for each hospital, consistent with published studies (Girling et al. 1997).

To check for the presence of sequence variants in normal individuals a panel of 50 women with gestational diabetes but with otherwise normal pregnancies who had no history of cholestasis was used. Forty women with obstetric cholestasis without a known raised GGT were also analysed.

3.2.2 Experimental methods

This section describes the methods used for the experiments described in this study. In general, protocols used were based upon those described in "A Laboratory Manual", by J. Sambrook, E. Fritsch and T. Maniatis, 2nd Edition, from the Cold Spring Harbour Press.

3.2.3 DNA preparation

Preparation of genomic DNA

Genomic extraction of DNA was performed using an adaptation of the method of Kunkel (1977); 10 ml blood containing anticoagulant (0.5M EDTA, pH 8) was divided between two 50 ml polypropylene tubes and 45 ml lysis buffer (precooled to 4^0C) added to each tube. Following centrifugation (1000g for 10 minutes at 4^0C) the supernatant was immediately discarded to avoid degradation of DNA by nucleases. The pellets were pooled and resuspended in 4.5 ml 75 mM NaCl / 24 mM EDTA pH 8. 2 mg/ml proteinase K (Boehringer Mannheim) and 0.5 ml 5% SDS was added to

each tube and digestion performed overnight at 37^{0}C. Each sample was phenol chloroform extracted to remove the digested protein: the samples were first mixed with 5 ml phenol (saturated with 10 mM Tris, pH 8, 1 mM EDTA) and centrifuged at 1000g for 15 minutes. The aqueous layer was transferred to another tube and the phenol extraction repeated if considered necessary based upon the purity of the aqueous layer. The samples were then further purified by extraction twice with 5 ml chloroform/isoamyl alcohol (24:1 v/v) which also removed any traces of phenol. The genomic DNA was precipitated by adding 0.5 ml 3M sodium acetate and 11ml of ethanol, the precipitate removed using a glass hook and resuspended in 1 ml TE pH 8. The DNA was allowed to dissolve overnight at 4°C before the yield was assessed by UV spectrophotometry.

Quantification of DNA

The concentration of DNA in aqueous solutions was determined by measuring the optical density (OD) of the sample at wavelengths 260 nm and 280 nm in the UV spectrum using a spectrophotometer. 5µl DNA was diluted 100 fold with TE, pH8, and placed in a quartz cuvette. The OD was measured against a "blank" sample (500µl TE, pH8) and the concentration determined. An OD of 1.0 at 260 nm corresponds to 50 mg/ml double stranded DNA or 20 mg/ml oligonucleotide. Therefore the concentration of a DNA sample (in mg/ml) is calculated by:

$OD^{260\ nm}$ x conversion factor (i.e. 50 or 20) x dilution factor (i.e. 100)

The ratio of the OD readings at 260 nm/280 nm provides an assessment of the purity of the DNA sample with pure samples having a ratio of greater than 1.8. A ratio of less than 1.5 indicates that the sample was contaminated with proteins and needed an extra extraction with phenol chloroform.

Preparation of DNA from plasmids

The small scale preparation of DNA (miniprep) is based on the alkaline lysis method (Birnboim et al. 1979) of DNA extraction. 10 ml of LB broth containing the appropriate antibiotic (kanamycin, tetracycline or ampicillin) at the appropriate concentration was inoculated with a single colony of transformed cells and incubated at 37°C overnight with agitation. The cells were pelleted at 3000g for 10 minutes, transferred to a microtitre tube, and lysed at room temperature for 10 minutes in 200 µl lysis buffer (50 mM glucose, 25 mM Tris pH 7.4, 10 mM EDTA). 400 µl of fresh 0.2M NaOH and 1% SDS was added, the mixture placed on ice for 5 minutes, 300 µl 3M sodium acetate, pH 4.8 then added and the mixture replaced on ice for 10 minutes. The precipitate was removed by centrifugation at 14000g for 5 minutes and the supernatant transferred to a new tube. This was repeated. The plasmid DNA was precipitated at -70°C for 10 minutes with 600 µl isopropanol and the precipitate pelleted by centrifugation. The supernatant was discarded and the pellet resuspended in 200µl 0.3M sodium acetate pH 7. Two phenol chloroform extractions were performed. 200 µl isopropanol was added for a final precipitation of the plasmid DNA. The pellet was washed in 70% ethanol, air dried and resuspended in 20 to 50µl. RNA was removed by the addition of 1ml RNAse (10µg/ml) and the solution left at 37°C for 1 hour. This DNA was then stored at 4°C prior to further use.

Plasmid manipulation

Preparation of Competent Cells An overnight culture of a suitable stain of cells (JM109) was grown from a single colony at 37°C in LB broth. These cells were then diluted 40 fold by transferring 2.5ml culture into 97.5ml sterile LB broth and incubated overnight at 37°C until an OD of 0.4 to 0.5 was achieved. The culture was then transferred to two Falcon 50ml polypropylene tubes and chilled in an ice water

bath for 10 minutes. The cells were pelleted at 4°C by centrifugation at 2000g for 10 minutes and the supernatant discarded. Each cell pellet was then resuspended in 25ml ice cold 100mM calcium chloride and left on ice for 30 minutes. The cells were again pelleted by centrifugation and resuspended in 2 ml ice cold calcium chloride, 15% glycerol. Aliquots of 200µl were transferred into 1.5ml microtitre tubes and left overnight at 4°C. The cells were then frozen in liquid nitrogen and stored at - 70°C.

Transformation of Plasmid DNA 50 to 100 ng plasmid DNA was added to 100 µl competent cells and the cells left on ice for 30 minutes. Following heat shock at 42°C for 2 minutes 1 ml LB broth was added to the cells and the cells incubated at 37°C for 1 hour. (This recovery period allows the development of antibiotic resistance.) Using a sterile spreader 100µl of cells was plated onto an LB agar plate containing the appropriate antibiotic and incubated overnight.

3.2.4 Restriction enzyme digestion of DNA

DNA was digested with a four-fold excess of the restriction endonuclease in the appropriate restriction enzyme buffer incubated at 37^0C, 55^0C, 60^0C or 65^0C for 4-12 hours, according to the suppliers specifications.

3.2.5 Amplification of DNA using the polymerase chain reaction

Amplification of genomic DNA by the polymerase chain reaction (PCR) was carried out using an automated DNA thermal cycler (MJ Research Tetrad) in a total volume of 50µl containing 50-100ng DNA template, 100 ng of each oligonucleotide primer (Table 8), 50 mM KCl, 10 mM Tris/HCl pH 8.3, 1-3 mM $MgCl_2$, 0.01% gelatin, 100 mM dNTP and 2 units *Taq* polymerase (Bioline). The reaction was overlaid with mineral oil, or a rubber lid was attached to the 96-well plate in which the PCR

reactions were performed, to prevent evaporation. The standard conditions for the reactions were as follows: 96°C for 3 minutes (denaturation step), 30 cycles of 30 seconds at 94°C (denaturation step), 30 seconds at 48°C - 62°C (annealing step) and 1 minute at 72°C (extension step), followed by a final extension of 10 minutes at 72°C. For >1 kb products, longer extension periods were used, increasing by 1 minute per kilobase. Positive (i.e. a sample known to contain the target sequence amplified by a particular set of primers) and negative controls (a water blank) were included in all PCR reactions.

Table 8. PCR primers used to amplify the coding exons of *MDR3*.

Exon	Sequence of forward primer	Sequence of reverse primer
2	5'AGGCTGTGGTTTCTCCTC	5'AGTGGCTGCTGGGATGT
3	5'GTGCTTGCTATGTTTGTTG	5'CATTTCACAGCTTACCAATG
4	5'GGAAGAGGAGAAATTCCATTC	5'CTGGAGTCAACCAGATATCC
5	5'CCTGGCAATGCCTATTAATAG	5'GGGTAAAGAGTACACGTT
6	5'GCCTTTCTCTTTTTTTTC	5'GACATTAACAATGTACCT
7	5'TTAGGTGTGATCTTTTTT	5'AAGGCCCAGCTTTCACAT
8	5'ACTGTTTCTTTTCTGTCC	5'GGCTAAAGAACCTTCCTG
9	5'GACTCGGAGTATGGATTGT	5'GTTCATCTTTCAAAAAGGAGCG
10	5'GCCAGAATGTGACTTAAAC	5'TAAAATAATAAATGACTT
11	5'ATGATGGAATAGTCATTAC	5'TGATTCAACAATCAACCTCAG
12	5'TTTTCTCATTTTCCTTAC	5'GCTTGGTTCTTCCCACTT
13	5'GAATGGTCCTGATACTTCAGC	5'CTCAGTCCTATGAGGTGA
14	5'GTTTTCTGTGTTAGAAATTT	5'GCTTTTTAGAGTCTACTG
15	5'CCACATTTTTGTTCTTTT	5'AATTCCTGAAAAGCAAGT
16	5'GCTTTATGATTCTAAACTTGGAGG	5'GAGTATGGCTCATAGTAGCAG
17	5'CCTATGTTTATATGTTCT	5'TGTGATGTTTGCAAACTT
18	5'GCCACTATTTATGAGAGG	5'CACTGTAAGAATTTGGAAGCTCC
19	5'CATGACTTGAGGATCCGTGGC	5'TCTCCAGCGCACACTCCT
20	5'ACGTGTATTTTATGCCAT	5'GTAGAGTGCAGTCTACCT
21	5'GACCAAGAGGCTAAGGCT	5'GTTGTAGTGGGCACAAAC
22	5'GGTCTATTCTGTGTTACC	5'GGAATCATATGGTTCATT
23	5'TATACTGATTTGCTTTTC	5'ATATTTCTAAAGCTACTT
24	5'ATTCCTCTACTTCTGTCT	5'TCAGGCATCAGAGAACTT
25	5'CTGGCACCAGAACTATACCA	5'TTATAAGGAAATGTGCTC
26	5'TTGTGGACTTTGGTTTTC	5'TCAGCTACTCTTTAACTT
27	5'ATAACTTGGTTAACTTGC	5'AGTTGGGAGGCCACACAC
28	5'TCTGTCTGATTTATCAAT	5'TTATTTTACAAGTCAGAT

Amplification of DNA using long range PCR to obtain novel intronic sequence for the MDR3 gene

Long-range PCR products covering parts of the *MRD3* gene were generated using primers designed from the published sequence (Linke et al. 1991). Products varying in size from 4kb to 12.3kb were amplified using the Expand long-range PCR kit (Boehringer Mannheim), and subcloned using the TOPO XL kit (Invitrogen), according to the manufacturers instructions. Multiple independent positive colonies were picked and DNA prepared using the Wizard minprep kit (Promega). Inserts were sequenced with vector specific and *MRD3* specific primers using the FS+ Dye Terminator sequencing kit (PE Applied Biosystems) according to the manufacturers instructions. Sequence information from these experiments was combined with previously published information (Linke et al. 1991) and with sequence information from the Washington University chromosome 7 sequencing project (http://genome.wustl.edu/gsc) to design 27 pairs of exon-specific primers for the *MDR3* gene (Table 8).

3.2.6 Agarose gel electrophoresis

DNA fragments were separated by electrophoresis on 0.8-2.5% agarose gels containing 0.5 μg/ml ethidium bromide. For the separation of small PCR products of 100-300 bp in size a 2% gel was used whereas for the separation of larger products, a gel with a lower agarose concentration was used. Prior to loading on a gel, 2-5 μl of loading dye (Sigma) was added to each DNA sample. The gels were cast in 1 x TBE buffer and run at a constant voltage. A size marker (1 Kb marker or 123bp ladder

(Gibco)) was electrophoresed alongside the samples to allow identification of DNA fragment sizes.

3.2.7 Photography of agarose gels

The ethidium bromide stained DNA was visualised by transillumination (ultraviolet 254 nm) and photographed using a Gel Doc 1000 (Bio Rad) onto thermal paper for Mitsubishi videocopy processor (model K655 HM), or with Polaroid 667 instamatic film using a Kodak 22A Wrattan filter.

3.2.8 Semi-Automated DNA Sequencing

PCR products were prepared for sequencing by gel purification in a 2.0% agarose gel. Individual bands were visualised under a long wave UV transilluminator and excised from the gel using a clean razor blade. Each product was spun through siliconised glass wool at low speed in a desktop centrifuge (6500rpm) and the DNA suspension made up to a final volume of 20 µl with dH_2O. Sequencing reactions were carried out using the FS+ Dye Terminator sequencing kit (PE Applied Biosystems), according to the protocol in the manual, and using a MJ Research Tetrad cycler. 10 µl of DNA (0.2-2.0 mg) was used with 8 µl of fluorochrome labelled dye terminator sequencing pre-mix (Amersham) and 2 µl oligonucleotide primer (1.6 pmol/µl). Cycling conditions for the sequencing reactions were as recommended by the manufacturers (25 cycles as follows: 96°C for 30 seconds, 45°C for 15 seconds and 60°C for 4 minutes). The 20 µl reaction was then transferred to a fresh tube containing 2.0 µl 3M sodium acetate pH5.5 and 50 µl 95% EtOH and the mixture placed on ice for 15 minutes to precipitate the DNA. Following centrifugation at high speed (13000 rpm) in a desktop centrifuge for 15 minutes, the pellets were washed in 70% EtOH

and dried under vacuum. Each pellet was resuspended with 4 µl formamide loading dye (for automated sequencing) and denatured for 2 minutes at 96 °C prior to gel electrophoresis on a 6% denaturing polyacrylamide gel using the ABI 373A system. The DNA sequence was collected using the Applied Biosystems Data Collection software.

3.2.9 Computer Analysis

DNA sequence analysis was carried out using ABI 373 Data Collection and Sequence Analysis software; alignments were carried out using the Sequence Navigator and AutoAssembler programmes, also from ABI. General sequence manipulation, such as restriction enzyme mapping, was carried out using the GCG package (University of Wisconsin), or the Cutter package (http://www.firstmarket.com/cutter/cut2.html) and homology searches such as BLAST analysis, through the MRC Human Genome Mapping Project, using a Power Macintosh computer via the telnet application, or via the World-Wide Web using Netscape Navigator.

Additional experiments that are discussed in the results section of this thesis are described in Appendix IV as they were performed in collaboration with Dr Kenneth Linton and Professor Christopher Higgins. The majority of this laboratory work was performed by Dr Linton, but Dr Linton and Dr Williamson analysed the results together. Otherwise, Dr Williamson did the majority of the experimental work described in this chapter, although two other scientists, Peter Dixon and Natasha Weerasekera assisted with the PCR-based experiments.

3.3 Results

3.3.1 Subject details.

DNA sequence analysis of the 27 coding exons of the *MDR3* gene, together with the respective exon/ intron boundaries, was performed in eight OC patients with raised GGT levels but no PFIC, together with a normal individual as a control. Clinical details of the cases used for this study are given in Table 9.

Table 9. Subject details for genetic studies of *MDR3*.

Case no.	Parity	Start of pruritus (gestational week)	Fetal distress	Gestation at delivery (weeks + days)	Max. ALT* (<28u/l)	Max. GGT (<30u/l)	Max. BA (<6μM)
1	1+0 (twins)	34	No	34+2	260	56	233
2	2+0	20	No	38	54	85	56
3	2+2	33	No	37+3	323	34	NT
4	4+4	36	Yes	38	163	36	45
5	3+4	31	Yes	36+2	691	74	34
6	1+0	32	No	36	NT	46	100
7	1+0	28	No	35+4	56*	134	236
8	2+2	15	Yes	35+6	163	50	237

Parity is expressed as the number of births that progressed to >24/40 gestation + other pregnancies not progressing beyond 24 weeks. Fetal distress was diagnosed if there was meconium staining of the amniotic fluid or fetal heart rate abnormality.
Key: ALT (alanine aminotransferase), GGT (gamma-glutamyl transpeptidase), BA (bile acids). NT (not tested). Normal ranges are given.
*the ALT was not available for patient 7, so the AST (aspartate aminotransferase) is shown (normal range in the 3rd trimester <29u/l).

3.3.2 Mutation detection

In patient 8, a heterozygous DNA base change was identified in exon 14, at the second nucleotide of codon 546 (Figure 10). In addition to the normal cytosine (C) at this position, an adenine (A) was also detected; this results in the substitution of the

wild-type alanine (A) with a mutant aspartic acid (D) (A546D). This mutation is in the highly conserved first nucleotide binding domain (NBD) of the MDR3 protein. The DNA base change introduces a *Sex*A1 restriction enzyme site, which facilitated confirmation of the mutation (Figure 10). This restriction enzyme change was used to exclude the presence of this mutation in two control panels consisting of 50 parous women without OC, and 40 OC women without a raised GGT during pregnancy. No other *MDR3* mutations were identified in the other 7 individuals with OC.

3.3.3 A546 is highly conserved in the NBDs of ABC transporters

A546 is a highly conserved residue, not only in the NBDs of proteins orthologous to the MDR3 protein in rat and mouse (Gros et al. 1986, Brown et al. 1993), but also in other members of the superfamily of ABC transporters, including human P-glycoprotein (P-gp1) (Chen et al. 1986) and the NBD of the histidine permease (HisP) of *Salmonella typhimurium.* While little is known about the structure of mammalian ABC transporters, the high resolution structure of HisP has been reported (Hung et al. 1998). From the crystal structure of HisP (Hung et al. 1998), A167 (the equivalent of the MDR3 protein A546) is located towards the carboxy terminus of α-helix 5 and, spatially, is in close proximity to the hydrophobic residues preceding the Walker B motif (Figure 11).

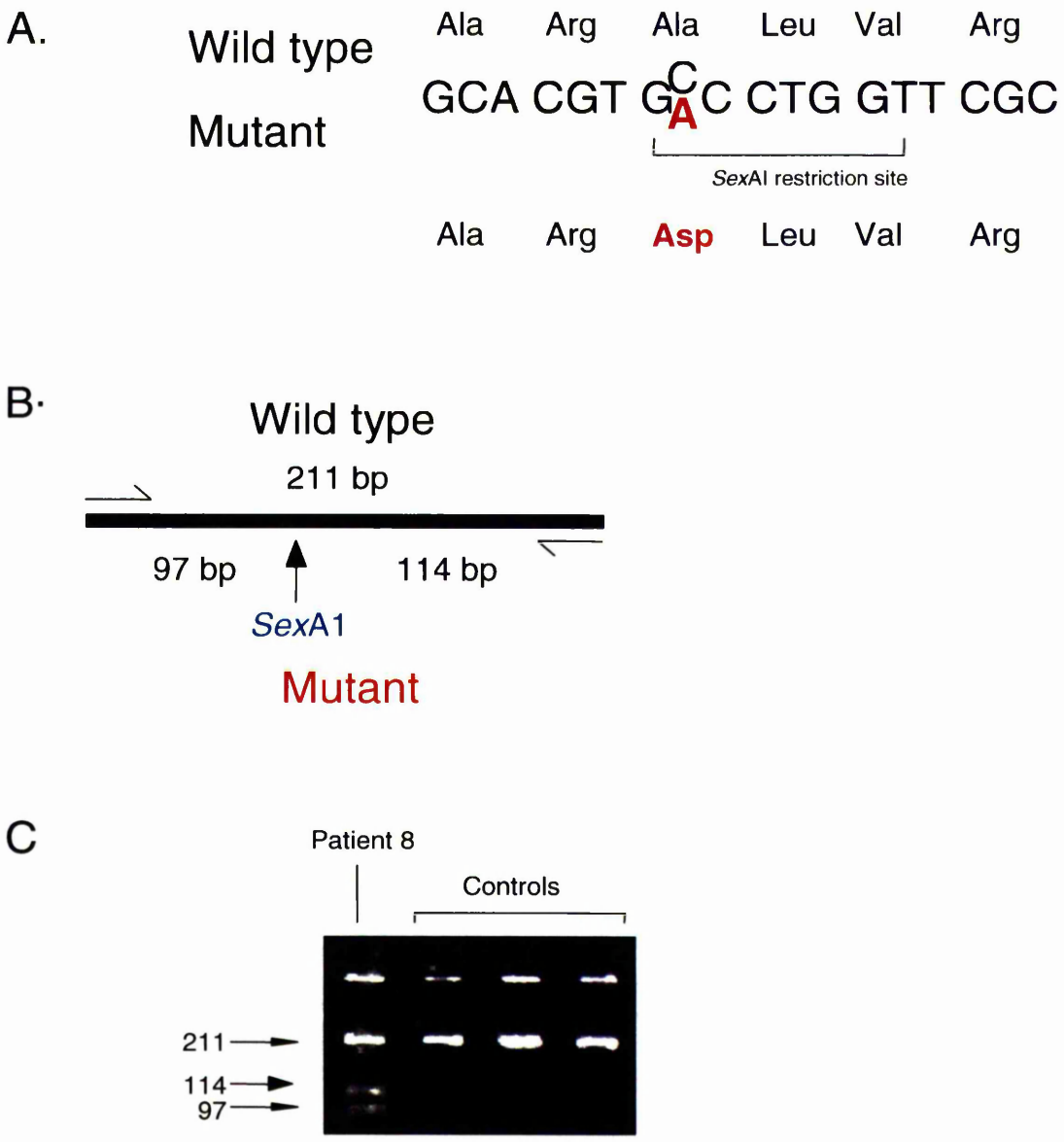


Figure 10. (a) shows the DNA sequence from patient 8 and a control patient. The C→A transversion results in the substitution of the wild type alanine with an aspartic acid. The transversion also generates a *Sex*AI restriction site (b). Amplification of exon 14 by PCR gives a 211 bp product. If the C to A mutation is present, digestion of this product with *Sex*A1 produces two smaller fragments of 97 bp and 114 bp (c). Thus, digestion of DNA from patient 8 with *Sex*AI reveals three products, the larger wild type product, and the two smaller fragments. In control subjects the 211 bp product is insensitive to cleavage by *Sex*AI (d).

3.3.4 Mature A544D-P-gp1 at the cell surface is functional

There are no established systems for quantification of PC translocation in mammalian systems making it currently not possible to study the function of the MDR3 protein. However, the transport of fluorescent substrates such as rhodamine by the multidrug transporter P-gp1, is relatively easy to assay in mammalian cell culture systems. The MDR3 protein shares 77% identity with P-gp1 and it has been shown that substitution of as few as three adjacent MDR3 protein residues in the first transmembrane domain by the equivalent residues from P-gp1 is sufficient to allow the chimeric molecule to transport substrates specific to P-gp1 (Zhou et al. 1999). The level of sequence identity between P-gp1 and the MDR3 protein increases to 87% in the NBDs. In addition, the NBDs of P-gp1 and the MDR3 protein, have been shown to be interchangeable (Buschman et al. 1991). We therefore introduced the equivalent A546D mutation into the first NBD of P-gp1 and studied the functional consequences of the change in transiently-transfected mammalian cells.

Alignment of the amino acid sequence of human P-gp1 with the MDR3 protein identified A544 as the P-gp1 equivalent of MDR3 protein A546. Site directed mutagenesis was used to alter codon 544 of *MDR1*, to encode an aspartic acid. The mutated fragment was subcloned into the plasmid pMDR1-wt to generate pMDR1-A544D, and used to transiently-transfect human epithelial kidney (HEK293T) cells.

Fluorescence activated cell sorting (FACS) of live cells was used to correlate the ability of transiently–transfected cells to extrude a fluorescent substrate of P-gp1, rhodamine 123 (R123). The presence of P-gp1 at the cell surface in the same batch of transfected cells was determined by the P-gp1-specific monoclonal antibody (mAb) UIC2 conjugated to phycoerythrin (UIC2-PE).

Cells transiently-transfected with pCIneo-βgal which does not encode P-gp1, all

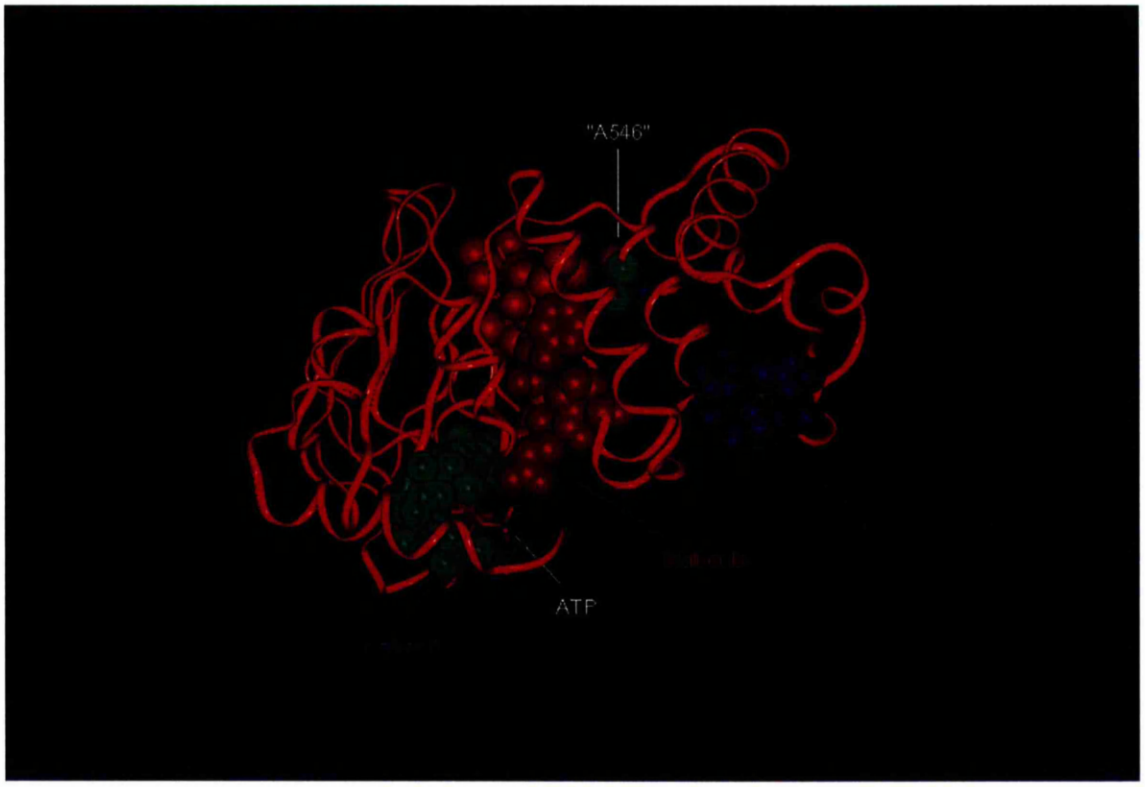


Figure 11. This figure shows a ribbon representation of the α-carbon backbone from the crystal structure of HisP (Hung et al. 1998), the NBD from the histidine permease of *Salmonella Typhimurium.* The side chains of A167 (the equivalent of A546 in MDR3 protein) (gold), the Walker A motif (green) and Walker B motif (the four hydrophobic residues at the amino-terminal end are shown in red and the carboxy-terminal residues in purple) and the ABC signature (blue) are shown space filled. The bound ATP is shown as ball and stick.

behaved in a similar way and accumulated R123 and failed to label with UIC2-PE (Figure 12A). In contrast, cells transiently-transfected with pMDR1-A544D, formed two distinct populations. The first population behaved similarly to the control cells with no UIC2-PE fluorescence and high R123 fluorescence (Figure 12B, lower right quadrant), consistent with failure to express A544D-P-gp1 at the cell surface (and thus accumulate R123). The second cell population was characterised by high UIC2-PE

fluorescence and no R123 fluorescence (Figure 12B, upper left quadrant) consistent with cell surface expression of A544D-P-gp1 and extrusion of R123. Further confirmation that the extrusion of R123 in the A544D-P-gp1-expressing cells is due to the function of the mutant P-gp was obtained by incubating the cells with the P-gp1-inhibitor cyclosporin A. Under these conditions the cells with a high UIC2-PE fluorescence accumulated high levels of R123 fluorescence (Figure 12C, upper right quadrant). These data are typical of cells expressing functional P-gp (Blott et al. 1999) and indicate that the A544D-P-gp1 mutant is functional if it reaches the cell membrane.

3.3.5 Evidence that A544D-P-gp1 is a trafficking mutant

The finding that substitution of an aspartic acid for an alanine in a highly conserved region of NBD1 did not alter the ability of the mature protein to transport R123 was unexpected and suggested that the equivalent A546D-MDR3 protein mutant would also be functional. However, the FACS analysis provided evidence that the mutant protein is inefficiently expressed when compared to the wild-type protein (Table 10). When transiently-transfected with pMDR1-wt, 97% of HEK293T cells expressed wild-type P-gp1 at the cell surface (compare the UIC2-PE labelling of intact cells transfected with pCIneo-βgal or pMDR1-wt; Figure 13). There were two populations of pMDR1-wt-transfected cells which differed by the amount of wild-type P-gp1 at the cell surface (Figure 13). The first population (M1) had a mean level of UIC2-PE fluorescence of 38 and the second population (M2) had a mean of 200. The overall mean level of fluorescence was 171, compared with a control value of 3. We suspect that those cells which have a lower level of P-gp1 at the cell surface (M1) took up the DNA shortly after mitosis and that this resulted in a lag phase of one cell cycle until

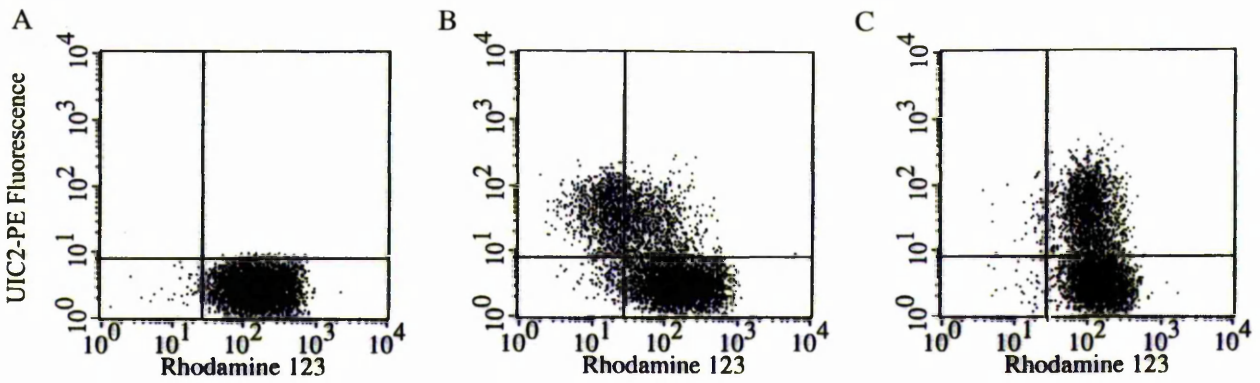


Figure 12. FACS analysis of HEK293T cells transiently-transfected with pCIneo-βgal (A), or pMDR1-A544D (B and C). Cells were incubated first with the P-gp1 substrate R123 in the presence (C) or absence (A and B) of the P-gp inhibitor CsA. The cells were then incubated with the P-gp1-specific mAb (UIC2-PE) before FACS.

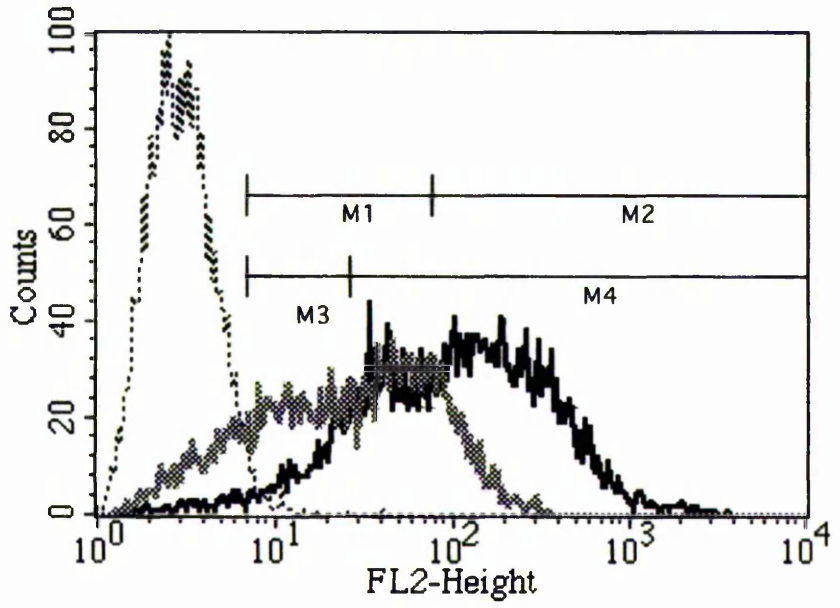


Figure 13. UIC2-PE antibody labelling of intact HEK293T cells transiently-transfected with pCIneo-βgal (grey intermittent line), pMDR1-wt (black) or pMDR1-A544D (grey). The transfected populations were observed to consist of two separate populations (M1 and M2 for pMDR1-wt, M3 and M4 for pMDR1-A544D) with different amounts of protein at the cell surface.

Table 10. Ratio of intracellular to cell-surface P-glycoprotein.

Mean fluorescence (relative arbitrary fluorescence units)	**A544D-P-gp1**	**Wt-P-gp1**	**HEK293T**
Intact cells	40	171	3
F&P then labelled with UIC2-PE	197	497	13
Labelled with UIC2-PE then F&P	32	144	3
Intracellular P-gp	*155*	*343*	-
Cell surface P-gp	*37*	*168*	-
Total P-gp	*192*	*511*	-

Summary of the mean observed fluorescence of intact cells (Figure 13), and of the mean fluorescence following fixation and permeabilisation (F&P) either before or after P-gp1-specific mAb UIC2-PE labelling (Figure 15). This allowed the intracellular, cell surface and total P-gp of wt-P-gp1 and A544D to be calculated (calculated values are shown in intalics).

the nuclear membrane dissolved to permit entry into the nucleus and expression of the *MDR1* gene. Effectively, these cells have had a delayed transfection time and so may have only had 24hrs for gene expression from the introduced plasmid; insufficient time to accumulate maximal levels of wild-type P-gp1 at the cell surface. The population of cells that express high levels of P-gp1 at the cell surface (M2) probably entered mitosis soon after the introduction of the pMDR1-wt DNA and so have effectively had double the expression time.

Cells transfected with pMDR1-A544D also formed two populations (Figure 13); the first population (M3) had a mean level of UIC2-PE fluorescence of 10 (barely above the mean (3) of the negative control cells, and the second population (M4) had a mean of 50. The overall mean fluorescence level was 40. These data indicate that the mutant protein is expressed at the cell surface at much lower levels than the wild-type protein (Table 10).

3.3.6 Western blot analysis of A544D-P-gp1

Transiently-transfected cells normally express two forms of P-gp1, discernible by polyacrylamide gel electrophoresis (PAGE) and western analysis: immature, non- or core-glycosylated P-gp1 and mature glycosylated P-gp1. All of the immature P-gp1 is found in intracellular compartments (Loo et al. 1995), and probably represents nascent polypeptide in the endoplasmic reticulum (ER). The glycosylation of the first extracellular loop of P-gp1 is completed in the distal cisternae of the Golgi apparatus. If the A544D mutation impairs the trafficking of the protein from the ER to the cell membrane then it might be expected to alter the ratio of immature to mature protein found in the cell. PAGE and western analysis (Figure 14) showed that wild-type P-gp1 was expressed at higher levels than A544D-P-gp1 and was predominantly present as the 170kDa mature, glycosylated form. In contrast, the A544D-P-gp1 was predominantly found in the 140kDa immature form. Reprobing of the blot with anti-actin mAb indicated that the same amount of cell protein was added to each lane (Figure 14).

3.3.7 The ratio of cell surface to intracellular P-gp1 is also altered in the A544D mutant

Additional evidence that A544D-P-gp1 is a trafficking mutant was provided by calculating the ratio of cell surface to intracellular P-gp1 by FACS analysis (Figure 15 and Table 10). Cells which were fixed with paraformaldehyde and permeabilised with saponin prior to incubation with antibody, provided an indication of the total P-gp in the cell (Figure 15, black trace). The intracellular P-gp fraction was then determined by subtraction from this figure of the cell surface P-gp determined by incubation of

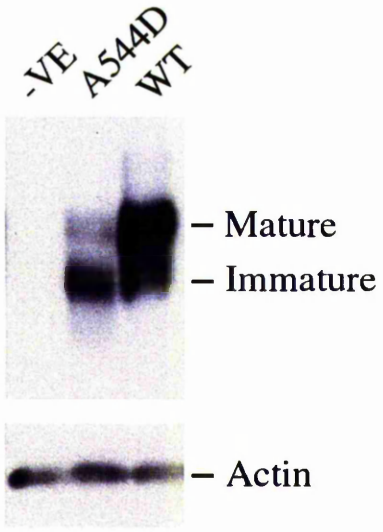


Figure 14. Western analysis of HEK293T cells transiently-transfected with pCIneo-βgal, pMDR1-A544D and pMDR1-wt. The blot was probed with anti-P-gp mAb, C219, and the 170kDa mature, glycosylated form and the 140kDa immature form of the protein are indicated. The blot was reprobed with anti-actin mAb to confirm that the same amount of protein (20μg) was added to each lane.

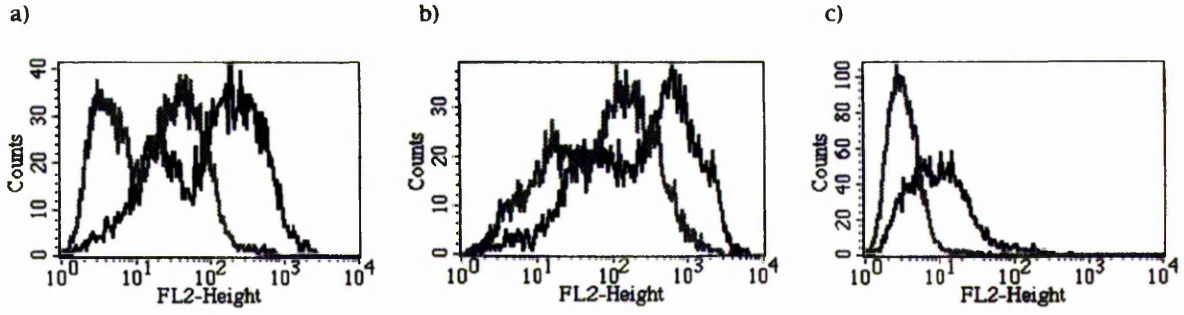


Figure 15. FACS analysis of cells transiently-transfected with pMDR1-A544D (A), pMDR1-wt (B) or pCIneo-βgal (C). Fluorescence from cells which have been fixed with paraformaldehyde and permeabilised with saponin, prior to incubation with antibody (UIC2-PE) is shown in black, and from cells incubated with antibody before fixation and permeabilisation shown in grey. The mean fluorescence for each population of cells is given in Table 10.

live cells with antibody before fixation and permeabilisation and FACS (Figure 15, grey trace); this controlled for the possible loss of cell surface antibody-antigen conjugate during the fixation and permeabilisation. It was also necessary to subtract the antibody bound non-specifically to cells as this fraction changes when intracellular antigens are exposed (compare black and grey traces of Figure 15). These data, obtained for cells expressing wild-type P-gp1 and for cells expressing the A544D mutant, are summarised along with the background levels of fluorescence in Table 10. Calculation of total P-gp1 level and the ratio of intracellular to cell surface P-gp clearly showed that cells transfected with pMDR1-A544D expressed a lower level P-gp1 than cells transfected with pMDR1-wt (total fluorescence 192 and 511, respectively), and that much less of it got to the cell surface (only 19% of the total made, compared with 33% of wild-type protein). The western analysis of cells expressing wt-P-gp1 demonstrated that there is more of the mature, glycosylated form than of the immature form of the protein. When considered together, these results show that not all of the mature protein is at the cell surface.

These results show that the *MDR3* mutation A546D causes abnormal protein glycosylation and trafficking when its equivalent is expressed in P-gp1. This is the first report of an *MDR3* mutation in OC without a family history of PFIC, and these are the only functional studies of a clinically important MDR3 mutation to date.

3.4 Discussion

Sequence analysis of the coding region of the *MDR3* gene in eight patients with OC and raised GGT revealed the presence of a mutation in exon 14 (A546D) in one individual. This mutation was not found in 50 parous controls without OC, nor in 40 OC women without a raised GGT, indicating that it is not a common polymorphism.

The location of this mutation within the highly conserved first NBD indicates that this is not likely to be a benign alteration, and the experiments reported in this chapter provide data showing that it interferes with protein trafficking. Thus OC without PFIC can be caused by mutations in *MDR3*. A mutation in *MDR3* was found in 1 of 8 women; this could be because other mutations are in untranslated regions, or in regulatory regions of the gene which were not screened in this study.

To date, only 7 other mutations of the *MDR3* gene have been published, all either in individuals with a family history of PFIC3 (de Vree et al.1998; Jacquemin 1999; 2001) or with an atypical form of cholelithiasis (Rosmorduc et al. 2001) (section 1.2.6). These mutations are believed to cause loss-of-function, as the majority (6/7) introduce premature stop codons. The occurrence of the A546D mutation, the substitution of a hydrophobic amino acid with a charged polar amino acid, in the highly conserved first NBD, suggested that this mutation would also result in loss-of-function of the MDR3 protein. In order to gain insight into the functional consequences of this mutation, we tested the effects of the equivalent mutation to A546D in P-gp1 (A544D). Transient expression of A544D-P-gp1 in HEK293T cells, and subsequent FACS and western analysis suggested that this mutation reduces the abundance of the protein and impairs protein trafficking to the cell membrane. This probably arises as a consequence of misfolding of the first NBD leading to retention of the protein in the ER and an increase in the rate of degradation. The A546D mutation in the MDR3 protein is likely to have a similar phenotype as there is considerable sequence identity between the proteins in this domain (87%), and because the NBDs of P-gp1 and the MDR3 protein have been shown to be functionally interchangeable (Buschman et al. 1991).

It is currently not clear whether *MDR3* mutations play a role in the aetiology of OC in a large proportion of cases. It is unlikely that coding sequence mutations contribute to many, as none were detected in any of the other patients in this series. Also, only 20-30% of cases of OC are associated with a raised GGT (Milkiewicz et al. 2001), and it is likely that other genes play a greater role in the aetiology of the condition in cases without a raised GGT. However, as OC has a complex aetiology, it is possible that more than one mutation contributes to the aetiology. Therefore DNA sequence variants in either the promoter or coding sequence of the gene may confer susceptibility to OC when co-inherited with other mutations that predispose women to developing the condition. We aim to collect a larger DNA resource from women with OC to allow a more extensive genome wide screen for susceptibility genes and loci.

A number of missense mutations have been identified in other genes that result in trafficking defects, including the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (Cheng et al. 1990; Smit et al. 1995), which encodes the CFTR protein, another member of the ABC transporter superfamily, and genes encoding the Wilson disease and Menkes disease proteins, both of which are P-type ATPases (Ambrosini et al. 1999; Payne et al. 1998). Such mutations in membrane transport proteins commonly result in reduced function in addition to abnormal protein trafficking (Ambrosini et al. 1999; Payne et al. 1998; Drumm et al. 1991). However, a disease-causing mutation has been reported in *CFTR* which is associated with protein mislocalisation, but normal CFTR function (Smit et al. 1995). The *MDR3* mutation we have identified may be similar, resulting in an increased proportion of mislocalised protein, but normal MDR3 protein function if it reaches the cell membrane. The results of our functional studies in P-gp1support this hypothesis.

Western and FACS analysis suggests that cells transiently-transfected with pMDR1-wt produce more of the mature, glycosylated form of wt-P-gp1, but that the majority of this is not localised at the cell surface. There is *in-vitro* evidence for the existence of cytoplasmic reservoirs of bile acid transporters in intracellular vesicles which are recruited to the hepatocyte canalicular membrane, resulting in increased bile acid transport (Boyer et al. 1995). This mechanism has not been specifically investigated in P-gp1, but it is possible that mature P-gp1 also resides in the Golgi apparatus and recycles to the cell surface. It has also been demonstrated that the mature Wilson disease protein, and the Menkes disease protein reside in the trans-Golgi apparatus and recycle to the cell surface when copper transport is required (Ambrosini et al. 1999; Payne et al. 1998).

The short term administration of oral oestrogens to women with a history of OC causes pruritus and abnormal LFTs (see sections 1.2.6 and 2.4.2), suggesting that predisposed women develop OC due to the cholestatic effect of raised oestrogens in pregnancy. Progestogens may also play a role (Bacq et al. 1995; 1997). Progestogens and oestrogens can inhibit MDR-catalysed drug efflux and interact with P-gp (Taylor et al. 1999). It is therefore possible that raised serum oestrogens and progestogens in normal pregnancy cause reduced function of the MDR3 protein, and that women who are heterozygotes for a trafficking mutation in the MDR3 protein have a reduced ability to compensate for this.

While the cause of fetal distress and intrauterine death in OC is not fully understood, some studies have reported a more frequent occurrence of fetal distress in cases with high maternal (Laatikainen et al. 1984) or fetal (Laatikainen et al. 1975c) bile acids levels. Loss of function of the MDR3 protein results in raised serum bile acids as a secondary effect, as is seen in children with PFIC3 (de Vree et al. 1998) and their

heterozygote mothers with OC (Jacquemin et al. 1999; 2001). Thus, *MDR3* mutations which result in raised serum bile acids, such as the one reported in this study, may predispose to fetal distress and subsequent unexplained intrauterine death.

Therapeutic interventions to improve protein trafficking are under investigation for the ΔF508 mutation in CFTR (Brown et al. 1997), and this approach may be useful for the treatment of protein trafficking mutations in OC.

In conclusion, we have demonstrated that OC in UK Caucasians has a genetic aetiology, and have demonstrated heterozygous missense mutation in the *MDR3* gene in a woman with OC and no family history of PFIC. Our analysis of this mutation in P-gp1 suggests that it results in abnormal protein trafficking and a subsequent lack of functional protein at the cell surface. These results confirm that *MDR3* mutations are responsible for OC in some women with a raised GGT. Identification and characterisation of further mutations in this gene will greatly increase our understanding of the role of *MDR3* in this subgroup of women with OC.

4. STUDY OF THE EFFECT OF THE PRIMARY BILE ACID TAUROCHOLATE ON RAT CARDIOMYOCYTES: A MODEL OF THE FETAL HEART IN OBSTETRIC CHOLESTASIS

4.1 Background and aims

The studies in chapter 3 demonstrate that OC can be caused by a loss-of-function mutation in a hepatic transporter that influences biliary bile acid transport. It is likely that a substantial proportion of cases of OC are caused by loss of function mutations in *MDR3* or other genes that influence bile acid transport. If this is the case, the fetus in an OC pregnancy has a 50% chance of inheriting this mutation, and this in turn may predispose the fetus to raised serum bile acids. This hypothesis is consistent with the results of two studies that demonstrated that although fetal serum bile acids are raised in OC, they are always lower than the corresponding maternal levels. In 50% of cases the levels were similar to the maternal serum bile acid level, while in 50% of cases they were considerably lower (Laatikainen 1975c, Shaw et al. 1982) (see section 1.2.7 and Figure 4). The hypothesis is also consistent with the results of the large studies of the prevalence of fetal distress in OC, i.e. rate of meconium staining 15-45% (section 1.2.3, Table 2).

Because the intrauterine death in OC occurs suddenly, and because an abnormal fetal heart rate ($\leq$ 100 or $\geq$ 180 beats/ minute) has been demonstrated in three studies (Laatikainen et al. 1984, Laatikainen 1975c; Reid et al. 1976), the studies in this chapter investigated the hypothesis that raised bile acids cause impaired fetal cardiomyocyte function resulting in fetal cardiac arrest. The protective effect of the therapeutic agents UDCA and dexamethasone were also studied.

4.1.1 Rat cardiomyocytes as a model of the fetal heart

It is not possible to investigate the effects of bile acids on the intact human fetal heart at a cellular level. Therefore the closest available model of fetal myocardium at term was used; a primary culture of neonatal rat cardiomyocytes in which cells beat synchronously (Kimura et al. 1995) and develop pacemaker activity (Soen et al. 1999).

Primary culture of neonatal rat myocytes as a model to investigate cardiac rate and ryhthm

Neonatal rat cardiomyocytes provide a unique *in-vitro* model for studying the rhythm, intracellular calcium (Ca^{2+}) dynamics and cell to cell interactions of cardiomyocytes (Husse et al. 1996, Barrigon et al. 1996, Hori 1992). Cardiomyocytes can be grown as primary cultures of single cells, each with its own independent rate of contraction, or as a network of cells that beat synchronously. When cultured as a network, the early culture consists of single myocytes which exhibit an unsynchronized beating pattern. Later, when any two spontaneously beating neonatal myocytes establish contact, they synchronize. Intercellular coupling via gap junction formation is crucial for this synchronization (Jongsma et al. 1987). Subsequently, the cells proliferate, migrate, and assemble into a monolayer network that beats synchronously (Soen et al. 1999).

It has been demonstrated that laser light can initiate reversible and irreversible cell damage (Furukawa et al. 1996), and this in turn can result in impaired synchronisation of contraction between cardiomyocytes within the network. The addition to cultured rat cardiomyocytes of ryanodine results in an increase in the cytosolic calcium concentration and a decrease in the amplitude of the Ca^{2+} transients. Similar effects were seen following addition of thapsigargin, an inhibitor of sarcoplasmic reticulum Ca^{2+}-ATPase (Husse et al. 1996). Taurine-conjugated bile acids have been shown to

act as Ca^{2+} ionophores, causing a dose-dependent increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Zimniak et al. 1991; Thibault et al. 1993). This is a result of transient permeabilisation of the endoplasmic reticulum to Ca^{2+}. To date, this effect has only been demonstrated in hepatocytes, and was not seen in platelets nor in a neuroblastoma cell line, both of which do not have specific bile acid transporters in the cell membrane (Thibault et al. 1993; Coquil et al. 1991). However, this led us to hypothesise that in OC pregnancies bile acids cause fetal cardiomyocyte damage and/ or raised intracellular Ca^{2+}, and that this may result in a fatal fetal dysrhythmia.

We therefore investigated the effects of the addition of the primary bile acid, taurocholate, to the calcium dynamics of primary cultures of neonatal rat cardiomyocytes. Taurocholate (TC) is the tauro-conjugate of cholic acid, the main primary bile acid that is raised in OC.

In summary, the aims of the studies reported in this chapter were to establish whether addition of TC to cardiomyocyte cultures resulted in altered rate or rhythm of contraction, or abnormal calcium dynamics.

4.2 Methods

The study protocol is summarised in a flow diagram (Figure 16).

4.2.1 Preparation of primary cultures of rat cardiomyoctes

Heart cells were obtained by Dr P Sugden at the National Heart and Lung Institute as described by Iwaki et al. (1990), and provided for use in these experiments on a collaborative basis. Cells were kept in a DMEM (Dulbecco's modified Eagle's minimum essential medium) (Gibco, Paisley, UK) with 5% fetal calf serum (Gibco, Paisley, UK), 1% streptomycin, 1% penicillin (Gibco, Paisley, UK), 5% NEAA (Non

Essential Amino Acids) (Gibco, Paisley, UK). Geniticin (Gibco, Paisley, UK) 100 μg/ml G418 was added to inhibit fibroblast growth. Cells were maintained at 37°C, in an atmosphere of humidified air plus 5% CO_2. Cells were used at 2 days (discrete cells) and 5 days (network of cells) after plating. The cardiomyocytes were cultured on Petri dishes or glass coverslips.

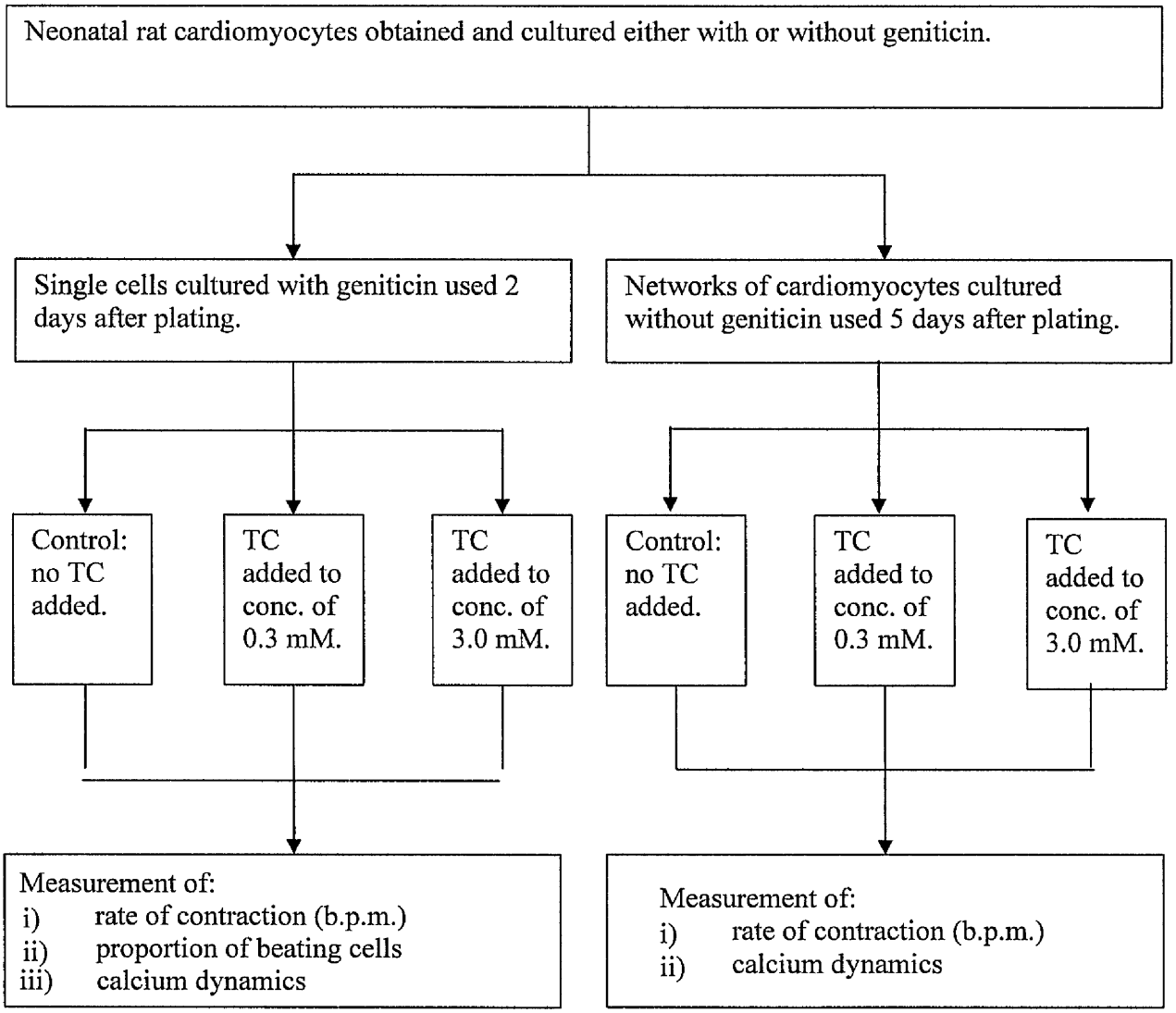


Figure 16. Flow diagram to summarise the study protocol for the cardiomyocyte experiments.

4.2.2 Investigation of the effect of TC on the rate of contraction and on the proportion of contracting cardiomyocytes

TC (Sigma, UK) was added to give a final concentration of 0.3 mM or 3.0 mM in the culture medium of single cardiomyocytes, and of the network of synchronously beating cells.

For cultures of single cells, the rate of contraction was calculated for 15 seconds in individual cells and expressed as beats per minute (b.p.m.). All measurements were made by a single observer to avoid operator bias. For the network of cardiomyocytes, the rate of contraction was measured in different parts of the network in the same way as for single cardiomyocytes. There were 3 and 4 separate cultures used for the single cell and network experiments respectively.

For cultures of single cells the percentage of contracting cardiomyocytes was calculated using separate groups of 25 individual cells. All measurements were made by a single observer.

The observer for the experiment described in this section was Dr Julia Gorelik.

4.2.3 Investigation of the effect of TC on cardiomyocyte cell death

To establish whether cells were living or dead fluorescein diacetate/ propidium bromide (Sigma) stain at a final concentration of 100 ng/ml was added to the cardiomyocyte cultures 3 minutes after TC administration (Miyamoto et al. 1999). The fluorescence of the cells was measured to determine whether cells were alive, indicated by a green colour, or dead, indicated by the nucleus staining red.

4.2.4 Calcium wave dynamics

The cardiomyocytes were loaded with the visible wavelength fluo-3 Ca^{2+} indicator by cell incubation with the esterified derivative of fluo-3 (fluo-3 acetoxymethyl, (Molecular probes)) in a medium containing equal volumes of Leibovitz's L-15 (Gibco) and HBSS (Hank's balanced salt solution) buffer (Gibco) at room temperature for 15 mins. Then cells were re-washed five times with the medium, followed by a postincubation period of 20 minutes to allow for complete intracellular dye cleavage (Lopez 1995, Williams 1992).

Scanning laser confocal microscopy was used to study the Ca^{2+} dynamics in the cardiac myocyte network. Scanning ion conductance microscopy (SICM) was developed by Dr Yuri Korchev with whom Dr Williamson collaborated to perform these studies. This was because we wanted to gain maximum information on cell rhythm and synchronisation while introducing minimum laser irradiation to the sample. SICM has a unique high resolution (< 50 nm) and enables direct visualization of living cells (Korchev et al., 2000). The images captured by SICM are similar to those produced by scanning electron microscopy with the significant difference that the cells remain viable and active. Scanning was performed, so that several Ca^{2+} elevations were visible in the adjacent cells in a single scan, allowing monitoring of the cell synchronization and the rhythm of Ca^{2+} wave propagation. The scanning laser confocal microscopy protocol has been described by Korchev et al. (2000). The low power (<0.05mW) laser beam rapidly scans the conglomerate of cells, chronologically, from the bottom up, allowing the Ca^{2+} transients to be recorded as bright horizontal bands. If the bands traverse all the cells in the field, and the time distances between horizontal bands are equal, this indicates synchronous rhythmic

beating of cells and adequate cell-to-cell conduction in the network (see results in section 4.3.4).

4.2.5 Investigation of the effect of preincubation with UDCA or dexamethasone on contraction and calcium dynamics

Cells were pre-incubated overnight with either 0.1 mM UDCA (Sigma-Aldrich, Dorset, UK) or 80 and 800 nM dexamethasone (David Bull Laboratories, Warwick, UK). The rate of contraction and SICM were performed as described in sections 4.2.2 and 4.2.4. In experiments where the therapeutic agents protected the cardiomyocytes from the effect of 3.0 mM TC, the higher dose of 4.5 mM was used. In the UDCA experiments the additional dose of 1.0 mM TC was used as UDCA protected cardiomyocytes from 0.3 mM TC, but not from 3.0 mM TC. There were 3 separate experiments for each condition.

4.2.6 Statistical analysis

Results are expressed as mean and standard deviation (S.D.). Comparisons were made using the unpaired *t*-test. The analysis was performed using the InStat statistics package (GraphPad Software).

The experimental work described in this chapter of this thesis was performed by Dr Julia Gorelik, but the experiments were designed by Dr Williamson and the results were analysed by Dr Williamson and Dr Gorelik together.

4.3 Results

4.3.1 Effect of TC on the rate of contraction and on the proportion of beating cardiomyocytes when cultured as single cells

Addition of TC to give a concentration of 0.3 mM in the culture medium of single cardiomyocytes caused a reduction in the mean rate of contraction after one hour, from 68.7 (± 5.7 b.p.m.) (mean (± S.D.)) to 31.4 (± 5.3 b.p.m) ($p < 0.0001$). The rate recovered to 58.0 b.p.m. (± 8.6) one hour after transfer to TC-free medium. A similar reduction in the rate of contraction was seen following the addition of TC to give a

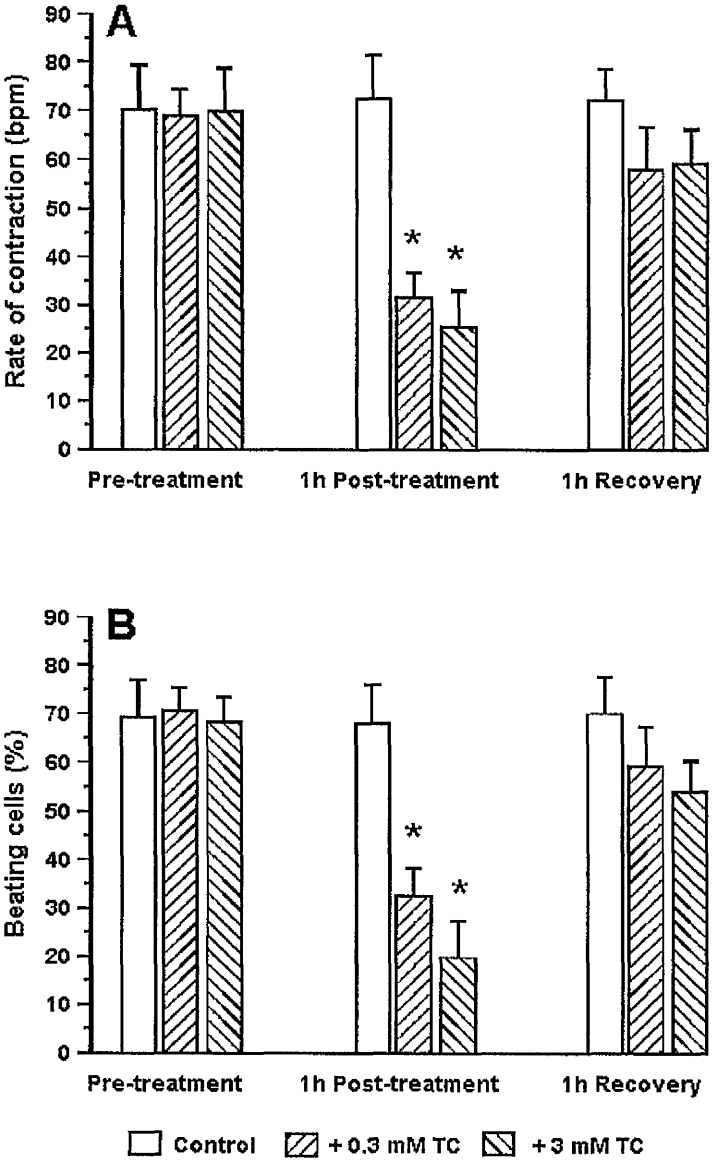


Figure 17. Effect of taurocholate (TC) on (A) the rate of contraction (beats per minute (b.p.m.)); and (B) the proportion, as a percent, of actively beating cardiomyocytes when cultured as single cells. Results are expressed as mean and standard deviation.

concentration of 3 mM; i.e. from 69.8 b.p.m. (± 8.9) to 25.4 b.p.m. (± 7.6) ($p < 0.0001$) and recovery to 59.2 b.p.m. (± 7.0) (Figure 17).

TC also affected the proportion of cells that demonstrated contractile activity. A concentration of 0.3 mM TC in the culture medium of single cardiomyocytes resulted in a reduction in the percentage of beating cells from 70.5% to 32.6% ($p < 0.0001$). This returned to 59.2% following transfer of the cells to TC-free medium. A similar but a greater reduction was seen with 3 mM TC (i.e. from 68.3% to 19.8% ($p < 0.0001$), and recovery to 54.2%) (Figure 17).

4.3.2 Effect of TC on the rate of contraction and integrity of networks of cardiomyocytes

A concentration of 0.3 mM TC in the culture medium of networks of cardiomyocytes caused a similar reduction in the rate of contraction to that seen with single cells. These changes were not reversible after transfer to TC-free medium. The higher concentration (3 mM TC) resulted in destruction of the integrity of the network, and cells ceased to beat synchronously. Transfer to TC-free medium resulted in recovery of the integrity of the network, but cells had a reduced rate of contraction. These changes are summarised in Table 11.

4.3.3 TC effects on cardiomyocytes do not cause cell death

The addition of TC to the culture medium of cardiomyoctyes when cultured either as single cells or as a network did not cause cell death. This was indicated by the reversible cessation of beating seen in the majority of cardiomyocytes following

Table 11. Effect of taurocholate (TC) on the rate of contraction of cardiomyoctes cultured as a network.

	Rate of Contraction (beats per minute)		
	Before adding TC	1h exposure to TC	1-2 h TC-free
Control	102.9 ± 6.7	104 ± 7.2	102.5 ± 8.0
+ 0.3 mM TC	103.2 ± 13	55.3 ± 3.5*	54.7 ± 2.0*
+ 3.0 mM TC	105.3 ± 11.8	L.I.	54.2 ± 5.5*

Values are Mean ± SD. L.I = Loss of integrity of the network. * = $p < 0.001$ when compared with control data.

transfer of cells to TC-free medium (Figure 17). Cells that did not recover contractile function were shown to be alive by the administration of fluorescein diacetate/ propidium bromide stain. No red staining was observed, consistent with TC not causing cardiomyocyte death.

4.3.4 Effect of TC on calcium wave dynamics

Addition of TC to single cells initially caused an increase in the frequency of Ca^{2+} waves. After approximately 6 minutes this was followed by either a decrease in wave frequency, or by Ca^{2+} overload. These changes are consistent with a reduced frequency of Ca^{2+} transients and were associated with a reduction in the rate of contraction and cessation of beating, as was observed 1 hour after addition of TC (Figure 17).

When TC was added to the network, the altered Ca^{2+} dynamics were different in adjacent cells, resulting in asynchronous beating. The intracellular Ca^{2+} dynamics of a 5 day old culture of newborn rat cardiomyocytes, after cell loading with Fluo-3, are shown in Figure 18. Standard bright-field microscopy revealed synchronous rhythmic contraction of the network of cells, and this was confirmed by the synchronous Ca^{2+}

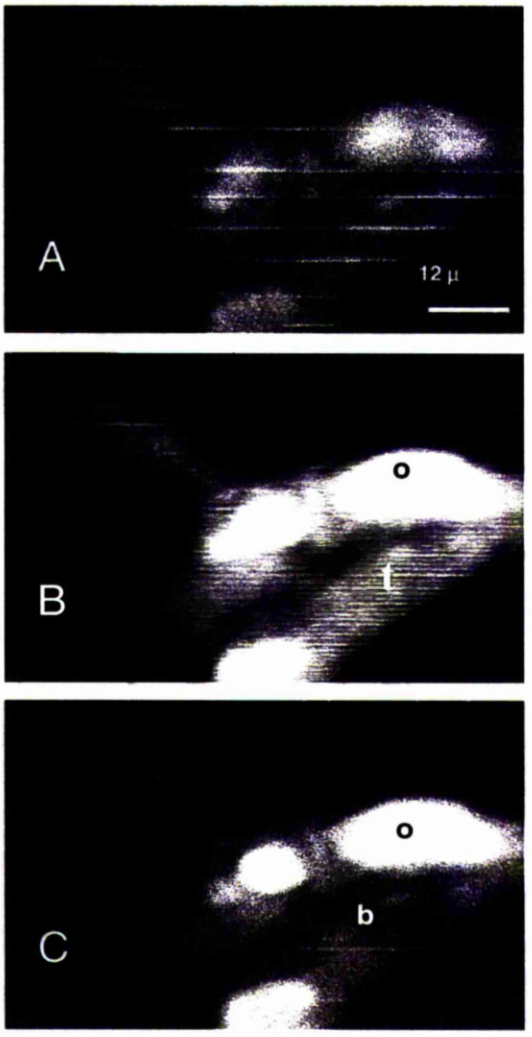


Figure 18. Effect of TC on calcium dynamics in cardiomyocytes. (A) Network of cardiomyocytes prior to the addition of TC to the culture medium. Horizontal lines represent calcium waves which extend across adjacent cells, consistent with synchronous beating. The spacing between the lines provides an indication of frequency. (B) The addition of 0.3 mM TC alters calcium wave dynamics, consistent with tachycardia – closely spaced lines (t), and with calcium overload with loss of contraction (O). The network of cells is no longer beating synchronously. (C) 6 minutes after tachycardia, bradycardia (b) and overload (O) are seen. There is no synchronous contraction. Each scan took 20 seconds to acquire.

transients throughout the cluster of cardiomyocytes. The Ca^{2+} transients were observed using SICM (see section 4.2.4).

A concentration of 0.3 mM TC in the culture medium resulted in altered Ca^{2+} wave dynamics, with an increased Ca^{2+} transient rate, and with Ca^{2+} overload and loss of contraction (Figure 18). The network of cells is no longer beating synchronously. Six minutes after the changes shown in Figure 18, bradycardia and overload were seen (Figure 18). Thus, a concentration of 0.3 mM TC in the culture medium resulted in altered intracellular Ca^{2+} dynamics and loss of synchronous contraction within this small cluster of cardiomyocytes. Similar changes were seen following the addition of TC to give a concentration of 3 mM.

4.3.5 Effect of preincubation of cardiomyocytes with UDCA or dexamethasone prior to addition of TC to culture medium.

Cells were pre-incubated overnight with either 0.1 mM UDCA or with either 80 nM or 800 nM dexamethasone and TC was then added to the culture medium. With dexamethasone or UDCA alone under standard bright-field microscopy cells displayed synchronous beating. After cell loading with calcium indicator fluo-4 synchronous calcium waves were observed that spanned the entire network of cells (Fig 19A; 20A), i.e. the cells were coupled. When TC was added to the culture medium of cells that had been pre-incubated with dexamethasone to give a final TC concentration ranging from 0.05 mM to 3 mM (the concentration that has been previously reported to cause severe disruption of cardiomyocyte contraction) there was no change in the rate or synchronisation of contraction (Figure 19B) (four experiments). Only when exposed to the higher dose of 4.5 mM TC did the cells show contractile irregularities (Figure 19C). Neighbouring cells had different patterns of

calcium wave propagation, consistent with abnormal cell-cell communication. As soon as the medium was changed to TC-free medium, the cells displayed signs of recovery. Although the rate of contraction did not return to that seen prior to TC addition, it became regular and calcium waves showed intercellular coupling (Figure 19D).

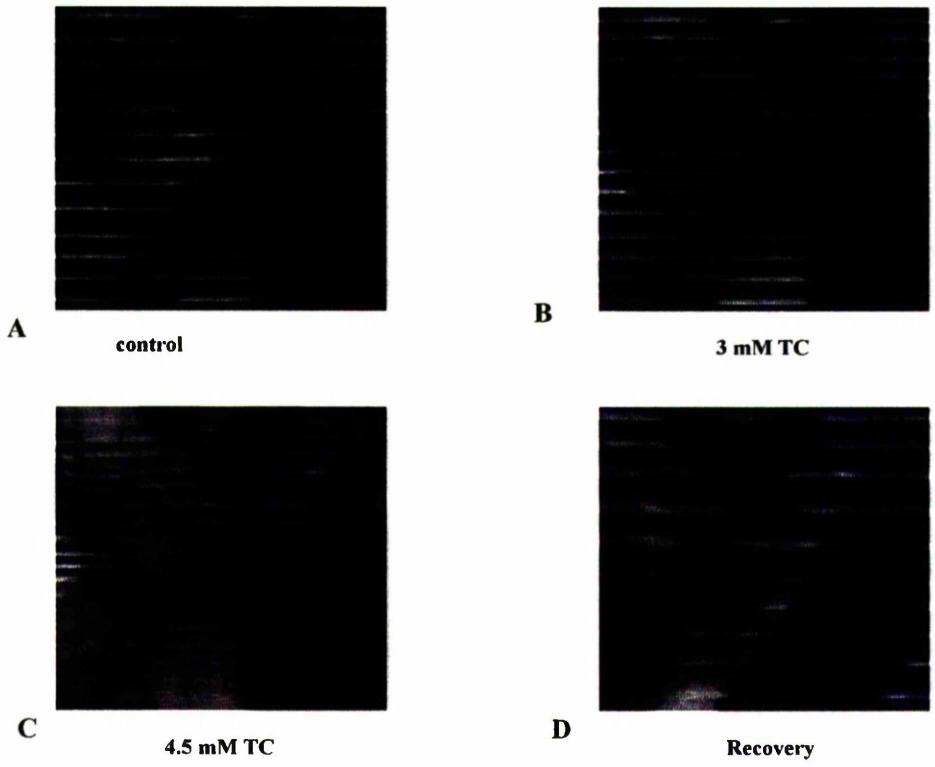


Figure 19. Effect of TC on calcium dynamics in cardiomyocytes pre-treated with dexamethasone. (A) The cardiomyocyte network was incubated for 16 hours with 800nM dexamethasone. Horizontal lines represent calcium waves which extend across adjacent cells, consistent with synchronous beating. The spacing between the lines provides an indication of frequency. (B) The addition of TC up to a concentration of 3 mM does not alter calcium dynamics. (C) Addition of TC to a concentration of 4.5 mM alters calcium dynamics. (D) Restoration of rhythmic contraction after transfer to TC-free medium.

Preincubation with UDCA protected cells from the effects of TC at concentrations ranging from 0.1 mM to 0.3 mM TC (Figure 20B) but a concentration of 3.0 mM TC produced fibrillation and loss of synchronous contraction (Figure 20C) and subsequent irreversible cellular overload with calcium (Figure 20D) (four experiments).

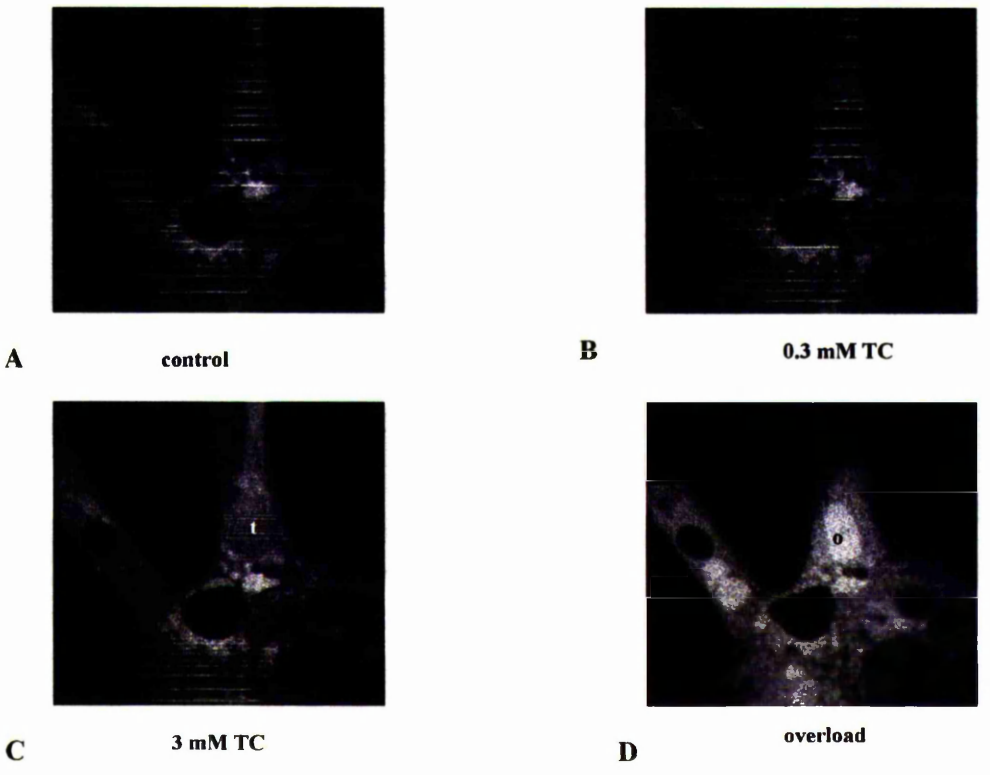


Figure 20. Effect of TC on calcium dynamics in cardiomyocytes pre-treated with UDCA. (A) Network of cardiomyocytes incubated for 16 hours with 0.1mM UDCA. (B) The addition of 0.3 mM TC does not alter calcium waves .(C) The addition of 3 mM TC alters calcium wave dynamics, consistent with tachycardia – closely spaced lines (t), (D) this did not return to normal following transfer to TC-free medium, and calcium overload (o) was seen with cessation of beating.

The protective effect of dexamethasone was dependent on both the dose of TC added and the concentration of dexamethasone in the preincubation medium (Table 12). Pre-incubation with 80 nM dexamethasone protected cells from the effect of 3.0 mM TC, but not from the higher dose of 4.5 mM. After transfer to TC-free medium the cells recovered but had a slower rate of contraction than before TC treatment. Addition of

Table 12. Effect of addition of dexamethasone (80 nM or 800 nM) to the culture medium, and overnight incubation, of cardiomyocytes prior to addition of 3.0 or 4.5 mM taurocholate. Rate of contraction is shown as beats per minute, LI = loss of integrity, TC = taurocholate.

10 minutes treatment

	No dexamethasone	**80 nM dexamethasone**	**800 nM dexamethasone**
Control	105.7±7.9	99.6±9.2	104.1+-13.0
3.0 mM TC	LI	88.2±1.5	96.5+-11.3
4.5 mM TC	LI	LI	62.1+-8.7

1 hour treatment

	No dexamethasone	**80 nM dexamethasone**	**800 nM dexamethasone**
Control	105.5±7.6	96.6+-8.4	100.2+-8.0
3.0 mM TC	LI	92.2+-12.8	96.8+-9.7
4.5 mM TC	LI	LI	61.0+-10.0

1 hour recovery

	No dexamethasone	**80 nM dexamethasone**	**800 nM dexamethasone**
Control	105.9±8.7	101.9+-7.2	99.0+-11.0
3.0 mM TC	46.1±8.0	93.7+-7.3	97.7+-9.1
4.5 mM TC	LI	67.7+-13.0	87.7+-10.0

800 nM dexamethasone to the preincubation medium protected cells from the effect of 0.3 mM and 3.0 mM TC concentrations, but cells exposed to 4.5 mM TC had a slower rate of contraction than before treatment. However, they made full recovery after TC was removed.

Table 13 shows that UDCA could only protect cells against TC concentrations up to

Table 13. Effect of addition of UDCA (0.1mM) to the culture medium, and overnight incubation, of cardiomyocytes prior to addition of 1.0 or 3.0 mM taurocholate. Rate of contraction is shown as beats per minute, LI = loss of integrity, TC = taurocholate.

10 minutes treatment

	No UDCA	**0.1 mM UDCA**
Control	88.0+-6.4	88.0+-7.4
1.0 mM TC	47.2+-4.5	84.0+-6.7
3.0 mM TC	LI	LI

1 hour treatment

	No UDCA	**0.1 mM UDCA**
Control	89.2+-4.6	85.5+-6.7
1.0 mM TC	43.2+-3.2	85.5+-5.2
3.0 mM TC	LI	LI

1 hour recovery

	No UDCA	**0.1 mM UDCA**
Control	90.5+-5.2	88+-7.4
1.0 mM TC	57.6+-6..0	86.5+-9.5
3.0 mM TC	53.3+-4.9	52.8+-3.9

1mM. When the cells were exposed to 3 mM TC there was loss of integrity and synchronous beating within the network of cells. When the cardiomyocytes were subsequently transferred to TC-free medium they recovered but had a slower rate of contraction than before treatment.

4.4 Discussion

The experiments reported in this chapter have demonstrated that the primary bile acid, taurocholate (TC), can alter the rate and rhythm of cardiomyocyte contraction and cause abnormal Ca^{2+} dynamics in this *in-vitro* system. These data are consistent with the hypothesis that raised fetal serum bile acids in OC may result in the development of a fatal dysrhythmia and sudden intrauterine death.

The mechanisms by which TC could cause a fatal fetal dysrhythmia in the intact heart include altered cardiomyocyte Ca^{2+} dynamics or impaired gap junction function, resulting in impaired propagation of conduction and subsequent loss of synchronous contraction. Taurine-conjugated bile acids have been shown to act as Ca^{2+} ionophores, causing a dose-dependent increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Zimniak et al. 1991; Thibault et al. 1993). This is a result of transient permeabilisation of the endoplasmic reticulum to Ca^{2+}. To date, this effect has only been demonstrated in hepatocytes. The study described in this chapter is the first investigation of the effect of TC on $[Ca^{2+}]_i$ in cardiomyocytes. Increased Ca^{2+} cycling, coupled with the reduction in pH which would accompany the rapid beating (Bountra et al. 1988), may also affect the function of intercellular gap junctions that provide low-resistance pathways for current flow between heart cells (Jongsma et al. 1987; White et al. 1985) and are thought to be crucial for beat synchronisation (Page et al. 1981; Rook et al. 1992), and for arrhythmia generation (Miyamoto et al. 1999; Lopez et al. 1995). Intracellular calcium, through altered dynamics and overload, also has a pivotal role in the generation of arrhythmia (Levy et al. 1991; Di Diego et al. 1994).

Sudden cardiac death is usually attributed to tachyarrhythmias, although asystole or severe bradycardia are also distinct possibilities. In the latter two cases one would have to postulate substantial sinoatrial slowing, or partial to complete atrioventricular

conduction block. We cannot, from our model, comment reliably on this type of conduction disturbance. We do see evidence of conduction block within the network. This cell to cell block could contribute to re-entry, an electrophysiological promoter of sustained tachyarrhythmias. Although our model is far from the clinical situation, our results would suggest ventricular tachycardia or fibrillation as the major contributors to cholestatically induced sudden death *in utero*.

Clinical studies of the fetal heart rate in OC pregnancies have demonstrated both tachycardia and bradycardia (Laatikainen et al. 1984, Laatikainen 1975c; Reid et al. 1976), consistent with the findings in this study. However, the intrauterine death in OC is a sudden event and fetal cardiotochography is not a reliable way of predicting the at-risk fetus (Fisk et al. 1988; Rioseco et al. 1994). This may be because there is a threshold above which the influence of bile acids, such as TC, is observed. In addition, there may be changes in cardiac function from 37 weeks gestation that result in an increased fetal cardiomyocyte susceptibility to the effects of bile acids. This suggestion is supported by clinical studies that have demonstrated that delivery by 37-38 weeks gestation has reduced the perinatal mortality rate in OC from 10-15% (Reid et al 1976; Reyes et al. 1982) to 2.0-3.5% (Reyes et al. 1982; Fisk et al. 1988; Rioseco et al. 1994).

Severe bradycardia has been reported in neonates following exchange transfusion (Abu-Ekteish et al. 2000), and bile duct ligation in adult male rats can cause bradycardia, increase in PR and QT intervals, and arrhythmia in association with raised serum bilirubin levels, accumulation of bilirubin in the myocardium and depletion of cardiac glycogen (Tajuddin et al. 1980). However, there are no *in vivo* studies that demonstrate bradycardia in association with raised bile acids.

The concentration of 0.3 mM TC was chosen initially because this is within the range of concentrations of total bile acids that has been observed in the serum of women with OC (Kenyon et al. 2001). A concentration of 3.0 mM was used for comparison. The concentration of TC or of other bile acids to which the fetal cardiomyocytes are exposed *in vivo* is difficult to quantify, and may be greater or smaller than the concentrations used in this study.

Following the experiments reported in this chapter it will be of interest to investigate effect of the same doses of TC on adult rat cardiomyocytes. Mothers with OC have not been reported to develop dysrhythmias. This suggests that the fetal heart is more susceptible to the development of dysrhythmias *in vivo*. The normal fetal heart beats faster than the maternal heart and this is consistent with more Ca^{2+} transients per unit time. Therefore a small increase in the Ca^{2+} wave frequency could result in the breakdown of synchronised contraction by the cellular mechanisms alluded to above.

The advantage of the experimental model used in this study is that it is possible to observe the effect of bile acids on single cells and on adjacent cells within the network. This is useful as a model of the cell-to-cell interactions that occur in the intact human heart. It will be of interest to extend the observations reported in this study, to investigate the effect of bile acids on the intact fetal and maternal heart in animals and humans. An advantage of *in vitro* experiments such as those reported in this study is that they provide a better understanding of the mechanisms by which bile acids may impair cardiomyocyte function prior to the commencement of *in vivo* experiments.

This study shows that the therapeutic agents dexamethasone and UDCA protect neonatal rat cardiomyocytes from the arrhythmogenic effects of the bile acid TC using this *in vitro* model. Dexamethasone was more protective than UDCA. This may

reflect different mechanisms of action. Dexamethasone has previously been shown to maintain the structural integrity of myocytes for periods of at least 45 days in the absence of any damaging agents (Muir et al. 1992). Dexamethasone also protects against the TC-induced reduction in rate of contraction. These data are in agreement with a report that demonstrated that dexamethasone protected against a decreased contraction rate and multiform arrhythmias that were induced in cultured rat myocardial cells following infection with Coxsackie B-2 virus (Yang et al. 1989, 1991). In addition, dexamethasone inhibits endotoxin-induced changes in calcium and contractility in rat isolated papillary muscle. Pretreatment of rats with dexamethasone prevented the endotoxin-induced decrease in peak tension and inhibited the elevation in resting $[Ca^{2+}]_i$, with a resultant maintenance of Ca^{2+} transient magnitude (Reilly et al. 1999). The effect of dexamethasone is often attributed to its ability to induce gene expression or stabilise mRNA in target cells (Reid et al. 1981).

The cardioprotective effect of UDCA may be a consequence of protection from the apoptosis that is induced by more hydrophobic bile acids or may be due to membrane stabilisation. Most of the studies of the protective effect of UDCA have compared it with more hydrophobic bile acids, e.g. glycochenodeoxycholic acid (GCDC). In rat and human hepatocytes, the mechanism of cytotoxicity of GCDC varies at different concentrations. At the higher concentrations that are seen in severe cholestasis, i.e. 0.5 mM, cytolytic cell destruction has been demonstrated (Galle et al. 1990). At lower pathological concentrations of 0.05-0.1 mM, apoptosis is the predominant mechanism of bile acid toxicity (Benz et al. 1998, 2000). Studies of human and rat hepatocytes have shown that UDCA protects against these cytotoxic effects of hydrophobic bile acids (Galle et al. 1990; Benz et al. 1998). UDCA at millimolar concentrations protects against membrane damage caused by more hydrophobic bile acids in *in vitro*

studies of rat and human hepatocytes, and in artificial membranes (Heuman et al. 1991a, 1991b, 1994).

UDCA may also exert its cardioprotective effect by increasing ATP levels. Studies of the mechanism by which UDCA promotes bile flow have demonstrated that it stimulates secretion of ATP in isolated rat hepatocytes (Nathanson et al. 2001). Separate studies of rat and mouse hearts have shown that both ATP and the P2X receptor agonist 2-meSATP stimulated large increases in the myocyte contractile amplitude (Mei et al. 2001). Thus it is possible that UDCA-induced rise of ATP in cardiomyocytes has a cardioprotective effect.

UDCA increases the density of the hepatic apical conjugal export pump, Mrp2, fourfold in rat canalicular membranes (Beuers et al. 2001). At the time of writing this thesis there are no reported studies of the effect of UDCA on the insertion of other potential bile acid transporters into the canalicular membrane.

It is possible that the cardioprotective effects of UDCA and dexamethasone are the result of altered expression of genes that influence bile acid transport or metabolism in cardiomyocytes. We are currently not aware of studies of the expression of these bile acid transporter genes in cardiomyocytes. If the transporters are expressed in cardiomyocytes, it is also possible that UDCA and dexamethasone could act by a direct effect on the function of the transporters.

Both UDCA and dexamethasone could have immediate effects on cell membrane transporters, or compete with bile acid and subsequent experiments can be designed to investigate this.

The experiments reported in this thesis suggest that dexamethasone may have a more potent cardioprotective effect on the fetus, although this study is relatively small. However, there is evidence from animal and human studies that fetal exposure to

glucocorticoids may have an adverse effect on subsequent blood pressure (Doyle et al. 2000) and brain maturation (Modi et al. 2001). Therefore I would not advocate using dexamethasone as a first line treatment for OC following the results of this study. However, these data have provided evidence that it may have a beneficial effect in the prevention of IUD in women who have not responded to UDCA.

5. FUTURE WORK

The results of the studies reported in this thesis demonstrate that OC is a common condition in the UK, and that it can have serious consequences, including premature delivery and intrauterine death. They also confirm that the aetiology of OC has a genetic component, and that the *MDR3* gene can contribute to this. The cardiomyocyte studies suggest a mechanism for the hitherto unexplained intrauterine death. The implications of the results of each study for our understanding of the aetiology of OC has been discussed in the relevant chapter of this thesis. This chapter will consider future experiments that will help develop this knowledge.

Understanding the aetiology of IUD and other fetal complications of OC

The study reported in chapter 2 is the largest collection of OC pregnancies complicated by IUD to date. However, one weakness of this study is that most of the data were obtained retrospectively. It will be of interest to scrutinise the hospital notes of the cases reported in this study to try to establish whether there are any features in the histories that would have allowed the IUD to have been predicted, and consent has been requested from these women to allow the hospital notes to be obtained. Also, the introduction of an international website for reporting OC-related IUDs will help overcome this.

Future studies of the management strategies for OC

Obstetricians frequently express concern about the risks of premature delivery in the infants of women delivered between 37 and 38 weeks gestation because of a diagnosis of OC. Overall the quoted risks of IUD in OC are higher than those of premature delivery (section 2.4.1). However, the risks for OC pregnancies were calculated for

cases that did not receive treatment with UDCA or dexamethasone (Reid et al. 1976; Reyes 1982). OC is now frequently diagnosed and managed with pharmacological agents and delivery by 38 weeks gestation in the UK. Therefore a subgroup of women are being induced by 38 weeks despite their symptoms and biochemical abnormalities having returned to normal. An interesting study that could be performed on this group is a randomised trial of induction of labour compared to expectant management of the pregnancy providing the serum bile acids remain normal on treatment.

Another valuable clinical study would be the evaluation of the intelligence and developmental assessments of the children of women who received treatment with UDCA in pregnancy. There are no reports of subsequent developmental delay in the offspring of pregnancies treated with UDCA, but this study has not been done. There are not sufficient numbers of pregnancies treated by dexamethasone in the UK to perform a similar study on the offspring of OC pregnancies where this drug was used. However, 12mg dexamethasone per day is a large dose and this must be considered when treating affected pregnancies.

It will be of interest to learn the outcome of a larger number of twin pregnancies complicated by OC. The results of the study reported in chapter 2 suggest that the risk of IUD before 37 weeks gestation is higher in these pregnancies. However, the numbers are small and it is possible that treatment with UDCA may reduce this risk.

Subsequent studies of the genetic aetiology of OC

The studies reported in chapters 2 and 3 demonstrate that the aetiology of OC clearly has genetic component. However, mutations in *MDR3* are likely to only contribute to the aetiology in a small proportion of cases. Even amongst raised GGT cases, not all will have genetic aetiology. For example women with viral hepatitis will also have

raised GGT. The genes coding for the other transporters that influence hepatic bile acid transport are also candidates for the condition. The merits of each gene as a candidate for OC are discussed below.

BSEP, the principal canalicular bile acid transporter, is implicated in cholestasis in pregnancy by animal experiments; i.e. reduced expression of *mBsep* following administration of ethinylestradiol (Green et al. 2000) and the demonstration of inhibition of Bsep following secretion of estradiol-17-beta-glucuronide into bile canaliculi by Mrp2 (Steiger et al. 2000; Huang et al. 2000). However, although homozygous mutations in *BSEP* cause PFIC2 (Strautnieks et al. 1998), heterozygote mothers do not develop cholestasis in pregnancy.

FIC1 is mutated in PFIC1 and BRIC, and some women with BRIC develop cholestasis in pregnancy. However, none of the reported cases with *FIC1* mutations, nor the heterozygote mothers of PFIC1 cases, had a history of OC. The mechanism by which *FIC1* mutations cause cholestasis is not known. It is possible that reduced function of the FIC1 protein causes abnormal phospholipid composition of cell membranes, and that this in turn impairs the function of the liver canalicular bile acid transporters. It is possible that raised oestrogens or progestogens alter *FIC1* expression and function, and that this in turn may cause cholestasis due to abnormal transport by other hepatic transporters.

MRP2 and *MRP3* may play a role in the aetiology of OC. *MRP2* plays a role in the inhibition of *Bsep* by estradiol-17-beta-glucuronide (Steiger et al. 2000; Huang et al. 2000). *MRP3* can transport bile acids (Hirohashi et al. 2000), and it is possible that mutations in this gene may result in cholestasis.

NTCP and the *OATP* genes are less likely to be directly implicated in the aetiology of OC, as cholic acid and chenodeoxycholic acid are the main bile acids that are raised in

the condition, and these primary bile acids are synthesised within the hepatocyte (i.e. before transport across the cananlicular membrane). However, ethinyl estradiol administration caused reduced RNA and protein expression of rat Ntcp and Oatp (Simon et al. 1996), so they should still be considered as candidates.

The genes that encode the intranuclear receptors FXR, RXR, PXR, LRH1 and SHP are all candidates for OC as these proteins all influence the expression of either bile acid transporters or enzymes that metabolise bile acids.

As with many complex traits, it is likely that several of the genes described above play a role in the aetiology of OC, and it is possible that genetic variants in more than one gene act simultaneously to confer susceptibility to cholestasis in pregnancy. It is also possible that the genetic variants that cause OC are not all in the coding sequence, so it will be important to also screen intronic sequence and the 3'and 5' untranslated regions of the genes of interest. It is time consuming and not cost effective to screen large numbers of candidate genes by direct sequence analysis, the technique that was used for the studies reported in chapter 3. Therefore, future studies of the genetic aetiology of OC will require experimental strategies that identify individual variation in genomic sequence (increasingly as single nucleotide polymorphisms – SNPs). Once variants (SNPs or other polymorphisms) have been identified in the region of genes of interest, their frequency can be compared in OC cases and controls. In most circumstances, linkage disequilibrium (LD) methods are then used to test these variants for their relationship to disease, allowing researchers to locate and characterise those variants with functional and aetiological roles.

There are two experimental strategies that can be used for SNP-based LD mapping – 'direct' and 'indirect' (Collins et al. 1997). In practice, gene discovery efforts increasingly involve implementation of both of these in parallel, and both can be

applied to the subsequent investigation of the aetiology of OC. As there are a small number of obvious candidate genes, as described above, the 'direct' approach could be tried in the first instance. However, this may not be sufficient to identify all of the genes that confer susceptibiliy to OC, and if this is the case, the 'indirect' method could also be used.

Direct LD mapping: In the 'direct' approach, attention is focused on the detection and evaluation of SNPs of presumed functional relevance, i.e. SNPs in the immediate vicinity of genes of interest. Increasing awareness of the vagaries of extent of LD (Chakravarti et al. 1998), of the complexity of haplotypic patterns (Nickerson et al. 1998, Clark et al. 1998) and the importance of regulatory sequence variation necessitate a more systematic approach to the assessment of candidate genes than have previously been used, involving the following steps:

- Screening of the gene (coding, regulatory, intronic regions) in a subset of individuals to provide an inventory of all common (and most infrequent) variants;
- Typing of these variants in a larger set of subjects (e.g. 50-100) to determine LD relationships between them, define the common haplotypes, and prioritise variants for genotyping in larger data sets, the objective being to select the subset of variants which collectively provide the strongest chance of picking up LD relationships with disease-susceptibility alleles in the region;
- Genotyping of variants in large case-control or family-based data-sets, looking for evidence of association between genomic variation and disease phenotypes.

Only through such a systematic approach, is it possible to be confident that no (frequent) disease-susceptibility variant has been left undetected.

When performing LD mapping it is important to allow for effects of ethnic heterogeneity. Given that OC occurs more commonly in women of Chilean,

Scandinavian and Asian origin, it will be important to subdivide samples from women with OC according to ethnic origin. For example, some genomic loci may only be polymorphic in certain populations (Shriver et al. 1998). If variants in a specific gene are found to be associated with OC, an inventory of all variants will be made and these will be investigated to establish whether they have functional effects.

Indirect LD mapping: in the indirect approach, a dense map of SNPs is typed across a region of interest in the expectation that disease-alleles will be detected through LD with one or more of these variants. Archetypically, 'indirect' markers are chosen solely on the basis of genomic map position, without regard to possible functional relevance. Whilst some have questioned the value of such approaches on theoretical grounds (Kruglyak et al. 1999), several successful examples are emerging where indirect mapping in such populations has led towards gene discovery (Loder 1999; Hanis et al. 1996).

At present it is not possible to use the pedigrees that have been identified for linkage studies. However, it is possible that a specific gene confers susceptibility to all cases within an individual pedigree. Therefore, it may be useful to perform linkage studies using polymorphic markers for specific candidate genes or loci in selected pedigrees. At present, confirmation of the diagnosis is not possible in most relatives of affected women. However, a useful study would be the investigation of a test of phenotype for relatives. The older studies in which the oral contraceptive pill was administered to women with a previous history of OC used doses of oestrogen that are now not justified for use in non-therapeutic investigations. However, these studies measured transaminase levels rather than the serum bile acids (Kreek et al. 1967; Holzbach et al. 1983). Therefore it may be possible to design a test of phenotype using lower concentrations of oestrogen and/ or progesterone and measuring the serum bile acids

rather than liver transaminases. This would allow the diagnosis of OC to be confirmed in the relatives of affected women, and may allow the pedigrees to be used for linkage studies (either conventional linkage, or affected sib-pair analysis). Another advantage of a reliable test of phenotype will be that sisters and daughters of affected women can be tested prior to conception if they would like to know their risk of developing OC.

Once genetic variants that predispose to OC have been identified, it will be necessary to perform in vitro transfection studies to establish their functional effects. The mutation described in chapter 3 of this thesis was functionally characterised using the closely related protein P-gp1. However, it is important to aim to characterise mutations using a functional assay of the corresponding protein where possible, in this case the MDR3 protein.

It will also be of interest to investigate whether addition of oestrogens and progestogens to the culture media used in such experiments, i.e. to mimic the endocrine changes of pregnancy, will influence the functional effect of molecular variants. A similar strategy was used to unmask the effects of a mutation in the thyrotrophin receptor gene that caused familial gestational hyperthyroidism. When transfected into COS-7 cells, basal production of cyclic AMP was similar in cells expressing the mutant receptor to that in cells expressing the wild-type receptor. However, addition of chorionic gonadotrophin at concentrations similar to those seen in the 1st trimester of pregnancy indicated that the mutant receptor was 1000 times less responsive (Rodien et al. 1998).

The role of fetal inheritance of mutations in genes that influence bile acid transport

If a subgroup of mothers with OC have a loss of function mutation in a gene that influences bile acid transport, 50% of fetuses will inherit this mutation. The normal

balance of bile acids between mother and fetus is maintained by transfer across the placenta from the fetus to the mother by specific transport systems located on either side of the plasma membrane of the trophoblast. Two studies of OC pregnancies have demonstrated increased bile acid levels in the fetus. However, they were uniformly lower than the corresponding maternal levels (Figure 4). This is consistent with the increased maternal serum bile acids being transferred from the mother to the fetus via the placenta. As the fetus cannot excrete bile acids via the gut, it is necessary to transport the bile acids back across the placenta to the mother. The efficiency of this transfer was markedly reduced in approximately 50% of the OC pregnancies in both studies (Figure 4), suggesting that an inherited placental bile acid transport abnormality may predispose to the development of raised bile acids in these offspring. This suggestion is of particular interest in the light of the results reported in chapter 4. There have been no studies of the placental expression of the genes that influence bile acid transport with the exception of one study of the multidrug resistance family in which *MRP2* was localised to the apical syncytiotrophoblast membrane, and *MRP3* was localised to the blood vessel endothelia with some evidence for expression in the apical syncytiotrophoblast (St Pierre et al. 2000). Otherwise, studies using trophoblast vesicles have demonstrated that bile acid transport into the placenta is mediated by a reversible anion: bile acid exchanger located at the basal (fetal) facing membrane of the human chorionic trophoblast (Marin et al. 1990), and by an ATP-dependent transport system at the maternal facing membrane (Marin et al. 1995). In OC pregnancies there is significant impairment of the rate and affinity of bile acid transport in the basal membrane, consistent with significantly reduced transport efficiently (Serrano et al. 1998). At the apical membrane the efficiency of transport is significantly reduced in spite of an enhancement in the capacity of ATP-dependent

bile acid transport. The changes observed in the OC patients were all reversed in placentas obtained from women treated with the therapeutic agent UDCA (Serrano et al. 1998). However, it has not been established whether the effect of UDCA is due to a direct effect on one or more of the transporters, or due to altered expression of the transporters.

Thus, it will be of interest to perform additional studies to investigate RNA and protein expression of the canalicular and sinusoidal transporters that influence bile acid transport in placentas from women with OC (UDCA treated and non-treated) and controls. It will also be of interest to investigate the localisation of the transporters using immunohistochemistry. In addition, transport experiments using placental explant cultures of control placentas (Kudo and Boyd 2001) will allow the assessment of whether UDCA influences bile acid transport due to a direct effect on the transporters, or whether it acts via altered expression.

Future experiments to extend understanding of the effects of bile acids on the fetal heart

The studies reported in chapter 4 demonstrate that taurocholate, the tauroconjugate of the main primary bile acid that is raised in OC, alters cardiomyocyte contraction and calcium dynamics in an *in vitro* rat model. The pharmacological agents ursodeoxycholic acid and dexamethasone both improve maternal serum bile acid levels and the clinical features of OC (Berkane et al. 2000; Brites et al. 1998a; Davies et al. 1995; Floreani et al. 1996; Mazzella et al. 1991, 2001; Nicastri et al. 1998; Palma et al. 1992, 1997 ; Hirvioja et al, 1992), but it is currently not known whether their use improves the fetal prognosis. Future experiments using this model will allow the evaluation of whether these agents may have a protective effect on fetal

cardiomyocytes. They will also have a protective effect secondary to reduction of maternal, and therefore fetal, serum bile acid levels. It will also be of interest to compare the effect of TC on adult and fetal cardiomyocytes, as mothers with OC have not been reported to develop dysrhythmias. An interesting clinical study could be to monitor 24 hour heart traces of mothers OC. It would, in principle, be of interest to simultaneously monitor the fetal heart rate, and it is likely that the technology to do this will be available in the near future.

It is also important to aim to perform these studies using human cardiomyocytes from adults and from term fetuses. In principle it will be possible to obtain small amounts of tissue from term infants that require heart surgery for conditions where there is a normal heart, e.g. transpostion of the great vessels.

A study reported in the 1997 Confidential Enquiry into Stillbirths and Deaths in Infancy (CESDI) report revealed that the serum bile acid levels were raised in 4.5% of cases of unexplained stillbirth. These data suggest that the spectrum of cholestasis may be larger than previously estimated. It will be of interest to perform a prospective study of the serum bile acid levels of the mothers of pregnancies complicated by unexplained IUD. If this is also consistent with the hypothesis that cholestasis is implicated in unexplained IUD, it will be of interest to extend the genetic studies pertinent to OC to try to establish the aetiology of other cases of IUD.

A hypothesis to explain the aetiology of obstetric cholestasis

The studies reported in this thesis, in combination with those of published investigations of the aetiology of OC allow the development of a hypothesis to explain the aetiology of OC.

The aetiology has a hereditary component, and this is likely to be complex with the interaction of several genes and environmental factors. The raised maternal bile acids cross the placenta and raised fetal bile acids are likely to be the cause of the fetal complications of OC. As a subgroup of women have mutations in genes that code for proteins that influence bile acid transport, it is likely that the inheritance of these mutations will cause some fetuses to have inefficient transfer of bile acids across the placenta to the maternal circulation. It is this subroup of fetuses that will be at risk of spontaneous prematurity, fetal distress and intrauterine death.

At present, the most important intervention to prevent IUD and the other fetal complications is delivery by 37-38 weeks gestation. It is likely that UDCA treatment is also of use, with beneficial effects for the fetus as well as for the mother.

REFERENCES

1. Abedin P, Weaver JB, Egginton E. Intrahepatic cholestasis of pregnancy: prevalence and ethnic distribution. Ethn.Health 1999; 4: 35-37.
2. Abu-Ekteish F, Daoud A, Rimawi H, Kakish K, Abu-Heija A. Neonatal exchange transfusion: a Jordanian experience. Ann Trop Paediatr 2000; 20: 57-60.
3. Alsulyman OM, Ouzounian JG, Ames-Castro M, Goodwin TM. Intrahepatic cholestasis of pregnancy: perinatal outcome associated with expectant management. Am J Obstet.Gynecol. 1996; 175: 957-960.
4. Ambrosini, L., Mercer, J.F.B. Defective copper-induced trafficking and localization of the Menkes protein in patients with mild and copper-treated classical Menkes disease. Hum. Mol. Genet. 1999; 8, 1547-1555.
5. Ananthanarayanan M, Balasubramanian N, Makishima M, Mangelsdorf DJ, Suchy FJ. Human bile salt export pump promoter is transactivated by the farnesoid X receptor/bile acid receptor. J Biol Chem. 2001; 276: 28857-2865.
6. Bacq Y, Myara A, Brechot MC, Hamon C, Studer E, Trivin F, Metman EH. Serum conjugated bile acid profile during intrahepatic cholestasis of pregnancy. J Hepatol 1995; 22: 66-70.
7. Bacq Y, Sapey T, Brechot MC, Pierre F, Fignon A, Dubois F. Intrahepatic cholestasis of pregnancy: a French prospective study. Hepatology 1997; 26: 358-364.
8. Barrigon S, Wang SY, Ji X, Langer GA. Characterization of the calcium overload in cultured neonatal rat cardiomyocytes under metabolic inhibition. J.Mol. Cell Cardiol. 1996; 28: 1329-1337.

9. Benz C, Angermuller S, Tox U, Kloters-Plachky P, Riedel HD, Sauer P, Stremmel W, Stiehl A. Effect of tauroursodeoxycholic acid on bile-acid-induced apoptosis and cytolysis in rat hepatocytes. J Hepatol 1998; 28: 99-106.

10. Benz C, Angermuller S, Otto G, Sauer P, Stremmel W, Stiehl A. Effect of tauroursodeoxycholic acid on bile acid-induced apoptosis in primary human hepatocytes. Eur J Clin Invest 2000; 30: 203-9.

11. Berg B, Helm G, Petersohn I, Tryding N. Cholestasis of pregnancy. Clinical and laboratory studies. Acta Obstet Gynecol Scand 1986; 65: 107-113.

12. Berkane N, Cocheton JJ, Brehier D, Merviel P, Wolf C, Lefevre G, Uzan S. Ursodeoxycholic acid in intrahepatic cholestasis of pregnancy. A retrospective study of 19 cases. Acta Obstet Gynecol Scand 2000; 79: 941-946.

13. Beuers U, Bilzer M, Chittattu A, Kullak-Ublick GA, Keppler D, Paumgartner G, Dombrowski F. Tauroursodeoxycholic acid inserts the apical conjugate export pump, Mrp2, into canalicular membranes and stimulates organic anion secretion by protein kinase C-dependent mechanisms in cholestatic rat liver. Hepatology 2001; 33: 1206-16.

14. Birnboim, H. C., and J. Doly. A rapid alakaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Research. 1979; 7: 1513-1523.

15. Blott, E.J., Higgins, C.F., Linton, K.J. Cysteine scanning mutagenesis provides no evidence for the extracellular accessability of the nucleotide-

binding domains of the multidrug resistance transporter P-glycoprotein. EMBO J. 1999; 18, 6800-6808.

16. Bossard R, Steiger B, O'Neill B, Fricker G, Meier PJ. Ethinylestradiol treatment induces multiple canalicular membrane transport alterations in rat liver. J Clin Invest 1993; 91: 2714-2720.

17. Bountra, C., Kaila, K., Vaughan Jones, R .D. Effect of repetitive activity upon intracellular pH, sodium and contraction in sheep cardiac Purkinje fibres. J Physiol Lond. 1988; 398, 341-360

18. Boyer, J.L., Soroka, C.J. Vesicle targeting to the apical domain regulates bile excretory function in isolated rat hepatocyte couplets. Gastroenterology 1995; 109:1600-1611.

19. Brites D, Rodrigues CM, Oliveira N, Cardoso M, Graca LM. Correction of maternal serum bile acid profile during ursodeoxycholic acid therapy in cholestasis of pregnancy. J Hepatol 1998a; 28: 91-98.

20. Brites D, Rodrigues CM. Elevated levels of bile acids in colostrum of patients with cholestasis of pregnancy are decreased following ursodeoxycholic acid therapy. J Hepatol 1998b; 29: 743-751.

21. Brown PC, Thorgeirsson SS, Silverman JA. Cloning and regulation of the rat mdr2 gene. Nucleic Acids Res. 1993; 21, 3885-91.

22. Brown CR, Hong-Brown LQ, Welch WJ. Strategies for correcting the delta F508 CFTR protein-folding defect. J. Bioenerg Biomembr 1997; 29, 491-502.

23. Bull LN, Carlton VE, Stricker NL, Baharloo S, DeYoung JA, Freimer NB, Magid MS, Kahn E, Markowitz J, DiCarlo FJ, McLoughlin L, Boyle JT, Dahms BB, Faught PR, Fitzgerald JF, Piccoli DA, Witzleben CL, O'Connell

NC, Setchell KD, Agostini RM Jr, Kocoshis SA, Reyes J, Knisely AS. Genetic and morphological findings in progressive familial intrahepatic cholestasis (Byler disease [PFIC-1] and Byler syndrome): evidence for heterogeneity. Hepatology 1997; 26, 155-64.

24. Bull LN, van Eijk MJ, Pawlikowska L, DeYoung JA, Juijn JA, Liao M, Klomp LW, Lomri N, Berger R, Scharschmidt BF, Knisely AS, Houwen RH, Freimer NB. A gene encoding P-type ATPase mutated in two forms of hereditary cholestasis. Nature Genet. 1998; 18, 219-224.

25. Buschman E, Gros P. Functional analysis of chimeric genes obtained by exchanging homologous domains of the mouse mdr1 and mdr2 genes. Mol. Cell. Biol. 1991; 11: 595-603.

26. Butler FA, Whanger PD, Tripp MM. Blood selenium and glutathione peroxidase activity in pregnant women. Comparative assays in primates and other animals. Am J Clin Nutr 1982; 32: 15-32.

27. Campos G, Guerra F, Israel E. Effects of cholic acid infusions in fetal lambs. Acta Obstet Gynecol Scand 1986; 65: 23-26.

28. Campos G, Guerra F, Israel E. Effecto de los acidos biliares sobre la contractibilidad miometral en utero gestante aislado. Rev Chile Obstet Ginecol 1988; 53: 229-233.

29. Chakravarti A. It's raining SNPs, hallelujah? Nature Genetics 1998; 19: 216-217.

30. Chen, C., Chin, J.E., Ueda, K., Clark, D.P., Pastan, I., Gottesman, M.M., Roninson, I.B. Internal duplication and homology with bacterial transport proteins in the mdr1/P-glycoprotein gene from multidrug resistant human cells. Cell 1986; 47, 381-389.

31. Cheng, S.H., Gregory, R.J., Marshall, J., Paul, S., Souza, D.W., White, G.A., O'Riordan, C.R., Smith, A.E. Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. Cell 1990; 63, 827-34.

32. Clark AG, Weiss KM, Nickerson DA, Taylor SL, Buchanan A, Stengård J, Salomaa V, Vartiainen E, Perola M, Boerwinkle E, Sing CF. Haplotype structure and population genetics inferences from nucleotide-sequence variation in human lipoprotein lipase. American Journal of Human Genetics 1998; 63: 595-612

33. Collins FS, Guyer MS, Chakravarti A. Variations on a theme: cataloging human DNA sequence variation. Science 1997; 278: 1580-1581.

34. Coquil JF, Berthon B, Chomiki N, Combettes L, Jourdon P, Schteingart C, Erlinger S, Claret M. Effects of taurolithocholate, a Ca2(+)-mobilizing agent, on cell Ca2(+) in rat hepatocytes, human platelets and neuroblastoma NG108-15 cell line. Biochem J. 1991; 273, 153-160

35. Costalo P, Ylikorkala O. Hepatosis of pregnancy: a clinical study of 107 patients. Ann Chir Gynaecol Fenn 1975; 64: 128-134.

36. Costoya A, Leontic E, Rosenberg H, Delgado M. Morphological study of placental terminal villi in intrahepatic cholestasis of pregnancy: histochemistry, light and electron microscopy. Placenta 1980; 1: 361-368.

37. Cotzias CS, Paterson-Brown S, Fisk NM. Prospective risk of unexplained stillbirth in singleton pregnancies at term: population based analysis. BMJ 1999; 319: 287-288.

38. Crawford JM, Barnes S, Stearns RC, Hastings CL, Godleski JJ. Ultrastructural localization of a fluorinated bile salt in hepatocytes. Lab Invest 1994; 71: 42-51.

39. Crawford JM. Role of vesicle-mediated transport pathways in hepatocellular bile secretion. Semin Liver Dis 1996; 16: 169-189.

40. Davies MH, da Silva RCMA, Jones SR, Weaver JB, Elias E. Fetal mortality associated with cholestasis of pregnancy and the potential benefit of therapy with ursodeoxycholic acid. Gut 1995; 37: 580-584.

41. Davis RA, Kern F Jr, Showalter R, Sutherland E, Sinensky M, Simon FR. Alterations of hepatic Na+,K+-atpase and bile flow by estrogen: effects on liver surface membrane lipid structure and function. Proc Natl Acad Sci USA 1978; 75: 4130-4134

42. Davis RA, Showalter R, Kern F. Reversal by Triton WR-1339 of ethinyl oestradiol-induced hepatic cholesterol esterification. Biochem J 1978b; 174: 45-51.

43. de Koning TJ, Sandkuijl LA, de Schryver JEAR, Hennekam EAM. Autosomal recessive inheritance of benign recurrent intrahepatic cholestasis. Am J Med Genet 1995; 57:479-482.

44. de Pagter AGF, van Berge Henegouwen GP, ten Bokkel Huinnk JA, Brandt KH. Familial benign recurrent intrahepatic cholestasis: interrelation with intrahepatic cholestasis of pregnancy and from oral contraceptives. Gastroenterology 1976; 71: 202-207.

45. de Vree JM, Jacquemin E, Sturm E, Cresteil D, Bosma PJ, Aten J, Deleuze JF, Desrochers M, Burdelski M, Bernard O, Oude Elferink RP, Hadchouel

M. Mutations in the *MDR3* gene cause progressive familial intrahepatic cholestasis. Proc. Natl. Acad. Sci. U.S.A. 1998; 95, 282-287.

46. Deleuze JF, Jacquemin E, Dubuisson C, Cresteil D, Dumont M, Erlinger S, Bernard O, Hadchouel M. Defect of multidrug resistance 3 gene expression in a subtype of progressive familial intrahepatic cholestasis. Hepatology 1996; 23: 904-908.

47. Denson LA, Sturm E, Echevarria W, Zimmerman TL, Makishima M, Mangelsdorf DJ, Karpen SJ. The orphan nuclear receptor, shp, mediates bile acid-induced inhibition of the rat bile acid transporter, ntcp. Gastroenterology 2001; 121: 140-147.

48. Di Diego, J.M., Antzelevich, C. High [Ca2+]o-induced electrical heterogeneity and extrasystolic activity in isolated canine ventricular epicardium. Phase 2 reentry. Circulation. 1994; 89, 1839-1850.

49. Doyle LW, Ford GW, Davis NM, Callanan C. Antenatal corticosteroid therapy and blood pressure at 14 years of age in preterm children. Clin Sci (Colch) 2000; 98: 137-42.

50. Drumm, M.L., Wilkinson, D.J., Smit, L.S., Worrell, R.T., Strong, T.V., Frizzell, R.A., Dawson, D.C., Collins, F.S. Chloride conductance expressed by delta F508 and other mutant CFTRs in Xenopus oocytes. Science 1991; 254, 1797-9.

51. Eppens EF, van Mil SWC, de Vree ML, Mok KS, Juijn JA, Oude Elferink RPJ, Berger R, Houwen RHJ, Klomp LWJ. FIC1, the protein affected in two forms of hereditary cholestasis, is localised in the cholangiocyte and the canalicular membrane of the hepatocyte. J Hepatol. 2001; 35: 436-443.

52. Fagan EA. Disorders of the liver, biliary system and pancreas. In: de Swiet M, ed. Medical disorders in Obstetric Practice 1995. 3rd edn. Blackwell Science Ltd, UK.

53. Fisk NM and Storey GNB. Fetal outcome in obstetric cholestasis. Br J Obstet Gynaecol 1988a; 95: 1137-1143.

54. Fisk NM, Bye WB, Storey GNB. Maternal features of obstetric cholestasis: 20 years experience at King George V Hospital. Aust NZ Obstet Gynaecol 1988b; 28: 172-176.

55. Floreani A, Paternoster D, Melis A, Grella PV. S-adenosyl methionine versus ursodeoxycholic acid in the treatment of intrahepatic cholestasis of pregnancy: preliminary results of a controlled trial. Eur J Obstet Gynecol Reprod Biol 1996; 67: 109-13.

56. Frezza M, Centini G, Cammareri G, Le Grazie C, Di Padova C. S-adenosyl methionine for the treatment of intrahepatic cholestasis of pregnancy. Results of a controlled clinical trial. Hepatogastroenterology 1990; 37 Suppl 2:122-5.

57. Furukawa T, Yamane T, Terai T, Katayama Y, Hirakoa M. Functional linkage of the cardiac ATP-sensitive K^+ channel to the actin cytoskeleton. Pflugers Arch 1996; 431: 504-512.

58. Gabbiani G, Montesano R, Tuchweber B, Salas M, Orci L. Phalloidin-induced hyperplasia of actin filaments in rat hepatocytes. Lab Invest. 1975; 33: 562-569.

59. Galle PR, Theilmann L, Raedsch R, Otto G, Stiehl A. Ursodeoxycholate reduces hepatotoxicity of bile salts in primary human hepatocytes. Hepatology 1990; 12: 486-91.

60. Gartung C, Ananthanarayanan M, Rahman MA, Schuele S, Nundy S, Soroka CJ, Stolz A, Suchy FJ, Boyer JL. Down-regulation of expression and function of the rat liver Na^+/bile acid cotransporter in extrahepatic cholestasis. Gastroenterology 1996; 110: 199-209.

61. Gerloff T, Stieger B, Hagenbuch B, Madon J, Landmann L, Roth J, Hofmann AF, Meier PJ. The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. J Biol Chem 1998; 273: 10046-10050.

62. Girling JC, Dow E, Smith JH. Liver function tests in pre-eclampsia: importance of comparison with a reference range derived for normal pregnancy. Br. J. Obstet. Gynaecol. 1997; 104, 246-250.

63. Gonzalez MC, Reyes H, Arrese M, Figueroa D, Lorca B, Andresen M, Segovia N, Molina C, Arce S. Intrahepatic cholestasis of pregnancy in twin pregnancies. J Hepatol 1989; 9: 84-90.

64. Goodwin B, Jones SA, Price RR, Watson MA, McKee DD, Moore LB, Galardi C, Wilson JG, Lewis MC, Roth ME, Maloney PR, Willson TM, Kliewer SA. A regulatory cascade of the nuclear receptors FXR, SHP-1 and LRH-1 represses bile acid biosynthesis. Mol Cell 2000; 6: 517-526.

65. Green RM, Hoda F, Ward KL. Molecuar cloning and characterisation of the murine bile salt export pump. Gene 2000; 241: 1177-123.

66. Gros, P., Ben Neriah, Y.B., Croop, J.M., Housman, D.E. Isolation and expression of a complementary DNA that confers multidrug resistance. Nature 1986; 323: 728-731.

67. Gumucio JJ, Accatino L, Macho AM, Contreras A.. Effect of phenobarbital on the ethinyl estradiol-induced cholestasis in the rat. Gastroenterology 1973; 65: 651-657.

68. Gumucio J, Valdivieso V. Studies on the mechanisms of ethinyl estradiol impairment of bile flow and bile salt excretion in the rat. Gastroenterology 1971; 61: 339-344.

69. Gylling H, Riikonen S, Nikkila K, Savonius H, Miettinen TA. Oral guar gum treatment of intrahepatic cholestasis and pruritus in pregnant women: effects on serum cholestanol and other non-cholesterol sterols. Eur J Clin Invest 1998; 28 : 359-363.

70. Hardison WG, Wood CA. Importance of bicarbonate in bile salt independent fraction of bile flow. Am J Physiol. 1978; 235: E158-164.

71. Hanis CL, Boerwinkle E, Chakraborty R, Ellsworth DL, Concannon P, Stirling B, Morrison VA, Wapelhorst B, Spielman RS, Gogolin-Ewens KJ, Shephard JM, Williams SR, Risch N, Hinds D, Iwasaki N, Ogata M, Omori Y, Petzold C, Rietzsch H, Schroder HE, Schulze J, Cox NJ, Menzel S, Boriraj VV, Chen X, Lim LR, Lindner T, Mereu LE, Wang YQ, Xiang K, Yamagata K, Yang Y, Bell GI. A genome-wide search for human non-insulin-dependent (type 2) diabetes genes reveals a major susceptibility locus on chromosome 2. Nature Genetics 1996; 13: 161-171

72. Heikkinen J, Maentausta O, Tuimala R, Ylostalo P, Janne O. Amniotic fluid bile acids in normal and pathologic pregnancy. Obstet Gynecol 1980; 56: 60-4.

73. Heikkinen J, Maentausta O, Ylostalo P, Janne O. Changes in serum bile acid concentrations during normal pregnancy, in patients with intrahepatic

cholestasis of pregnancy and in pregnant women with itching. Br J Obstet Gynaecol 1981; 88: 240-245.

74. Heikkinen J, Maentausta O, Ylostalo P, Janne O. Serum bile acid levels in intrahepatic cholestasis of pregnancy during treatment with phenobarbital or cholestyramine. Eur J Obstet Gynecol Reprod Biol 1982; 14: 153-162.

75. Heikkinen J. Serum bile acids in the early diagnosis of intrahepatic cholestasis of pregnancy. Obstet Gynecol 1983; 61: 581-587.

76. Heinonen S, Kirkinen P. Pregnancy outcome with intrahepatic cholestasis. Obstet Gynecol 1999; 94: 189-193.

77. Herre HD, Engelmann C, Wiken HP. Diagnosis and therapy of blood coagulation disorders in intrahepatic cholestasis. Zentralbl Gynakol 1976; 98: 217-219.

78. Heuman DM, Pandak WM, Hylemon PB, Vlahcevic ZR. Conjugates of ursodeoxycholate protect against cytotoxicity of more hydrophobic bile salts: in vitro studies in rat hepatocytes and human erythrocytes. Hepatology 1991a; 14: 920-6.

79. Heuman DM, Mills AS, McCall J, Hylemon PB, Pandak WM, Vlahcevic ZR. Conjugates of ursodeoxycholate protect against cholestasis and hepatocellular necrosis caused by more hydrophobic bile salts. In vivo studies in the rat. Gastroenterology 1991b; 100: 203-11.

80. Heuman DM, Bajaj R. Ursodeoxycholate conjugates protect against disruption of cholesterol- rich membranes by bile salts. Gastroenterology 1994; 106: 1333-41.

81. Higgins, C.F., Hiles, I.D., Salmond, G.P., Gill, D.R., Downie, J.A., Evans, I.J., Holland, I.B., Gray, L., Buckel, S.D., Bell, A.W., et al. A family of related ATP-binding subunits coupled to many distinct biological processes in bacteria. Nature 1986; 323: 448-50.

82. Higgins, C.F. ABC transporters: from microorganisms to man. Ann. Rev. Cell. Biol. 1992; 6, 67-113.

83. Hirohashi T, Suzuki H, Takikawa H, Sugiyama Yl. ATP-dependent transport of bile salts by rat multidrug resistance-associated protein 3 (Mrp3). J Biol Chem 2000; 275: 2905-2910.

84. Hirvioja ML, Tuimala R, Vuori J. The treatment of intrahepatic cholestasis of pregnancy by dexamethasone. Br J Obstet Gynaecol 1992; 99: 109-111.

85. Hirvioja ML, Kivinen S. Inheritance of intrahepatic cholestasis of pregnancy in one kindred. Clin Genet 1993;43:315-7.

86. Hofmann AF. The syndrome of ileal disease and the broken enterohepatic circulation: cholerhetic enteropathy. Gastroenterology 1967; 52: 752-757.

87. Hofmann AF. The continuing importance of bile acids in liver and intestinal disease. Arch Int Med. 1999; 159: 2647-2658.

88. Holzbach R, Sivak DA, Braun WE. Familial recurrent intrahepatic cholestasis of pregnancy: a genetic study providing evidence for transmission of a sex-limited, dominant trait. Gastroenterology 1983;85:175-9.

89. Hori M, Sato H, Iwai K, Takashima S, Sato H, Inoue M, Kitabatake A, Kamada T. Disruption of microtubules in cultured neonatal rat cardiomyocytes during rapid contractions; protective effects of beta-adrenoceptor antagonist. Jpn Circ J 1992; 56: 62-68.

90. Huang L, Smit JW, Meijer DK, Vore M. Mrp2 is essential for estradiol-17-β (β-D-glucuronide)-induced choelstasis in rats. Hepatology 2000; 32: 66-72.

91. Hung, L-W., Wang, I.X., Nikaido, K., Liu, P-Q., Ames, G.F-L., Kim, S-H. Crystal structure of the ATP- binding subunit of an ABC transporter. Nature 1998; 396, 703-707.

92. Husse B, Wussling M. Developmental changes of calcium transients and contractility during the cultivation of rat neonatal cardiomyocytes. Mol and Cell Biochem. 1996; 163-164: 13-21.

93. Ishikawa T, Muller M, Klunemann C, Schaub T, Keppler D. ATP-dependent transport of cysteinyl leukotrienes across liver canalicular membranes. J Biol Chem. 1990; 265: 19279-19286.

94. Israel E, Guzman M, Campos G. Maximal response to oxytocin of the isolated myometrium from pregnant patients with intrahepatic cholestasis. Acta Obstet Gynecol Scand 1986; 65: 581-582.

95. Iwaki, K., Sukhatme, V.P., Shubeita, H.E., Chein, K.R. α and β - adrenergic stimulation induces distinct patterns of immediate early gene expression in neonatal rat myocardial cells. J. Biol.Chem. 1990; 265, 13809-13817.

96. Jacquemin, E., Crestil, D., Manouvier, S., Boute, O., Hadchouel, M. Heterozygous non-sense mutation in the MDR3 gene in familial intrahepatic cholestasis of pregnancy. Lancet 1999; 353, 210-211.

97. Jacquemin E, De Vree JM, Cresteil D, Sokal EM, Sturm E, Dumont M, Scheffer GL, Paul M, Burdelski M, Bosma PJ, Bernard O, Hadchouel M, Elferink RP. The wide spectrum of multidrug resistance 3 deficiency: from

neonatal cholestasis to cirrhosis of adulthood. Gastroenterology 2001; 120: 1448-1458.

98. Janowski BA, Willy PJ, Devi TR, Falck JR, Mangelsdorf DJ. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. Nature 1996; 383: 728-731.

99. Jansen PLM. The pathophysiology of cholestasis with special reference to primary biliary cirrhosis. *Baillière's Clin Gastroenterol* 2000; **14**: 571-583.

100. Jedlitschky G, Leier I, Buchholz U, Barnouin K, Kurz G, Keppler D. Transport of glutathione, glucuronate, and sulfate conjugates by the MRP gene-encoded conjugate export pump. Cancer Res. 1996; 56: 988-994.

101. Johnson P, Samsioe G, Gustafson A. Studies in cholestasis of pregnancy II. Serum lipids and lipoproteins. Acta Obstet Gynec Scand 1975; 54: 105-111.

102. Jongsma HJ, Masson-Pevet M, Tsijernina L. The development of beat-rate synchronization of rat myocyte pairs in cell culture. Basic Res Cardiol. 1987; 82: 454-464.

103. Kaar K, Jouppila P, Kuikka J, Luotala H, Toivanen J, Rekonen A. Intervillous blood flow in normal and complicated late pregnancy measured by means of and intravenous 133Xe method. Acta Obstet Gynecol Scand 1980; 59: 7-10.

104. Kartenbeck J, Leuschner U, Mayer R, Keppler D. Absence of the canalicular isoform of the MRP gene-encoded conjugate export pump from the hepatocytes in Dubin-Johnson syndrome. Hepatology 1996; 23: 1061-1066.

105. Kauppila A, Korpela H, Makila UM, Yrjanheikki E. Low serum selenium concentration and glutathione peroxidase activity in intrahepatic cholestasis of pregnancy. BMJ 1987; 294: 150-152.

106. Kenyon A, Nelson Piercy C, Girling J, Williamson C, Tribe RM, Shennan AH. Obstetric cholestasis, outcome with active management: a series of 70 cases. Br.J Obstet.Gynaecol. 2002; 109: 1-7.

107. Keppler D, Konig J. Hepatic secretion of conjugated drugs and endogenous substances. Semin Liver Dis. 2000; 20: 265-272.

108. Kimura H, Oyamada Y, Ohshika H, Mori M, Oyamada M. Reversible inhibition of gap junctional intercellular communication, synchronous contraction, and synchronism of intracellular Ca^{2+} fluctuation in cultured neonatal rat cardiac myocytes by heptanol. Exp Cell Res. 1995; 220: 348-56.

109. Korchev YE, Gorelik J, Lab MJ, Sviderskaya EV, Johnston CL, Coombes CR, Vodyanoy I, Edwards CR. Cell volume measurement using Scanning Ion Conductance Microscopy. Biophys. J. 2000; 78, 451-457.

110. Kouzuki H, Suzuki H, Steiger B, Meier PJ, Sugiyama Y. Characterization of the transport properties of Organic Anion Transporting Polypeptide 1 (oatp 1) and Na+/Taurocholate Cotransporting Polypeptide (Ntcp): comparative studies on the inhibitory effect of their possible substrates in hepatocytes and cDNA-transfected COS-7 cells. J Pharmacol Exp Ther 2000; 292: 505-511.

111. Kreek MJ, Weser E, Sleisennger MH, Jeffries GH. Idiopathic cholestasis of pregnancy: The response to challenge with the synthetic estrogen, ethinyl estradiol. N Engl J Med 1967; 277: 1391-1395.

112. Kretowicz E, McIntyre HD. Intrahepatic cholestasis of pregnancy, worsening after dexamethasone. Aust N Z J Obstet Gynaecol 1994; 34: 211-213.

113. Kruglyak L. Prospects for whole-genome linkage disequilibrium mapping of

common disease genes. Nature Genetics 1999; 22: 139-144.

114. Kudo Y and Boyd CAR. The role of L-tryptophan transport in L-tryptophan degradation by indoleamine 2,3-dioxygenase in human placental explants. J Physiol 2001; 531: 417-423.

115. Kullak-Ublick GA., Ismair MG., Stieger B., Landmann L., Huber R., Pizzagalli F., Fattinger K., Meier PJ. and Hagenbuch B. Organic anion-transporting polypeptide B (OATP-B) and its functional comparison with three other OATPs of human liver. Gastroenterology 2001; 120, 525-533.

116. Kunkel, L. M., K. D. Smith, S. H. Boyer, D. S. Borgaonkar, S. S. Wachtel, O. J. Miller, W. R. Breg, H. W. Jones, and J. M. Rary.. Analysis of human Y chromosome-specific reiterated DNA in chromosome variants. Proceedings of The National Academy of Sciences, U. S. A. 1977; 74: 1245-1249.

117. Laatikainen T and Hesso A. Determination of serum bile acids by glass capillary gas-liquid chromatography. Clin Chim Acta 1975a; 64: 63-68.

118. Laatikainen T, Ikonen E. Fetal prognosis in obstetric hepatosis. Ann Chir Fenn 1975b; 64: 155-164.

119. Laatikainen T. Fetal bile acid levels in pregnancies complicated by maternal intrahepatic cholestasis. Am J Obstet Gynecol 1975c;122: 852-6.

120. Laatikainen T, Ikonen E. Serum bile acids in cholestasis of pregnancy. Obstet Gynecol 1977; 50: 313-318.

121. Laatikainen T. Effect of cholestyramine and phenobarbital on pruritus and serum bile acid levels in cholestasis of pregnancy. Am J Obstet Gynecol 1978; 132: 501-506

122. Laatikainen T, Tulenheimo A. Maternal serum bile acid levels and fetal distress in cholestasis of pregnancy. Int J Gynaecol Obstet 1984; 22: 91-94.

123. Larsson-Cohn U, Stenram U. Jaundice during treatment with oral contraceptive agents. JAMA 1965; 193: 422-427.

124. Lee JM, Trauner M, Soroka CJ, Stieger B, Meier PJ, Boyer JL. Expression of the bile salt export pump is maintained after chronic cholestasis in the rat. Gastroenterology 2000; 118: 163-172.

125. Levy, M.N., Wiseman, M.N. Electrophysiologic mechanisms for ventricular arrhythmias in left ventricular dysfunction: electrolytes, catecholamines and drugs. J Clin Pharmacol. 1991 ; 31, 1053-1060

126. Linke, C. R., Smit, J. J. M., van der Velde-Koerts, T., Borst, P. Structure of the Human MDR3 gene and Physical Mapping of the Human MDR3 Locus. J. Biol. Chem. 1991; 266: 5303-5310.

127. Locatelli A, Roncaglia N, Arreghini A, Bellini P, Vergani P, Ghidini A. Hepatitis C virus infection is associated with a higher incidence of cholestasis of pregnancy. Br J Obstet Gynaecol 1999; 106: 498-500.

128. Loder N. Genetic variations can point the way to disease genes. Nature 1999; 401: 734.

129. Loo TW, Clarke DM. Membrane topology of a cysteine-less mutant of human P-glycoprotein. J. Biol. Chem. 1995; 270, 838-848.

130. Lopez JR, Jovanovic A, Terzic A. Spontaneous Calcium waves without contraction in cardiac myocytes. Biochem & Biophys Res Com. 1995; 214, 781-787

131. Lu TT, Makishima M, Repa JJ, Schoonjans K, Kerr TA, Auwerx J, Mangelsdorf DJ. Molecular Cell Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. 2000; 6: 507-515.

132. Macdonald IA, Hutchison DM, Forrest TP. Formation of urso- and ursodeoxy-cholic acids from primary bile acids by *Clostridium absonum.* J Lipid Res 1981; 22: 458-466.

133. Lunzer M, Barnes P, Byth K, O'Halloran M. Serum bile acid concentrations during pregnancy and their relationship to obstetric cholestasis. Gastroenterology 1986; 91: 825-829.

134. Lunzer MR. Jaundice in pregnancy. Baillieres Clin Gastroenterol 1989; 3: 467-483.

135. Madar J, Richmond S, Hey E. Surfactant-deficient respiratory distress after elective delivery at 'term'. Acta Paediatr. 1999; 88: 1244-1248.

136. Maggiore G, Bernard O, Riely CA, Hadchouel M, Lemonnier A, Alagille D. Diagnostic value of serum gamma-glutamyl transpeptidase activity in liver diseases in children. J. Pediatr. Gastroenterol. Nutr. 1991; 12: 21-26.

137. Magowan BA, Bain M, Juszczak E, McInneny K. Neonatal mortality amongst Scottish preterm singleton births (1985- 1994). Br.J Obstet.Gynaecol. 1998; 105: 1005-1010.

138. Makashima M, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, Hull MV, Lustig KD, Mangelsdorf DJ, Shan B. Identification of a nuclear receptor for bile acids. Science 1999; 284: 1362-1365.

139. Marin JJ., Serrano MA., el Mir MY., Eleno N. and Boyd R. Bile acid transporter by basal membrane vesicles of human term placental trophoblast. Gastroenterology 1990; 99: 1431-1438.

140. Marin JJ., Bravo P., el Mir MY. and Serrano MA. ATP-dependent bile acid transport across microvillous membrane of human term trophoblast. Am.J.Physiol 1995; 268: G685-G694.

141. Mauricio AC, Slawik M, Heitzmann D, von Hahn T, Warth R, Bleich M, Gregor R. Deoxycholic acid (DOC) affects the transport properties of distal colon. Pflugers Arch 2000; 439: 532-540.

142. Mazzella G, Rizzo N, Salzetta A, Iampieri R, Bovicelli L, Roda E. Management of intrahepatic cholestasis in pregnancy. Lancet 1991; 338: 1594-1595.

143. Mazzella G, Rizzo N, Azzaroli F, Simoni P, Bovicelli L, Miracolo A, Simonazzi G, Colecchia A, Nigro G, Mwangemi C, Festi D, Roda E, Nicola R, Francesco A, Patrizia S, Luciano B, Anna M, Giuliana S, Antonio C, Giovanni N, Constance M, Davide F, Enrico R. Ursodeoxycholic acid administration in patients with cholestasis of pregnancy: effects on primary bile acids in babies and mothers. Hepatology 2001; 33: 504-8.

144. Mei Q, Liang BT. P2 purinergic receptor activation enhances cardiac contractility in isolated rat and mouse hearts. Am J Physiol Heart Circ Physiol 2001; 281: H334-H341.

145. Meier PJ, Knickelbein R, Moseley RH, Dobbins JW, Boyer JL. Evidence for carrier-mediated chloride/bicarbonate exchange in canalicular rat liver plasma membrane vesicles. J Clin Invest 1985; 75: 1256-1263.

146. Metherall JE, Li H, Waugh K. Role of multidrug resistance P-glycoproteins in cholesterol biosynthesis. J Biol Chem 1996; 271: 2634-2640.

147. Meyers M, Slikker W, Vore M. Steroid D-ring glucoronides: characterisation of a new class of cholestatic agents in the rat. J Pharmacol Exp Ther 1981; 218: 63-73.

148. Milkiewicz P, Gallagher R, Eggington E, Kilby M, Weaver JB, Elias E. Obstetric cholestasis with elevated GGT: incidence, presentation and treatment. Hepatology 2001; 34: A364.

149. Misra PS, Evanov FA, Wessely Z, Rosenblum GA, Singh P. Idiopathic intrahepatic cholestasis of pregnancy. Report of an unusual case and review of the literature. Am J Gastroenterology 1980; 73: 54-59.

150. Miyamoto, Y., Umebayashi, Y., Nishisaka, T. Comparison of phototoxicity mechanism between pulsed and continuous wave irradiation in photodynamic therapy. J Photochem Photobiol B. 1999; 53, 53-59.

151. Modi N, Lewis H, Al Naqeeb N, Ajayi-Obe M, Dore CJ, Rutherford M. The effects of repeated antenatal glucocorticoid therapy on the developing brain. Pediatr Res 2001; 50: 581-5.

152. Morrison JJ, Rennie JM, Milton PJ. Neonatal respiratory morbidity and mode of delivery at term: influence of timing of elective caesarean section. Br.J Obstet.Gynaecol. 1995; 102: 101-6.

153. Muir TM, Hair J, Inglis GC, Dow JW, Lindop GB, Leckie BJ. Dexamethasone-induced differentiation of atrial myocytes in culture. Am J Physiol 1992; 263: H722-H729.

154. Nathanson MH, Burgstahler AD, Masyuk A, LaRusso NF. Stimulation of ATP secretion in the liver by therapeutic bile acids. Biochem J 2001; 358: 1-5.

155. Nicastri PL, Diaferia A, Tartagni M, Loizzi P, Fanelli M. A randomised placebo-controlled trial of ursodeoxycholic acid and S-adenosylmethionine in the treatment of intrahepatic cholestasis of pregnancy. Br J Obstet Gynaecol 1998; 105: 1205-7.

156. Nickerson DA, Taylor SL, Weiss KM, Clark AG, Hutchinson RG, Stengård J, Salomaa V, Vartiainen E, Boerwinkle E, Sing CF. DNA sequence diversity in a 9.7kb region of the human lipoprotein lipase gene. Nature Genetics 1998; 19: 233-240.

157. Oude Elferink RPJ, van Berge Henegouwen GP. Cracking the genetic code for benign recurrent and progressive familial intrahepatic cholestasis. J Hepatol. 1998; 29: 317-320.

158. Page, E., Shibata, Y. Permeable junctions between cardiac cells. Ann Rev Physiol. 1981; 43, 431-441

159. Palma J, Reyes H, Ribalta J, Iglesias J, Gonzalez MC, Hernandez I, Alvarez C, Molina C, Danitz AM. Effects of ursodeoxycholic acid in patients with intrahepatic cholestasis of pregnancy. Hepatology 1992; 15: 1043-1047.

160. Palma J, Reyes H, Ribalta J, Hernandez I, Sandoval L, Almuna R, Liepins J, Lira F, Sedano M, Silva O, Toha D, Silva JJ. Ursodeoxycholic acid in the treatment of cholestasis of pregnancy: a randomized, double-blind study controlled with placebo. J Hepatol 1997; 27: 1022-8.

161. Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kleiwer SA, Stimmel JB, Willson TM, Zavacki AM, Moore DD, Lehmann JM. Bile acids: natural ligands for an orphan nuclear receptor. Science 1999; 284: 1365-1368.

162. Payne, A.S., Kelly, E.J., Gitlin, J.D. Functional expression of the Wilson disease protein reveals mislocalisation and impaired copper-dependent trafficking of the common H1069Q mutation. Proc. Natl. Acad. Sci. U.S.A. 1998; 95, 10854-10859.

163. Peet DJ, Turley SD, Ma WM, Janowski BA, Lobaccaro J-MA, Hammer RE, Mangelsdorf DJ. Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXRα. Cell 1998a; 93: 693-704.

164. Peet DJ, Janowski BA, Mangelsdorf DJ. The LXRs: a new class of oxysterol receptors. Curr Opin Genet Dev 1998b; 8: 571-575.

165. Perreau P, Rouch R. Ictere cholestatique recidivant de la grosseusse. Gynecol Obstet 1961; 60: 161-179.

166. Putterman C, Keidar S, Brook JG. Benign recurrent intrahepatic cholestasis – 25 years of follow-up. Postgrad Med J 1987; 63: 295-296.

167. Rauramo I, Forss M. Effect of exercise on placental blood flow in pregnancies complicated by hypertension, diabetes or intrahepatic cholestasis. Acta Obstet Gynecol Scand 1988; 67: 15-20.

168. Reid R, Ivey KJ, Rencoret RH, Storey B. Fetal complications of obstetric cholestasis. Br Med J 1976;1:870-2.

169. Reid L, Morrow B, Jubinsky P, Schwartz E, Gatmaitan Z. Regulation of growth and differentiation of epithelial cells by hormones, growth factors, and substrates of extracellular matrix. Ann N Y Acad Sci 1981; 372: 354-70.

170. Reilly AM, Sun X, Williams DA, Dusting GJ. Dexamethasone inhibits endotoxin-induced changes in calcium and contractility in rat isolated papillary muscle. Cell Calcium 1999; 26: 1-8.

171. Reizer J, Reizer A, Saier MH. A functional superfamily of sodium/ solute symporters. Biochim Biophys Acta 1994; 1197: 133-166.

172. Reyes H, Ribalta J, Gonzalez-Ceron M. Idiopathic cholestasis of pregnancy in a large kindred. Gut 1976;17:709-13.

173. Reyes H, Gonzalez MC, Ribalta J. Prevalence of intrahepatic cholestasis of pregnancy in Chile. Ann Int Med 1978; 88: 487-493.

174. Reyes H, Taboada G, Ribalta J. Prevalence of intrahepatic cholestasis of pregnancy in La Paz, Bolivia. J Chron Dis 1979; 32: 499-504.

175. Reyes H. The enigma of intrahepatic cholestasis of pregnancy: lessons from Chile. Hepatology 1982; 2: 87-96.

176. Reyes H, Radrigan ME, Gonzalez MC, Latorre R, Ribalta J, Segovia N, Alvarez C, Andresen M, Figueroa D, Lorca B. Steatorrhea in patients with intrahepatic cholestasis of pregnancy. Gestroenterology 1987; 93: 584-590.

177. Reyes H. The spectrum of liver and gastrointestinal disease seen in cholestasis of pregnancy. Gastroenterol Clin North Am 1992; 21: 905-921.

178. Reyes H, Ribalta J, Hernandez I, Arrese M, Pak N, Wells M, Kirsch RE. Is dietary erucic acid hepatotoxic in pregnancy? An experimental study in rats and hamsters. Hepatology 1995; 21: 1373-1379.

179. Reyes H. Intrahepatic cholestasis. A puzzling disorder of pregnancy. Gastroenterol Hepatol. 1997; 12: 211-216.

180. Reyes H, Baez ME, Gonzalez MC, Hernandez I, Palma J, Ribalta J, Sandoval L, Zapata R. Selenium, zinc and copper plasma levels in intrahepatic cholestasis of pregnancy, in normal pregnancies and in healthy individuals in Chile. J Hepatol. 2000; 32: 542-549.

181. Ribalta J, Reyes H, Gonzalez MC, Iglesias J, Arrese M, Poniachik J, Molina C, Segovia N. S-adenosyl-L-methionine in the treatment of patients with intrahepatic cholestasis of pregnancy: a randomized, double-blind, placebo-controlled study with negative results. Hepatology 1991; 13: 1084-1089.

182. Riikonen S, Savonius H, Gylling H, Nikkila K, Tuomi AM, Miettinen TA. Oral guar gum, a gel-forming dietary fiber relieves pruritus in intrahepatic cholestasis of pregnancy. Acta Obstet Gynecol Scand 2000; 79: 260-264

183. Rioseco AJ, Milenko B, Manzur A, Hamed F, Kato SR, Parer JT, Germain AM. Intrahepatic cholestasis of pregnancy: a retrospective case-control study of perinatal outcome. Am J Obstet Gynecol 1994;170: 890-895.

184. Rodien P, Bremont C, Sanson MLR, Parma J, van Sande J, Costagliola S, Luton JP, Vassart G, Duprez L. Familial gestational hyperthyroidism caused by a mutant thyrotropin receptor hypersensitive to human chorionic gonadotropin. N Engl J Med. 1998; 339: 1823-1826.

185. Rook, M.B., van Ginneken, A.C., de Jonge, B., el Aoumari, A., Gros, D., Jongsma, H.J. Differences in gap junction channels between cardiac myocytes, fibroblasts, and heterologous pairs. Am J Physiol. 1992; 263, C959-77

186. Rosmorduc O, Hermelin B, Poupon R. MDR3 gene defect in adults with symptomatic intrahepatic and gallbladder cholesterol cholelithiasis. Gastroenterology 2001; 120: 1459-67.

187. Ruetz, S., Gros, P. Phosphatidylcholine translocase: a physiological role for the mdr2 gene. Cell 1994; 77, 1071-81.

188. Sadler LC, Lane M, North R Severe fetal intracranial haemorrhage during treatment with cholestyramine for Intrahepatic cholestasis of pregnancy. Br J Obstet Gynaecol 1995 ; 102 : 169-170.

189. Schreiber AJ, Simon FR. Estrogen-induced cholestasis; clues to pathogenesis and treatment. Hepatology 1983; 3: 607-613.

190. Sepulveda WH, Gonzalez C, Cruz MA, Rudolph MA. Vasoconstrictive effect of bile acids on isolated human placental chorionic veins. Eur J Obstet Gynecol Reprod Biol 1991; 42: 211-215.

191. Serrano MA, Brites D, Larena MG, Monte MJ, Bravo MP, Oliveira N, Marin JJ. Beneficial effect of ursodeoxycholic acid on alterations induced by cholestasis of pregnancy in bile acid transport across the human placenta. J Hepatol. 1998; 28: 829-839.

192. Shaw D, Frohlich J, Wittmann B, Willms M. A prospective study of 18 patients with cholestasis of pregnancy. Am J Obstet Gynecol 1982; 142: 621-625.

193. Shriver MD, Smith MW, Jin L, Marcini A, Akey JM, Deka R, Ferrell RE. Ethnic-affiliation estimation by use of population-specific DNA markers. American Journal of Human Genetics 1998; 60: 957-964.

194. Simon FR, Gonzalez M, Sutherland E, Accatino L, Davis RA. Reversal of ethinyl estradiol-induced bile secretory failure with Triton WR-1339. J Clin Invest 1980; 65: 851-859.

195. Simon FR, Fortune J, Iwahashi M, Gartung C, Wolkoff, Sutherland E. Ethinyl estradiol cholestasis involves alterations in expression of liver sinusoidal transporters. Am J Physiol 1996; 34: G1043-G1052.

196. Sjovall K, Sjovall J. Serum bile acid levels in pregnancy with pruritus. Clin Chim Acta 1966; 13: 207-211.

197. Smit JJM, Schinkel AH, Ouse Elferink RPJ, Groen AK, Wagenaar E, van Deemter L, Mol CAAM, Ottenhoff R, van der Lugt NMT, van Roon MA, van der Valk MA Offerhaus GJA, Berns AJM, Borst P. Homozygous disruption of the murine *mdr2* P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. Cell 1993; 75: 451-462.

198. Smit LS, Strong TV, Wilkinson DJ, Macek Jnr M, Mansoura MK, Wood DL, Cole JL, Cutting GR, Cohn JA, Dawson DC. Missense mutation (G480C) in the CFTR gene associated with protein mislocalisation but normal chloride channel activity. Hum. Mol. Genet. 1995; 4, 269-273.

199. Smith, A.J., Timmermans-Hereijgers, J.L., Roelofsen, B., Wirtz, K.W., van Blitterswijk, W.J., Smit, J.J., Schinkel, A.H., Borst, P. The human MDR3 P-glycoprotein promotes translocation of phosphatidylcholine through the plasma membrane of fibroblasts from transgenic mice. FEBS Lett. 1994; 354, 263-6.

200. Smith, AJ, de Vree ML, Ottenhoff R, Oude Elferink RPJ, Schinkel AH, Borst P. Hepatocyte specific expression of the human *MDR3* P-glycoprotein gene restores the biliary phosphatidylcholine excretion absent in *Mdr1* (-/-) mice. Hepatology 1998; 28: 530-536.

201. Soen Y, Cohen N, Lipson D, Braun E. Emergence of spontaneous rhythm disorders in self-assembled networks of heart cells. Physical Review Letters 1999; 82: 3556-3559.

202. St-Pierre M.V., Serrano MA Macias RIR Dubs U Hoechli M Lauper U Meier PJ & Marin JJG. Expression of members of the multidrug resistance protein family in human term placenta. Am J Physiol Regulatory Integrative Comp Physiol. 2000; 279: R1495-R1503.

203. Steiger B, Fattinger K, Madon J, Kullak-Ublick, Meier PJ. Drug- and estrogen-induced cholestasis through inhibition of the hepatocellular bile salt export pump (Bsep) of rat liver. Gastroenterology 2000; 118: 422-430.

204. Steiger B. FIC1: another bile salt carrier within the enterohepatic circulation? J Hepatol. 2001; 35: 522-524.

205. Strautnieks SS, Kagalwalla AF, Tanner MS, Gardiner RM, Thompson RJ. Locus heterogeneity in progressive familial intrahepatic cholestasis. J Med Genet 1996; 33: 833-836.

206. Strautnieks SS, Kagalwalla AF, Tanner MS, Knisely AS, Bull L, Freimer N, Kocoshis SA, Gardiner RM, Thompson RJ.. Identification of a locus for progressive familial intrahepatic cholestasis PFIC2 on chromosome 2q24. Am J Hum Genet 1997; 61: 630-633.

207. Strautnieks SS, Bull LN, Knisely AS, Kocoshis SA, Dah N, Arnell H, Sokal E, Dahan K, Childs S, Ling V, Tanner MS, Kagalwalla AF, Nemeth A, Pawlowska J, Baker A, Mieli-Vergani G, Freimer NB, Gardiner RM, Thompson RJ. A gene encoding a liver-specific ABC transporter is mutated in progressive familial intrahepatic cholestasis. Nature Genet. 1998; 20, 233-238.

208. Svanborg A, Ohlsson S. Recurrent jaundice of pregnancy. Am J Med 1959; 27: 40-49.

209. Tajuddin M, Tariq M, Bilgrami NL, Kumar S. Biochemical and pathological changes in the heart following bile duct ligation. Adv Myocardiol 1980; 2: 209-212.

210. Taniguchi K, Wada M, Kohno K, Nakamura T, Kawabe T, Kawakami M, Kagotani K, Okumura K, Akiyama S, Kuwano M. A human canalicular multispecific organic anion transporter (cMOAT) gene is overexpressed in cisplatin-resistant human cancer cell lines with decreased drug accumulation. Cancer Res. 1996; 56: 4124-4129.

211. Taylor, J.C., Ferry, D.R., Higgins, C.F., Callaghan, R. The equilibrium and kinetic drug binding properties of the mouse P-gp1a and P-gp1b P-glycoproteins are similar. Br. J. Cancer 1999; 81, 783-789.

212. Thibault, N., Ballet, F. Effect of bile acids on intracellular calcium in isolated rat hepatocyte couplets. Biochem Pharmacol. 1993 ; 45, 289-293.

213. Toh S, Wada M, Uchiumi T, Inokuchi A, Makino Y, Horie Y, Adachi Y, Sakisaka S, Kuwano M. Genomic structure of the canalicular multispecific organic anion-transporter gene (*MRP2/ cMOAT*) and mutations in the ATP-binding cassette region in Dubin-Johnson syndrome. Am J Hum Genet. 1999; 64: 739-746.

214. Twisk J, de Wit EC, Princen HM. Suppression of sterol 27-hydroxylase mRNA and transcriptional activity by bile acids in cultured rat hepatocytes. Biochem J. 1995; 305: 505-11.

215. Tygstrup N, Jensen B. Intermittent intrahepatic cholestasis of unknown aetiology in five young males from the Faroe Islands. Med Scand. 1969; 185: 523-530.

216. Ursini F, Maiorino M, Gregolin C. The selenoenzyme phospholipid hydroperoxide glutathione peroxidase. Biochim Biophys Acta 1985; 839; 62-70.

217. van Helvoort, A., Smith, A.J., Sprong, H., Fritzsche, I., Schinkel, A.H., Borst, P., van Meer, G. MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. Cell 1996; 87, 507-17.

218. Vore M, Hadd H, Slikker W. Ethinylestradiol-17β-D-ring glucoronide conjugates are potent cholestatic agents in the rat. Life Sci 1983; 32: 2989-2993.

219. Vore M, Slikker W. Steroid D-ring glucoronides: a new class of cholestatic agents. J Trends Pharmacol Sci 1985; 6: 256-259.

220. Wang H, Chen J, Hollister K, Sowers LC, Forman BM. Endogenous bile acids are ligands for the nuclear receptor FXR/ BAR. Mol Cell 1999; 3: 543-553.

221. Weber AM, Tuchweber B, Roy CC. Neonatal intrahepatic cholestasis in North American Indians: a new entity? Gastroenterology 1979; 77: A46.

222. Weber AM, Tuchweber B, Yousef I, Brochu P, Turgeon C, Gabbiani G, Morin CL, Roy CC. Severe familial cholestasis in North American Indian children: a clinical model of microfilament dysfunction? Gastroenterology 1981; 81: 653-662.

223. White, R.L., Spray, D.C., Schwartz, G.J., Wittenberg, B.A., Bennet, M.V.L. Some electrical and pharmacological properties of gap junctions between adult ventricular myocytes. AmJ Physiol. 1985; 249, C447-455

224. Williams, D.A., Delbridge, L.M., Cody, S.H., Harris, P.J., Morgan, T.O. Spontaneous and propagated calcium release in isolated cardiac myocytes viewed by confocal microscopy .Am.J. Physiol. 1992; 262, C731-742.

225. Froome VA, Forbes S, Robinson S, Johnston DG, de Swiet M and McCarthy MI, Williamson C. The cholestatic effect of raised circulating oestrogen: clinical features and demonstration of the genetic aetiology of obstetric cholestasis in a UK cohort. Journal of Endocrinol. 1998; 156 (Suppl): P51.

226. Williamson and Nelson-Piercy Liver disease in pregnancy. Br J Hosp Med 1997; 58: 213-216.

227. Wojcicka-Jagodzinska J, Kuczynska-Sicinska J, Czajkowksi K, Smolarczyk R. Carbohydrate metabolism in the course of intrahepatic cholestasis of pregnancy. Am J Obstet Gynecol 1989; 161: 959-964.

228. Yang YZ, Guo Q, Jin PY, Yan GC, Pan BS, Pu SY, Chen HZ, Shen JY, Pang BZ, Gong ZX. [Effect of dexamethasone on Coxsackie B-2 virus-infected rat beating heart cells in culture]. Zhongguo Yao Li Xue Bao 1989; 10: 346-9.

229. Yang YZ, Yuan WL, Guo Q, Gong ZX, Zhow TS, Shen JY, Chen HZ. Effect of dexamethasone on Coxsackievirus B2-infected rat beating heart cells in culture. Eur Heart J 1991; 12 Suppl D: 39-43.

230. Yoon YB, Hagey LR, Hofmann AF, Gurantz D, Michelotti EL, Steinbach JH. Gastroenterology 1986; 90: 837-852.

231. Zhou Y, Gottesman MM, Pastan I. Studies of human MDR1-MDR2 chimeras demonstrate the functional exchangeability of a major transmembrane segment of the multidrug transporter and phosphatidylcholine flippase. Mol. Cell. Biol. 1999; 19, 1450-1459.

232. Zimniak, P., Little, J.M., Radominska, A., Oelberg, D.G., Anwer, M.S., Lester, R. Taurine-conjugated bile acids act as Ca2+ ionophores. Biochemistry 1991; 30, 8598-8604.

APPENDIX

I. Questionnaire sent to women with OC

OBSTETRIC CHOLESTASIS QUESTIONNAIRE

We are trying to find the cause of obstetric cholestasis (OC). This condition is characterised by itching, raised bile acids and abnormal liver function tests in pregnant mothers. In addition, some of their children have complications which include fetal distress, premature labour and, in severe cases, stillbirth.

Please help us by giving us some information about your family. All information provided is in confidence.

First, could you answer these questions about yourself?

TODAY'S DATE			
YOUR SURNAME		**YOUR FIRST NAME**	
YOUR HOSPITAL		YOUR HOSPITAL **NUMBER (if known)**	
YOUR DATE OF BIRTH			
YOUR ADDRESS			
DAYTIME TELEPHONE			
EVENING TELEPHONE			
YOUR GP			

Now some questions on your immediate family (please tick the appropriate box).

We are interested in which of your relatives may have had OC, this includes relatives who had itching or jaundice during pregnancy, or who had babies who were born prematurely, or had stillbirths.

1. How many LIVING sisters do you have?			
2. How many have been pregnant?			
3. How many might have had OC?			
	YES	**NO**	**DON'T KNOW**
4. Is you mother alive?			
5. Might she have had OC?			
6. Is your father alive?			
7. Is your mother's mother alive?			
8. Might she have had OC?			
9. How many of your mother's sisters are alive?			
10. How many might have had OC?			
11. Do you have any other relatives who might have had OC?			
12. Please give details, e.g. cousin, niece, daughter, etc.			
13. Have any of your partner's family had OC?			
14. Please give details.			

Since OC may have different causes in people from different parts of the world, we need to know something about the origin of your family. Tick which of these boxes describes this best (you can tick more than one if that seems appropriate)

From Britain/Ireland	
From Scandinavia	
From elsewhere in Europe	
From India	
From Pakistan	
From Bangladesh	
From the Caribbean	
From the Middle East/N Africa	
From Central/ Southern Africa	
From South East Asia	
From China/Hong Kong	
From Latin America	
None of the above	

COMMENTS

Now, some questions about your previous pregnancies

1. *How many pregnancies have you had?*
2. *Did you have severe nausea/ vomiting in pregnancy?*
3. *How many live children do you have?*
4. *In how many pregnancies might you have had OC?*

Please give details

Finally, some questions about your personal and family medical history.

Have you or any of your relatives ever had any of the following conditions?

Condition	Who affected? You	Relative	Please give details
Itch when taking oral contraceptive pill			
Itch during menstrual cycle			
Hepatitis			
Gallstones			
Eczema			
Other			

If your family appears suitable, we may want to contact you for further details. Are you happy for us to do this?

YES	NO

Thank you for taking the time to complete this questionnaire.

II. Phenotype data sheet

OBSTETRIC CHOLESTASIS CLINICAL STUDY DATA SHEET

NAME: **Case no:**

D.O.B:

CLINICAL FEATURES OF INDEX CASE:

Parity ____

Gravidity ____

	Y/N
OC in all pregnancies	___

Clinical features outside pregnancy:

Cyclical itch	___
Oral contraceptive pill-induced itch	___
Gallstones	___

GENETICS:	Y/N	Details
1st degree relative with symptoms suggestive of OC	___	
1st degree relative with gallstones	___	

DRAW FAMILY TREE HERE:

Complete a separate sheet with clinical details for each pregnancy and attach to the back of this document.

Pregnancy number: ____

Sex of the fetus: ____

	Y/N	Gestation (weeks)	Duration
Itch	___	_____	_____
Biochemical abnormalities			
• Abnormal LFT	___	_____	_____
• Abnormal BA	___	_____	_____
• Other	___	_____	_____
Treatment			
• piriton	___	_____	_____
• UDCA	___	_____	_____
• dexamethasone	___	_____	_____
• other	___	_____	_____
Prematurity			
• Spontaneous	___	_____	
• Iatrogenic	___	_____	
Fetal distress			
• Meconium stained liquor	___	_____	
• CTG abnormalities	___	_____	
• SCBU for > 3 days	___		
Intrauterine Death	___	_____	
Mode of delivery			
• Vaginal delivery	___		
• Elective CS	___		
• Emergency CS	___		
Any future health problems for the child (please give short description)	___		

III. OC pedigrees from all cases with a positive family history of pruritus in pregnancy.

KEY:

= Female

= Male

= Obstetric cholestasis

= Gallstones

= Cyclical pruritus

= Oral contraceptive pill-induced pruritus

= Probable OC (no biochemical confirmation of diagnosis)

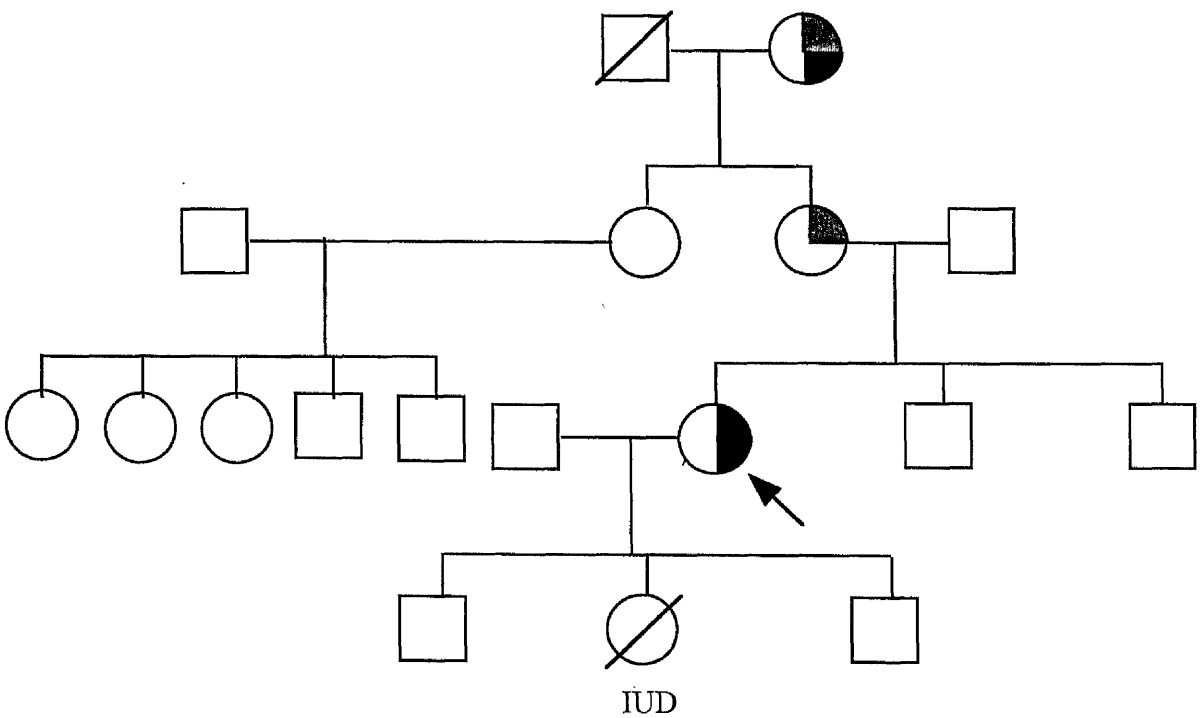
= Gallstones in a male relative

IUD = Intrauterine Death

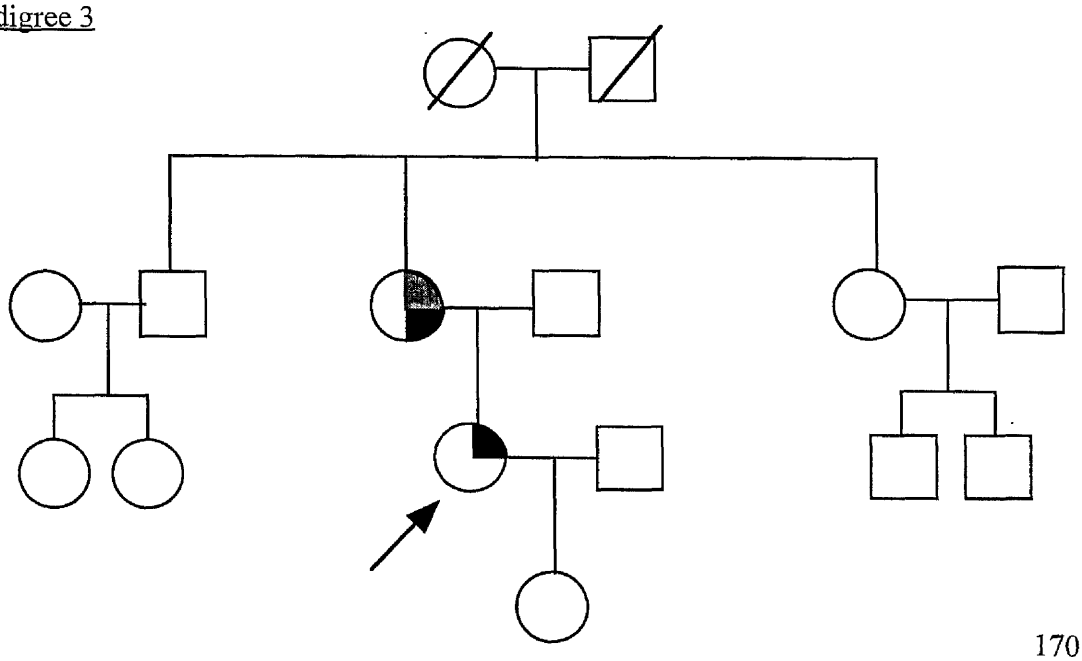
= Gender not known

Pedigree 1: see Figure 9, page 67.

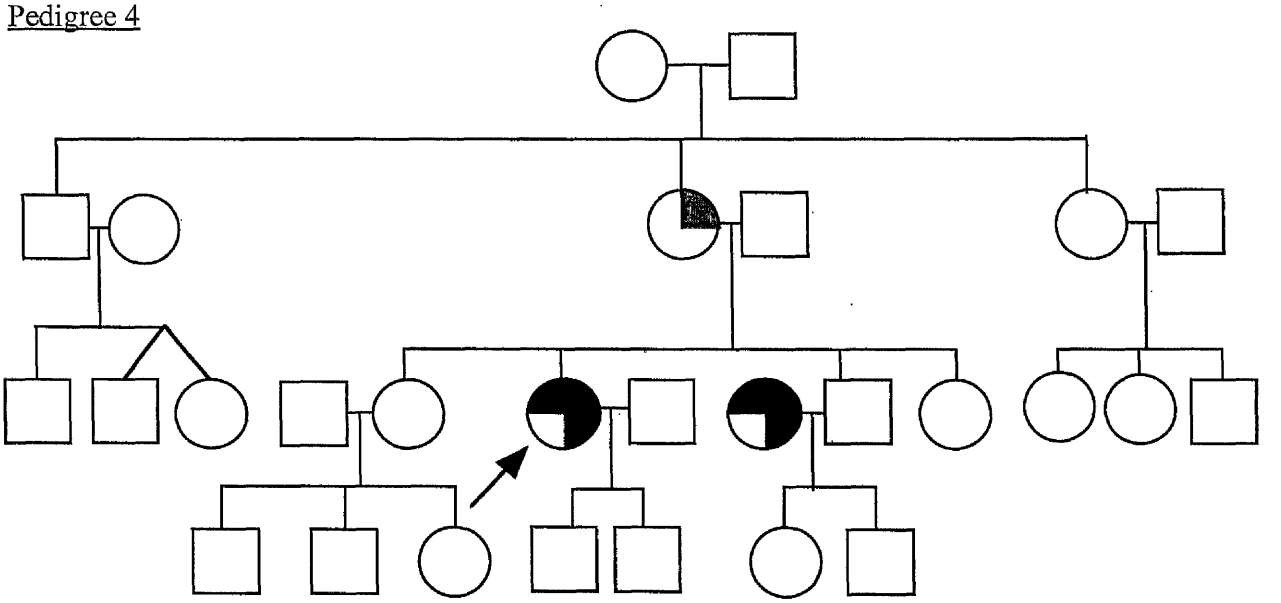
Pedigree 2



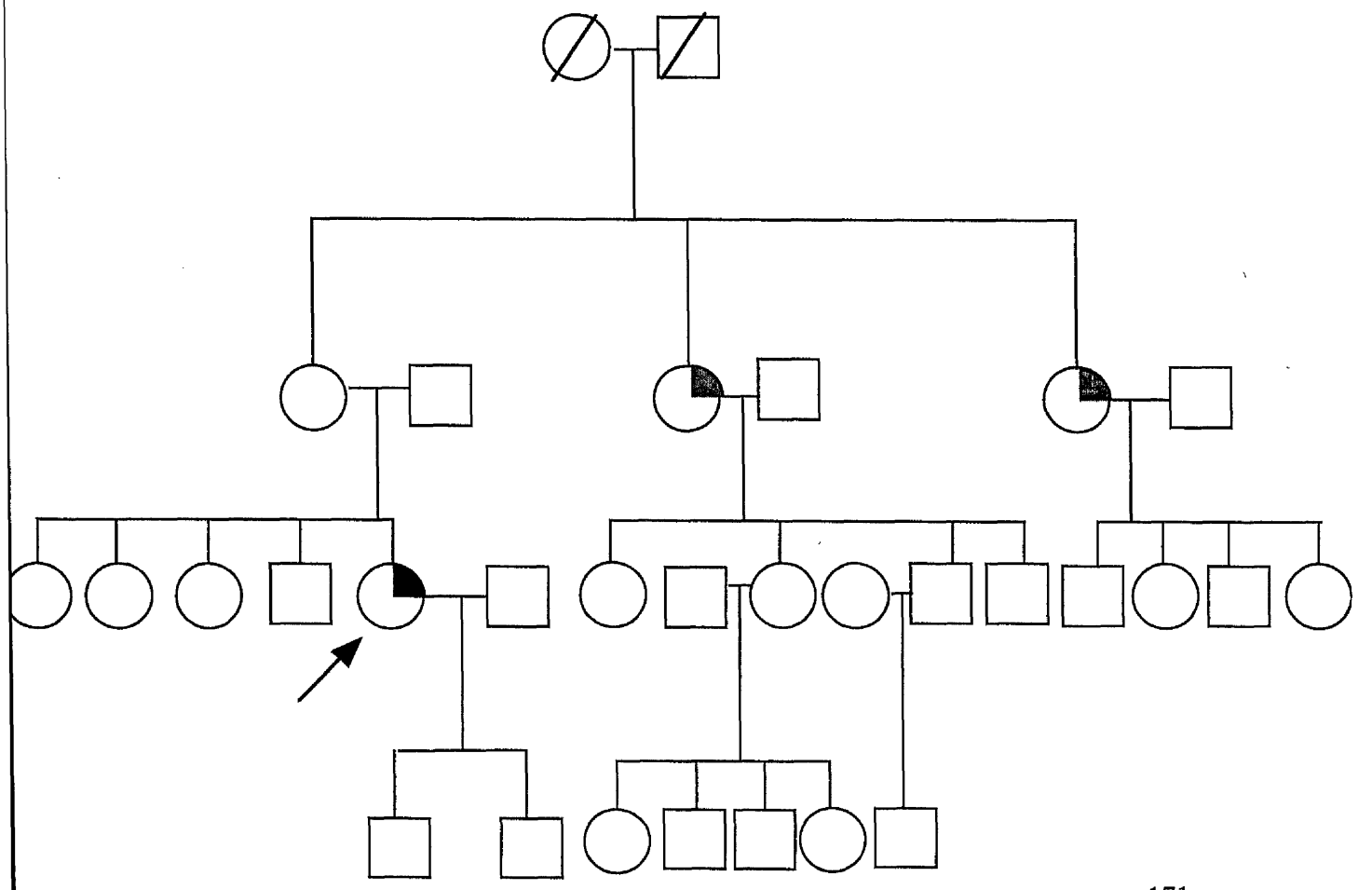
Pedigree 3



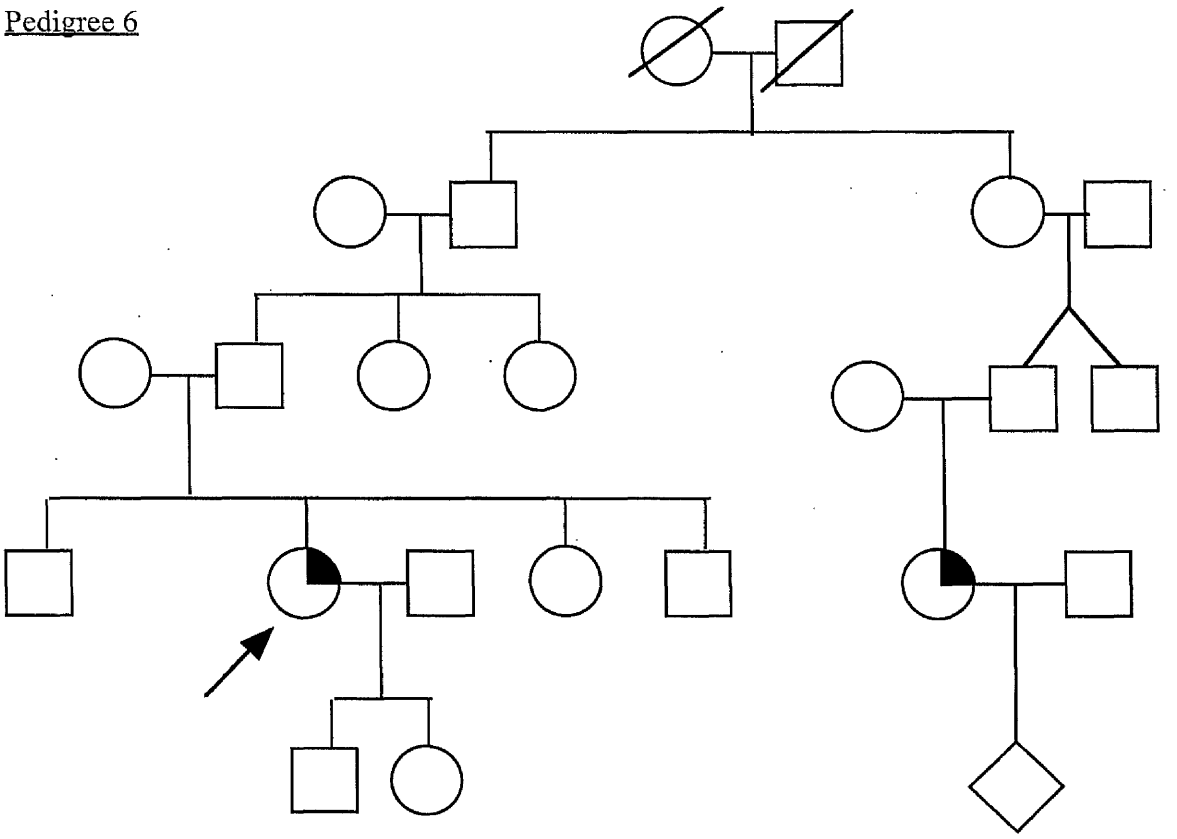
Pedigree 4



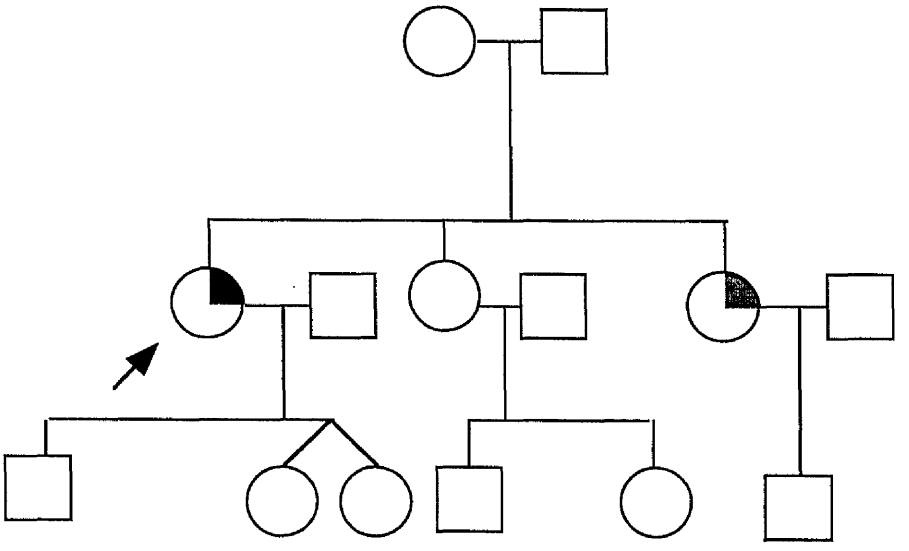
Pedigree 5



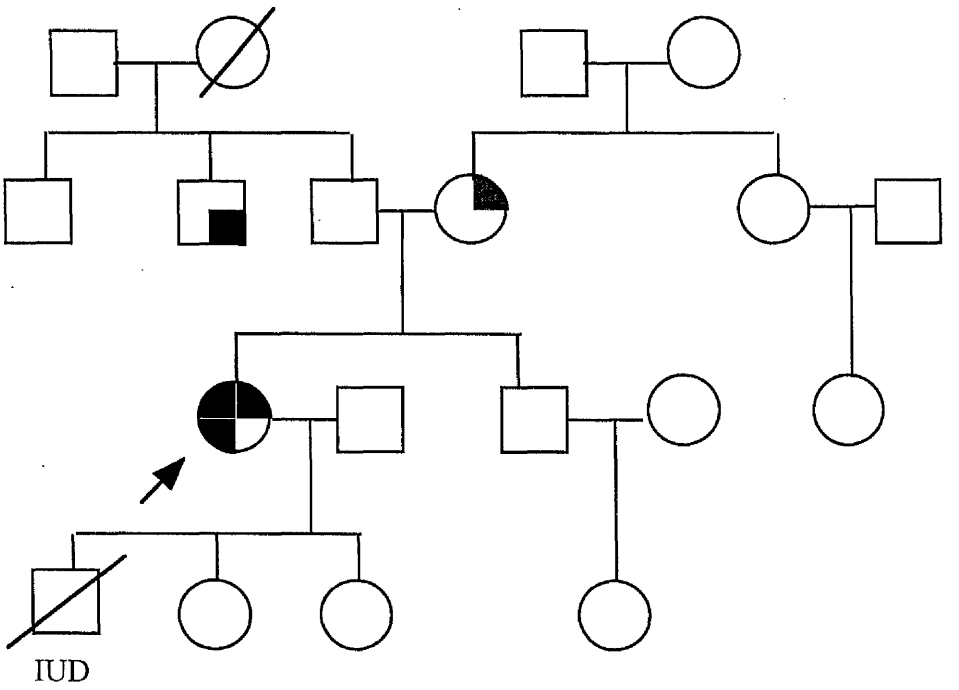
Pedigree 6



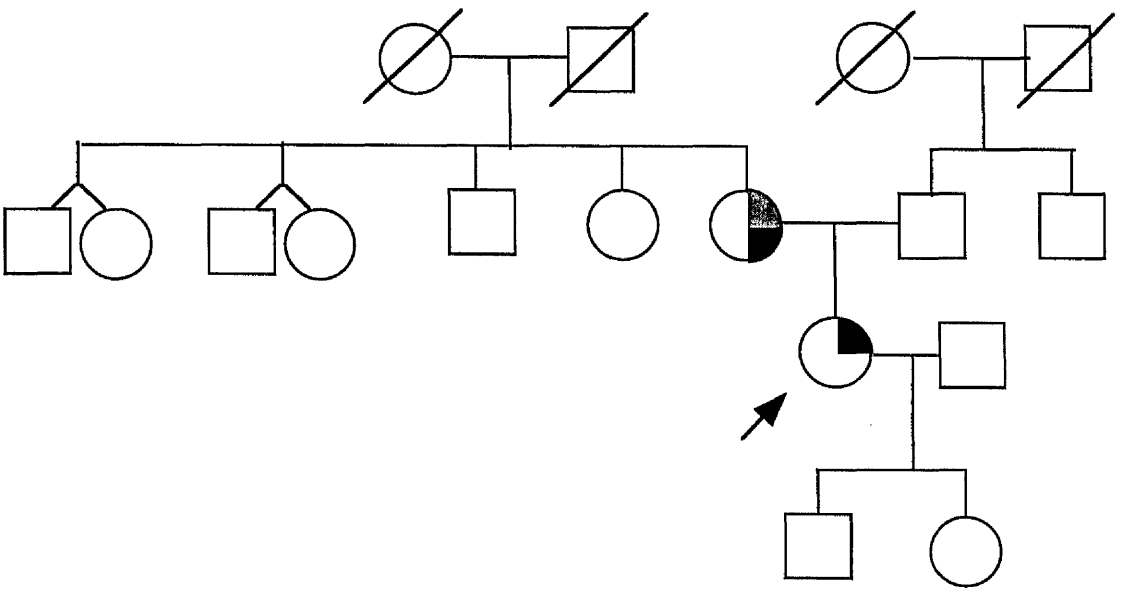
Pedigree 7



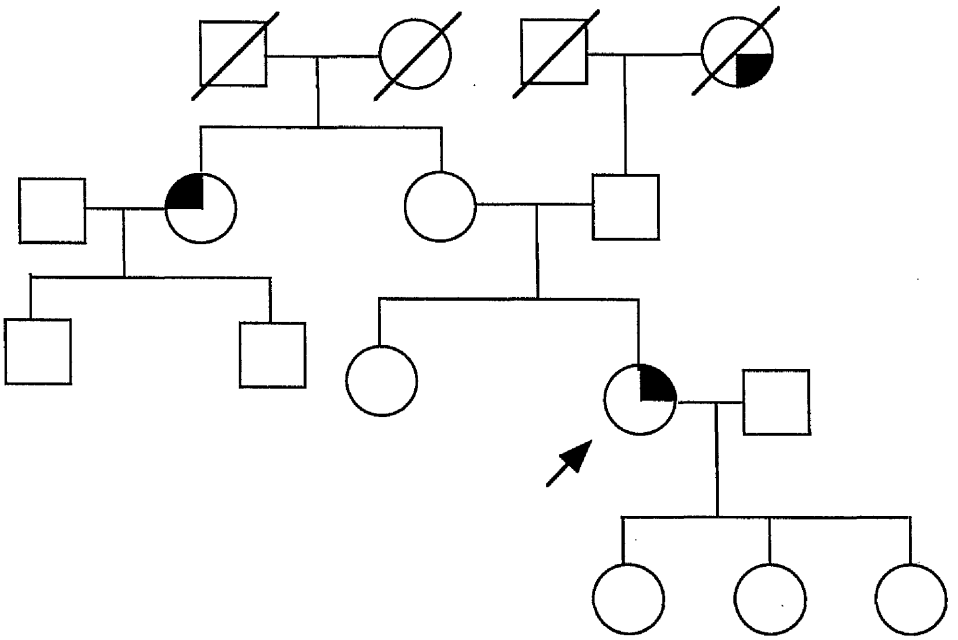
Pedigree 8



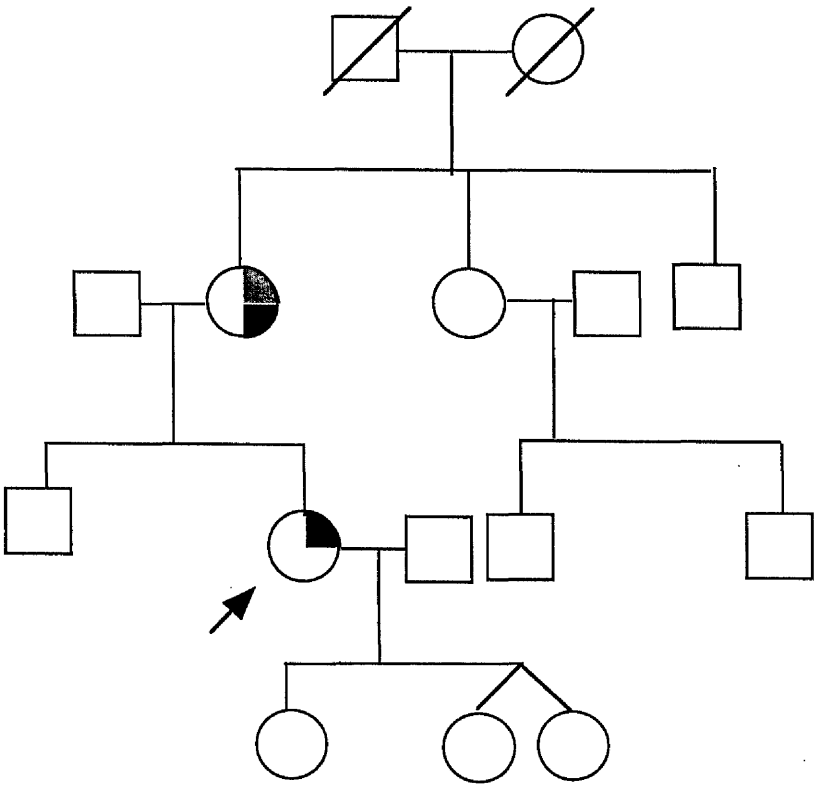
Pedigree 9



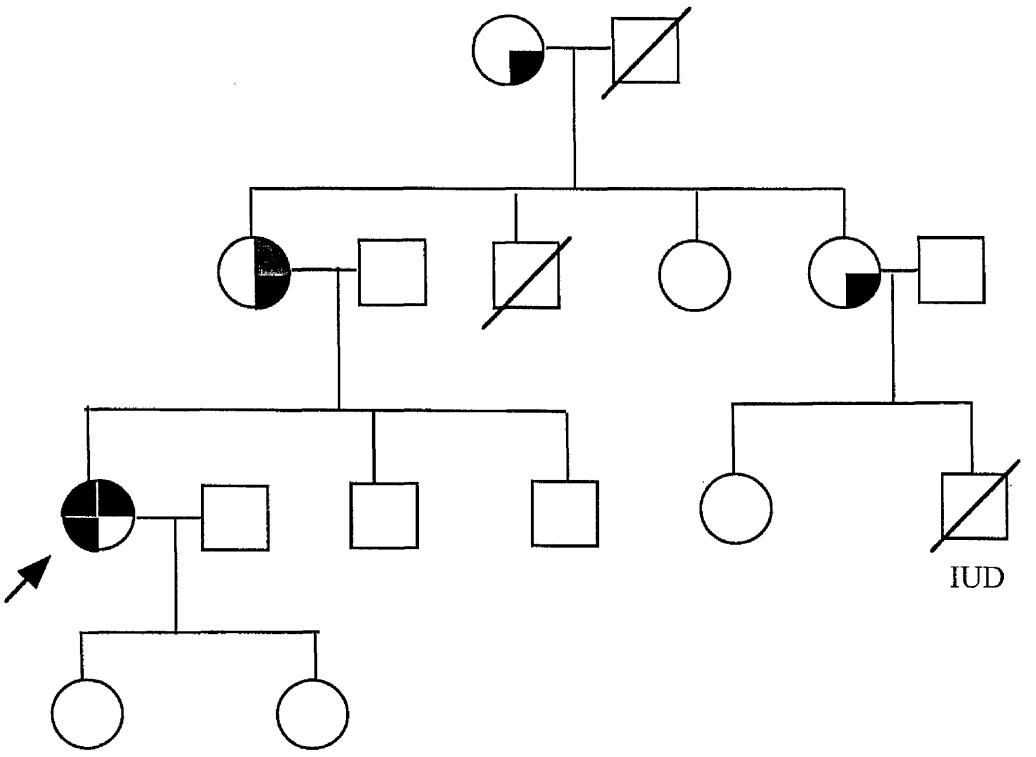
Pedigree 10



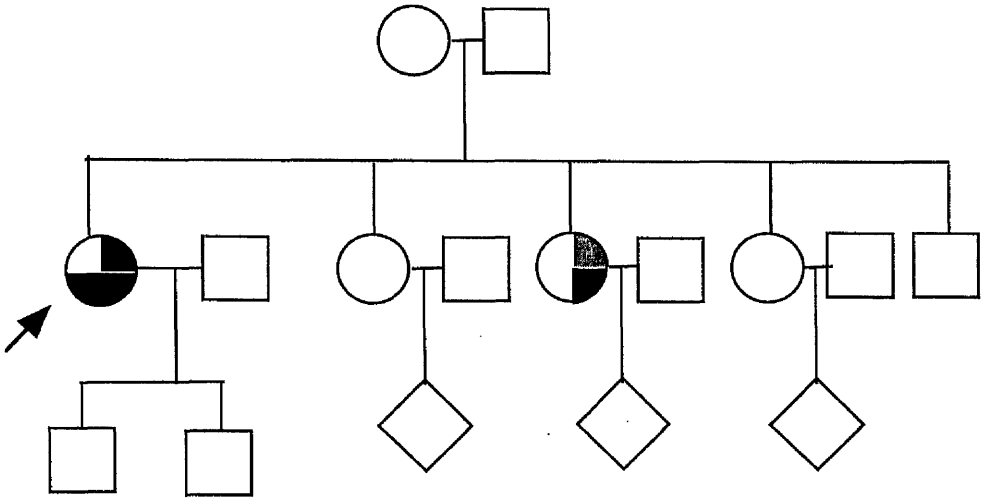
Pedigree 11



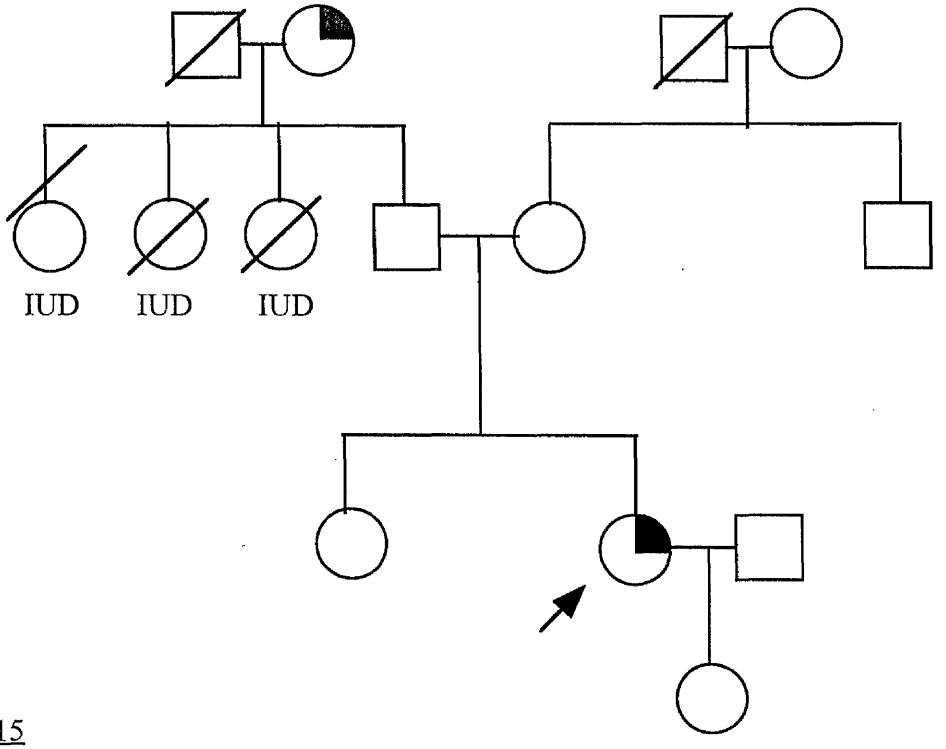
Pedigree 12



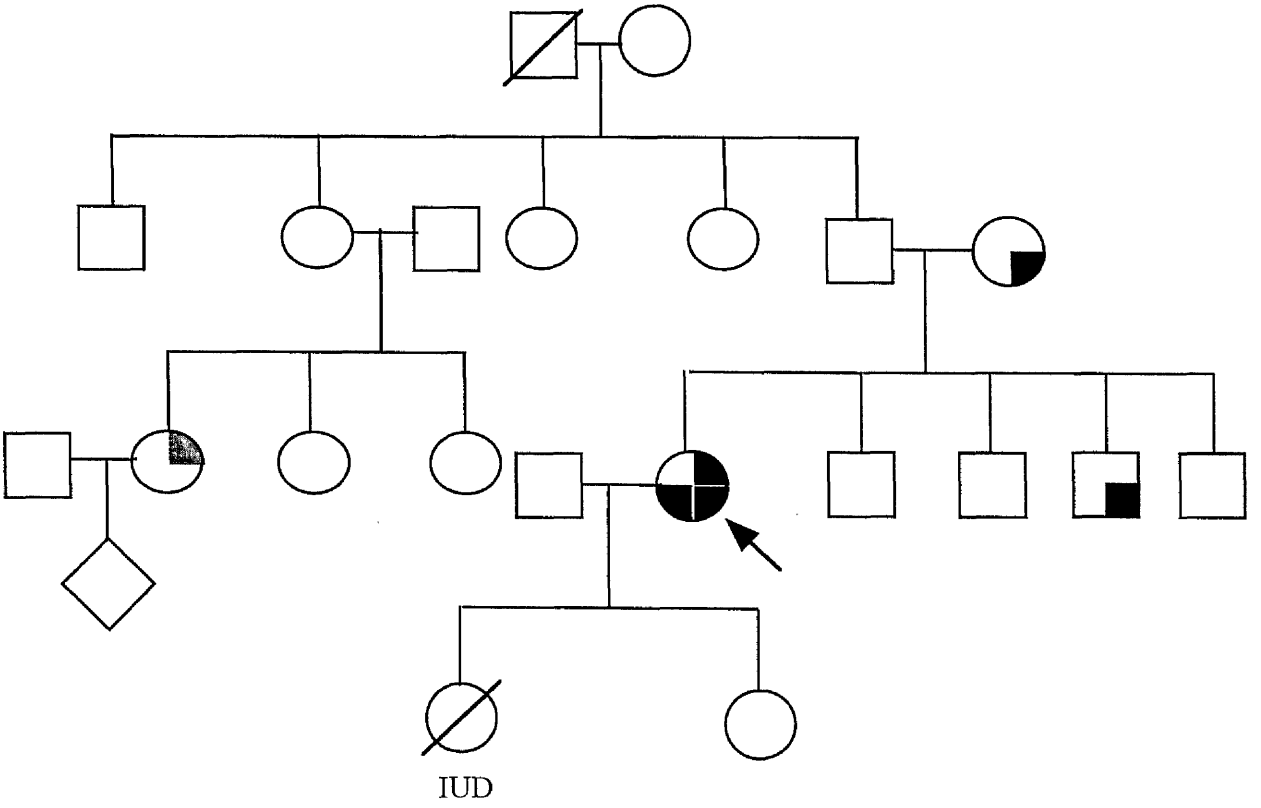
Pedigree 13



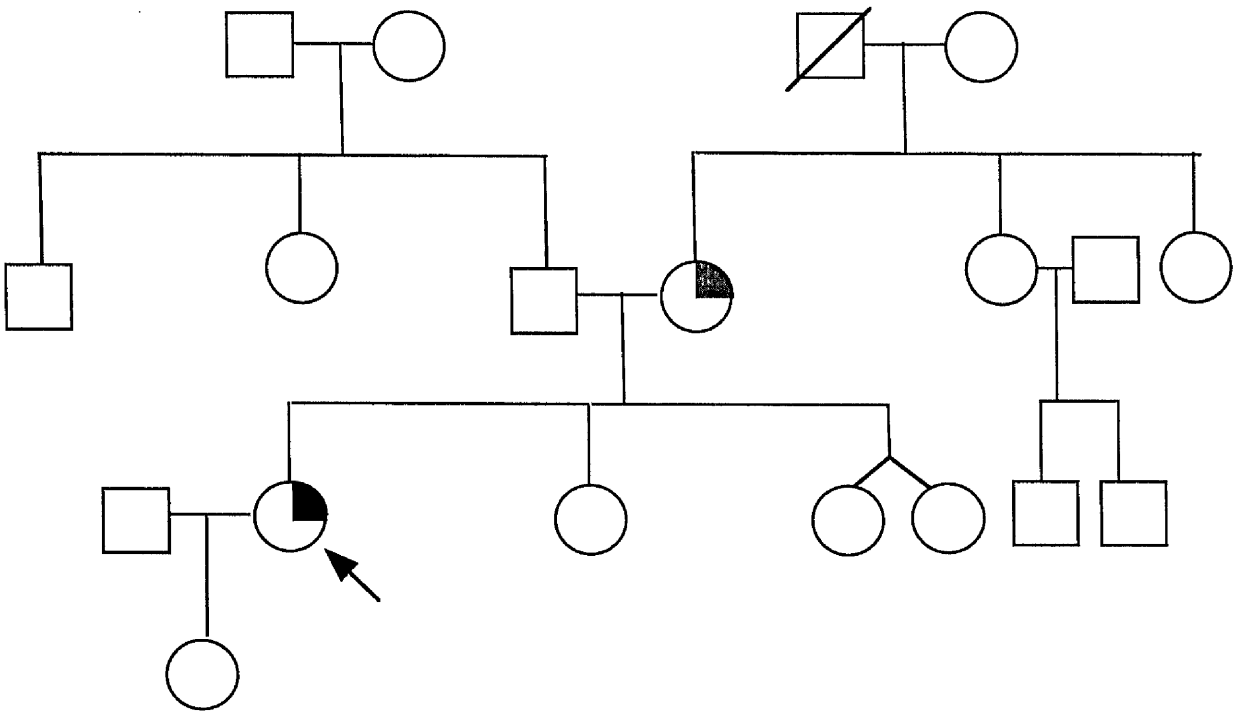
Pedigree 14



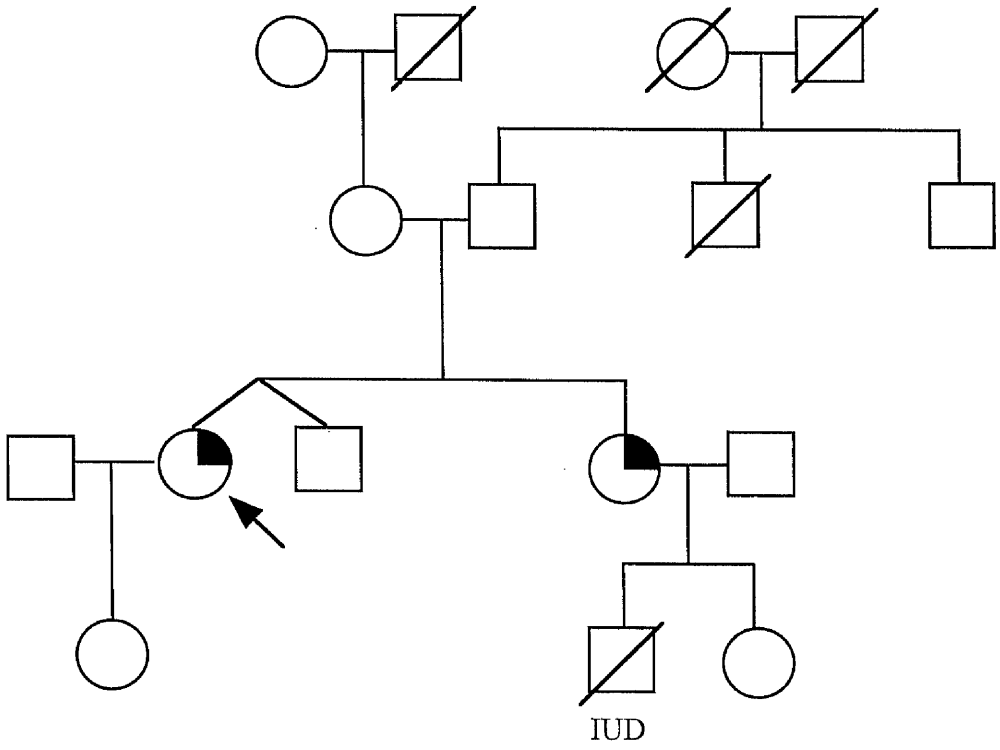
Pedigree 15



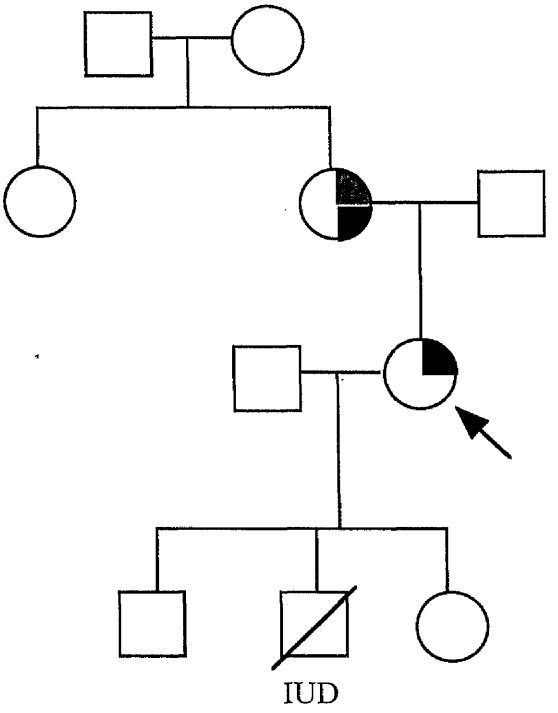
Pedigree 16



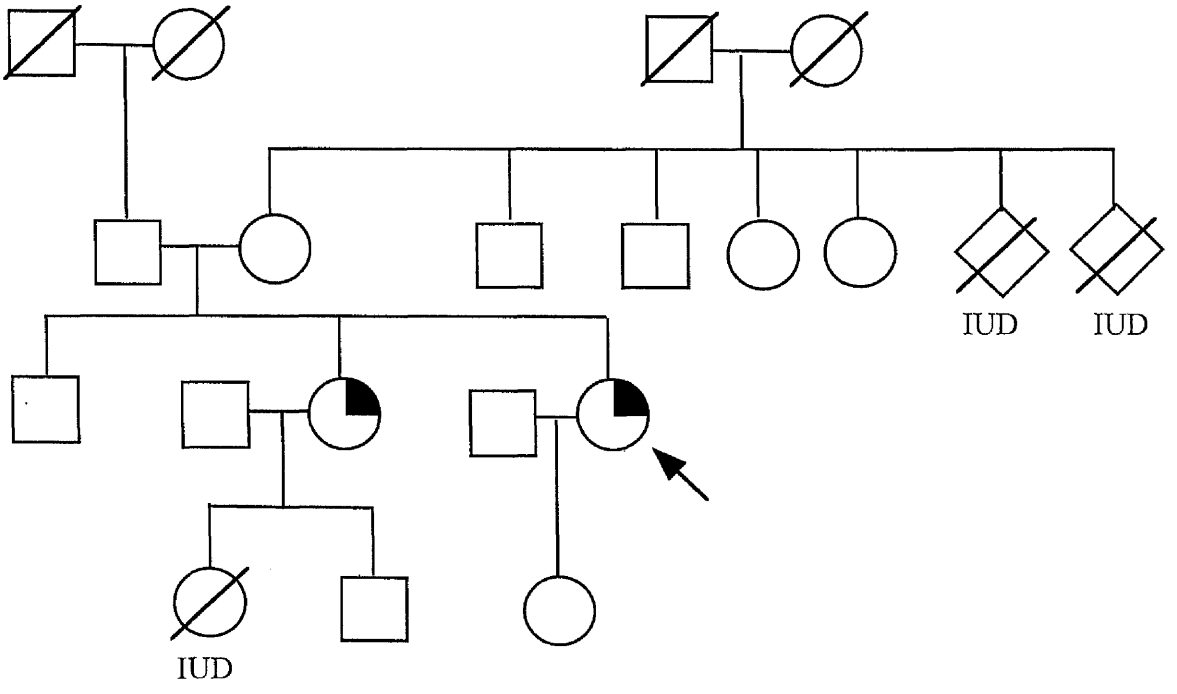
Pedigree 17



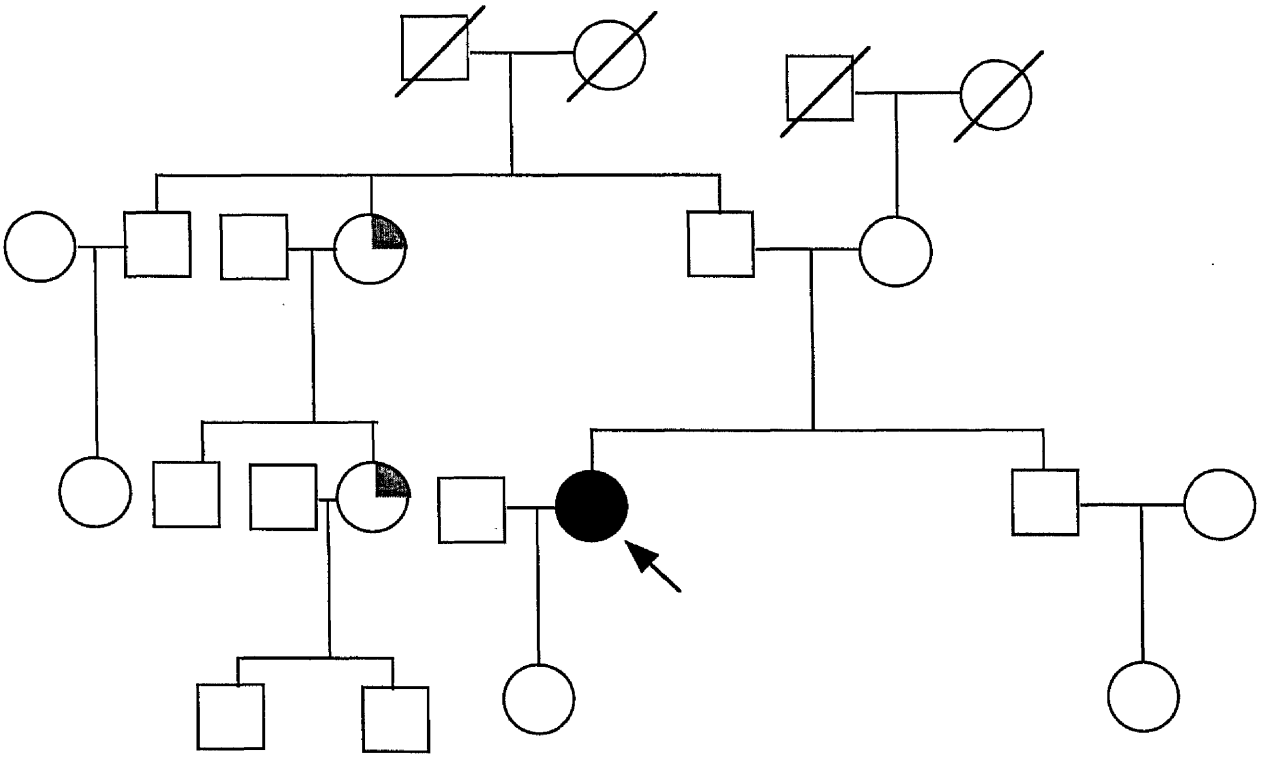
Pedigree 18



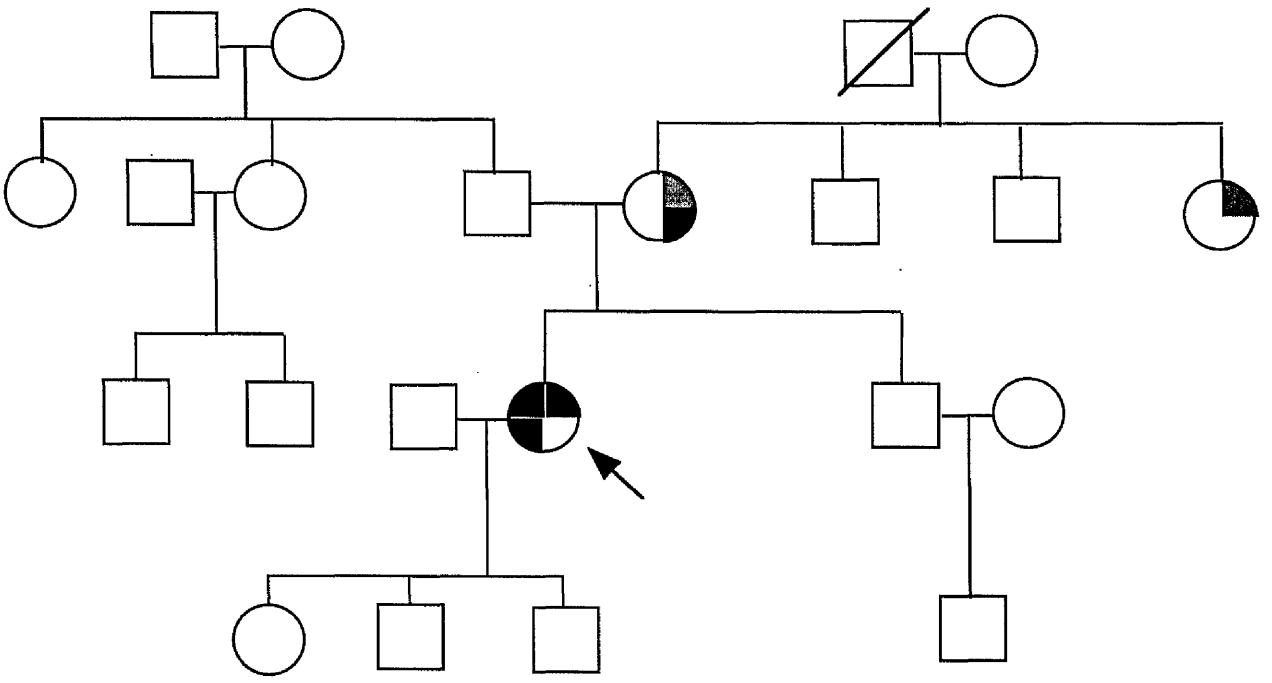
Pedigree 19



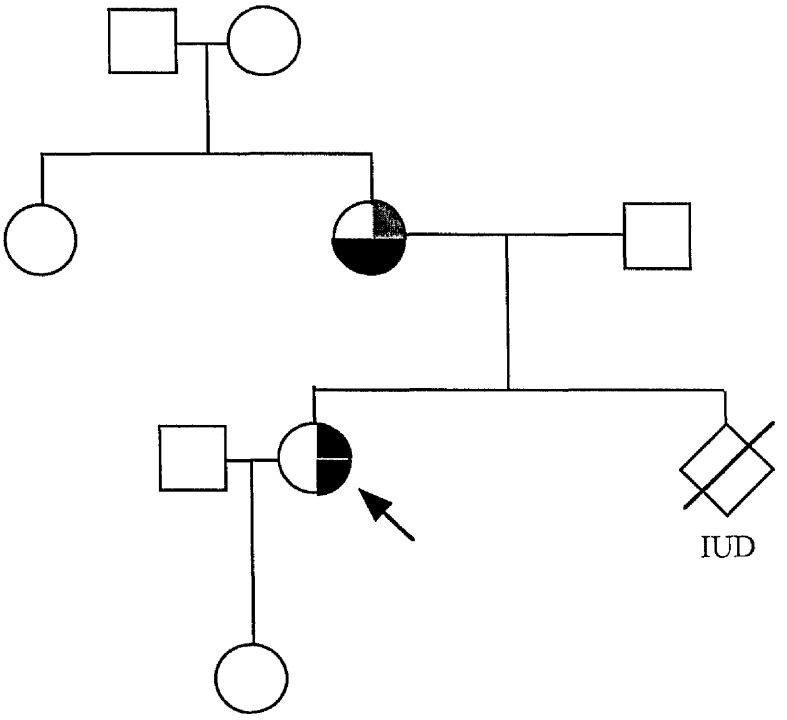
Pedigree 20



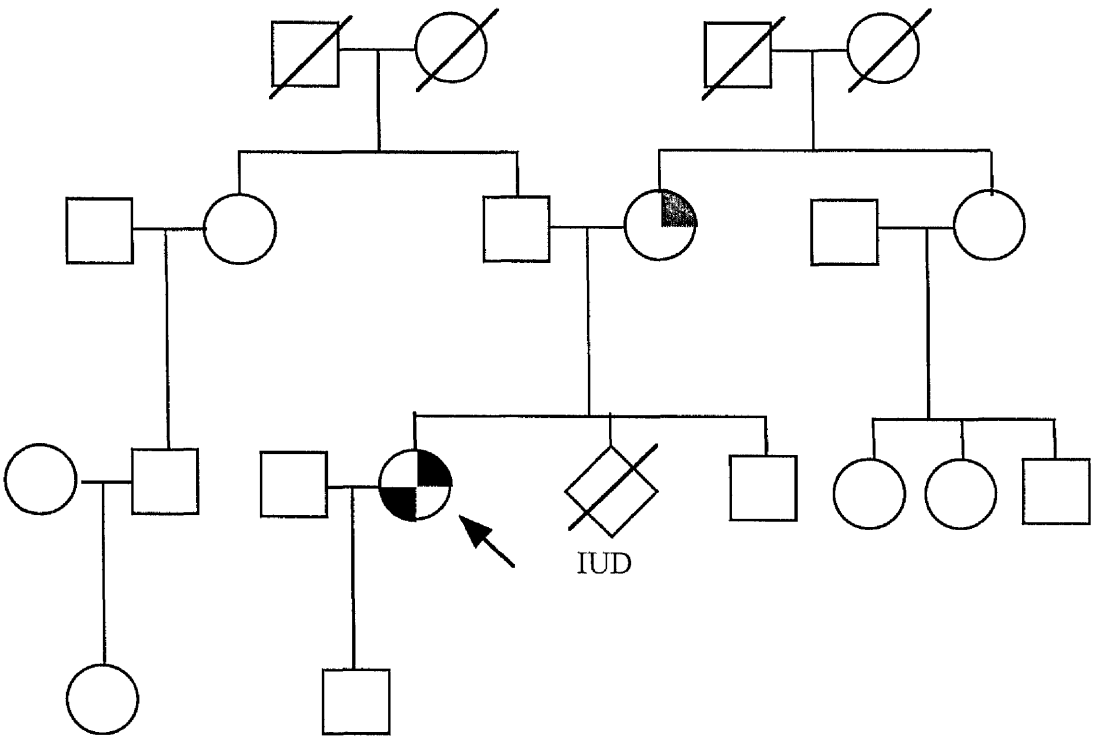
Pedigree 21



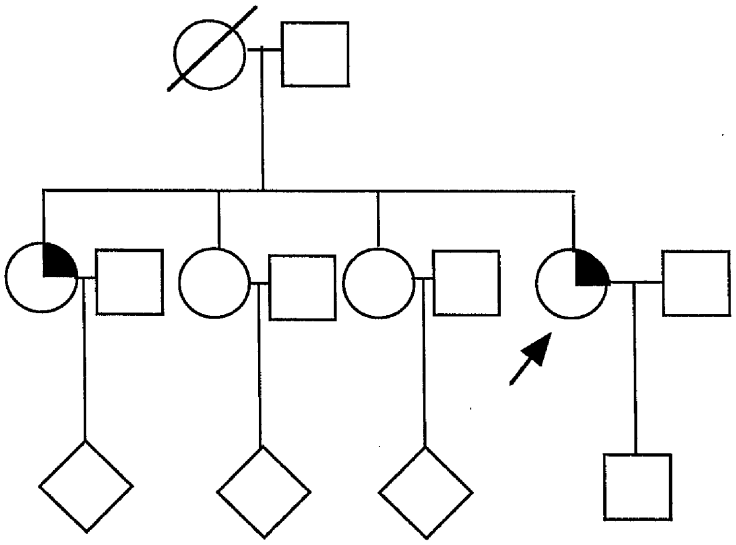
Pedigree 22



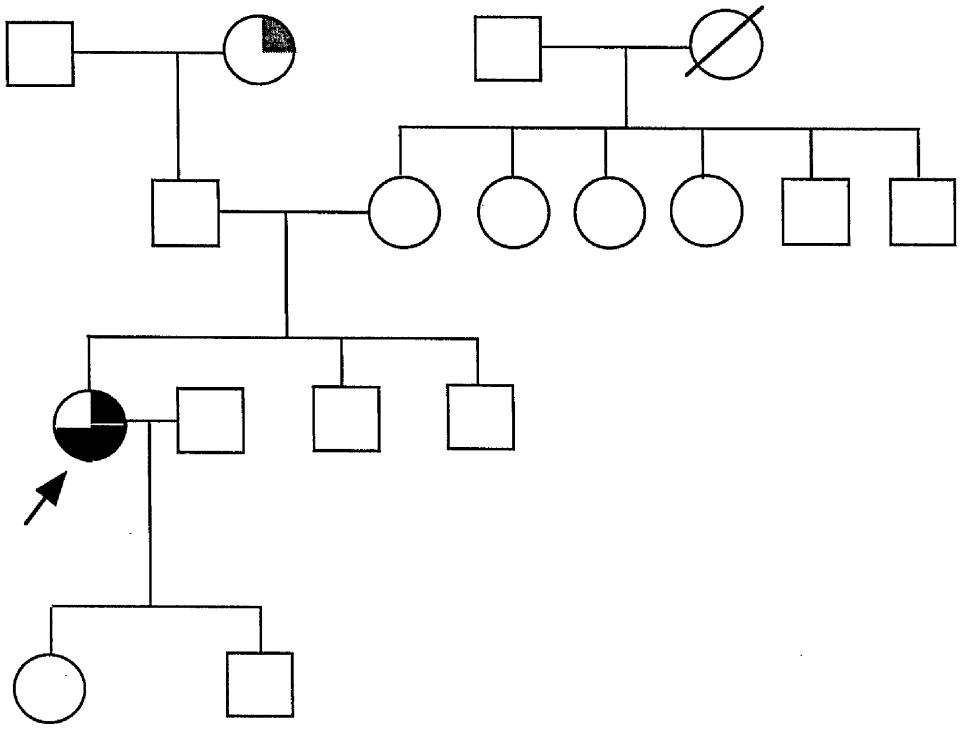
Pedigree 23



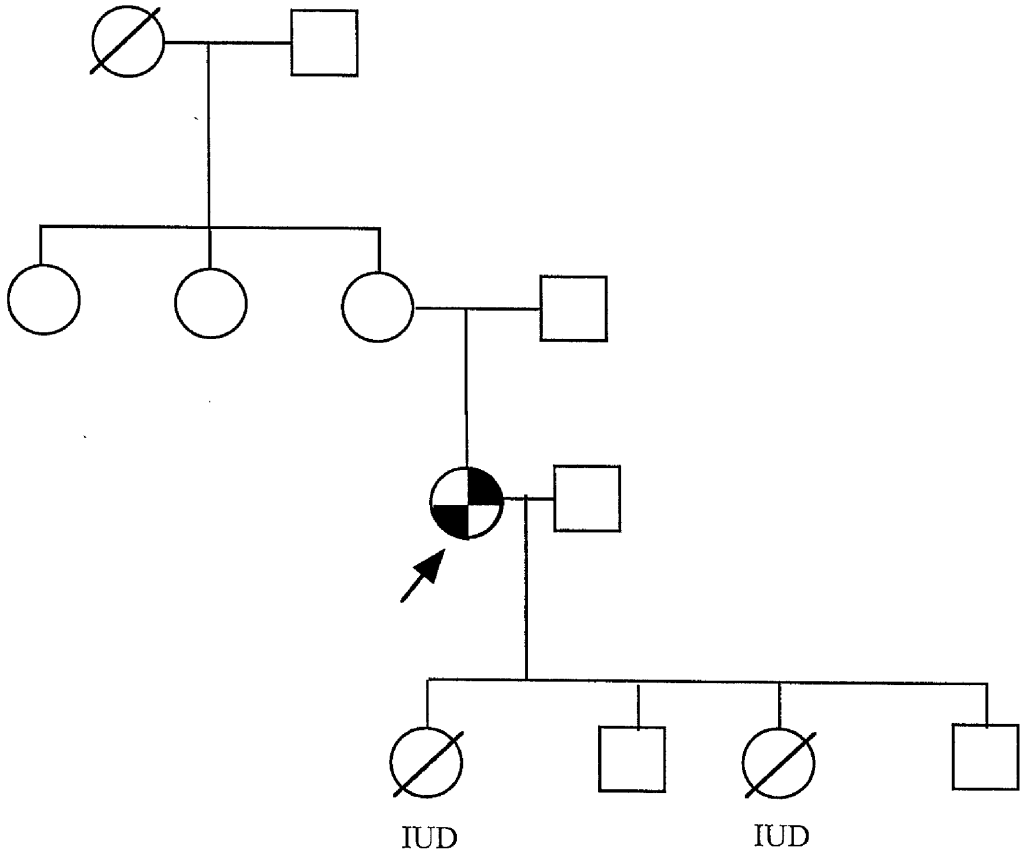
Pedigree 24



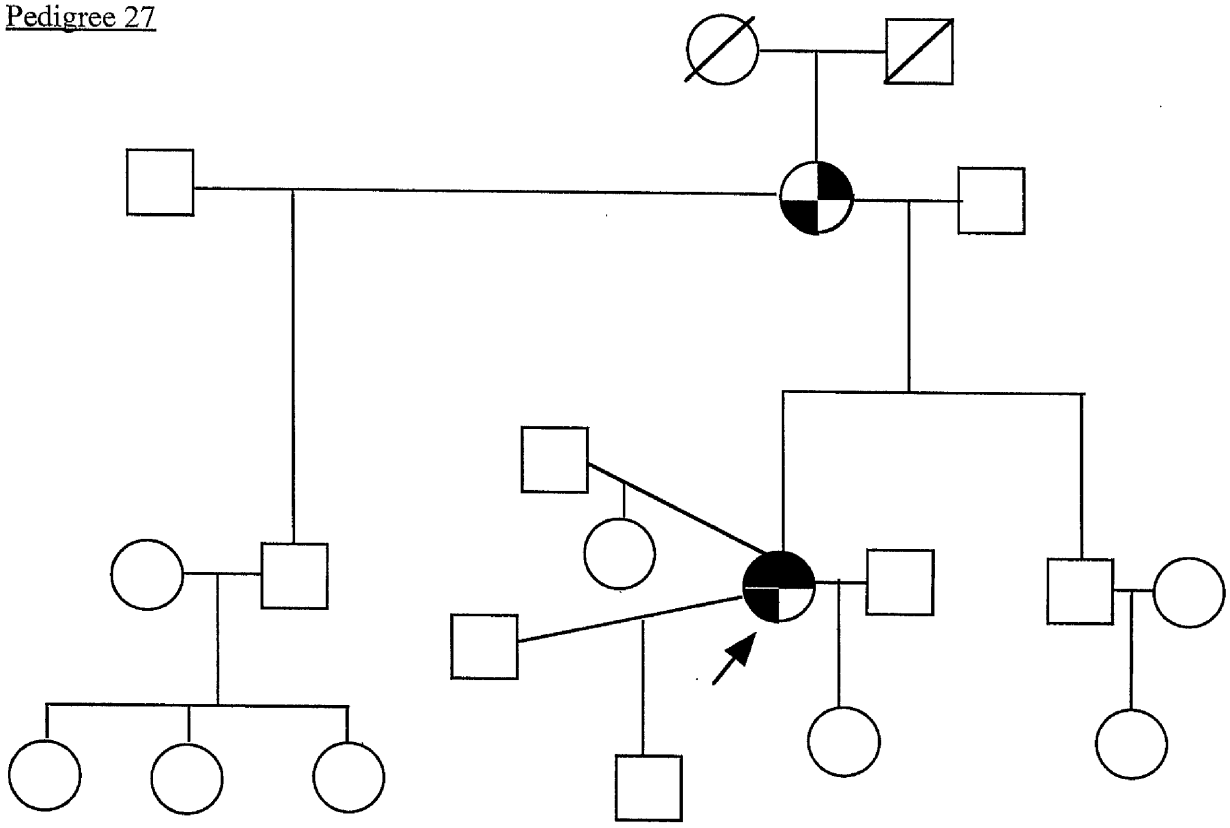
Pedigree 25



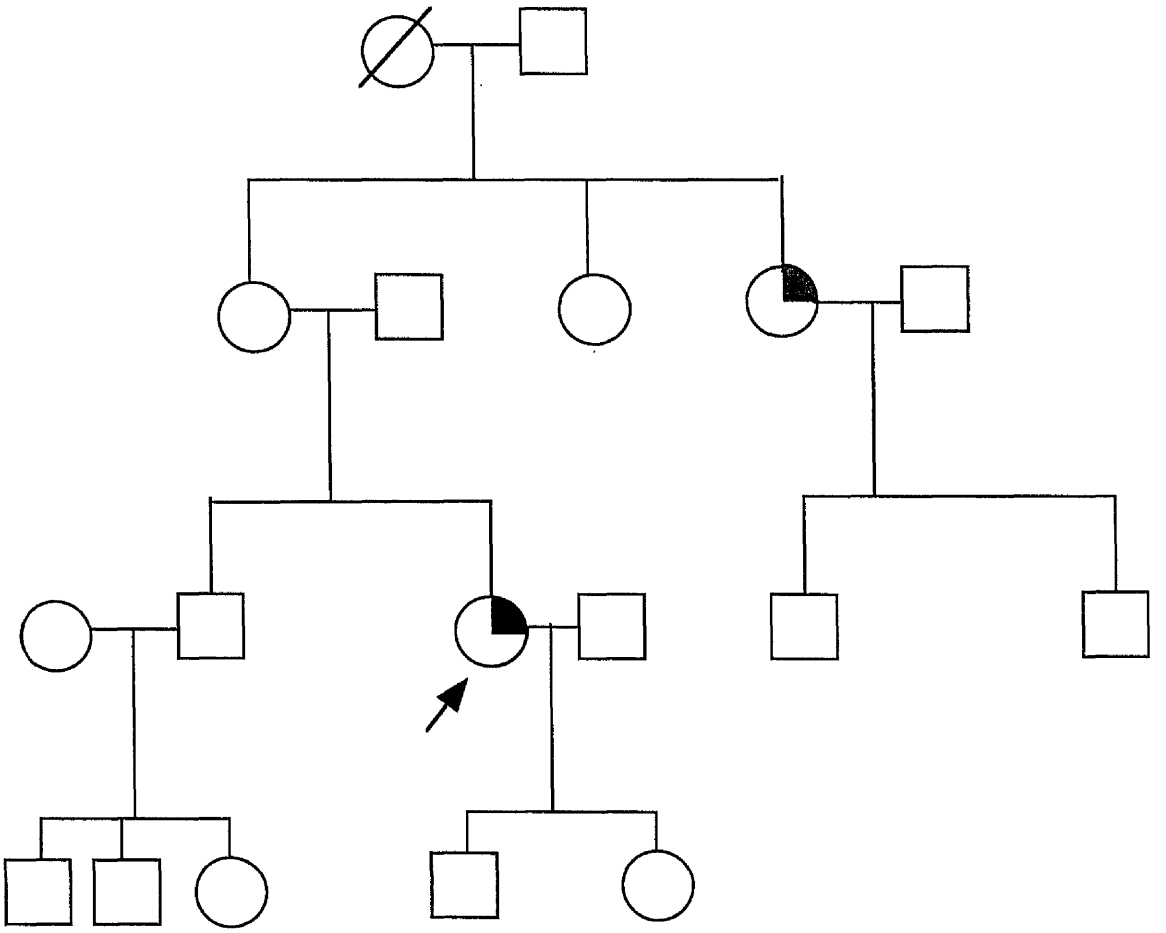
Pedigree 26



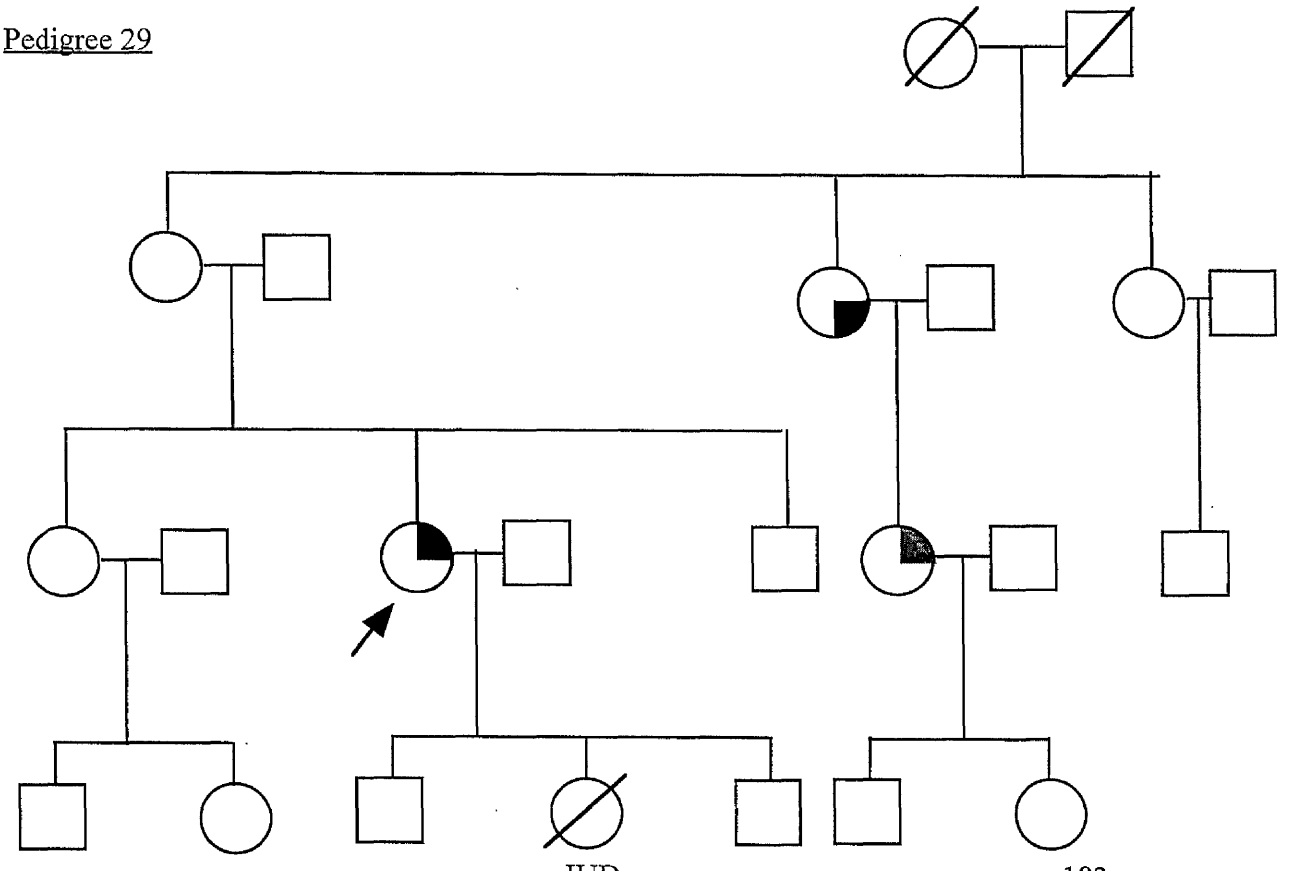
Pedigree 27



Pedigree 28

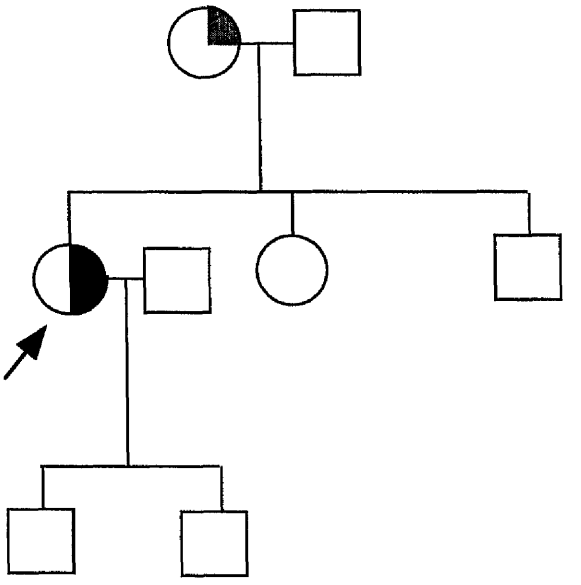


Pedigree 29

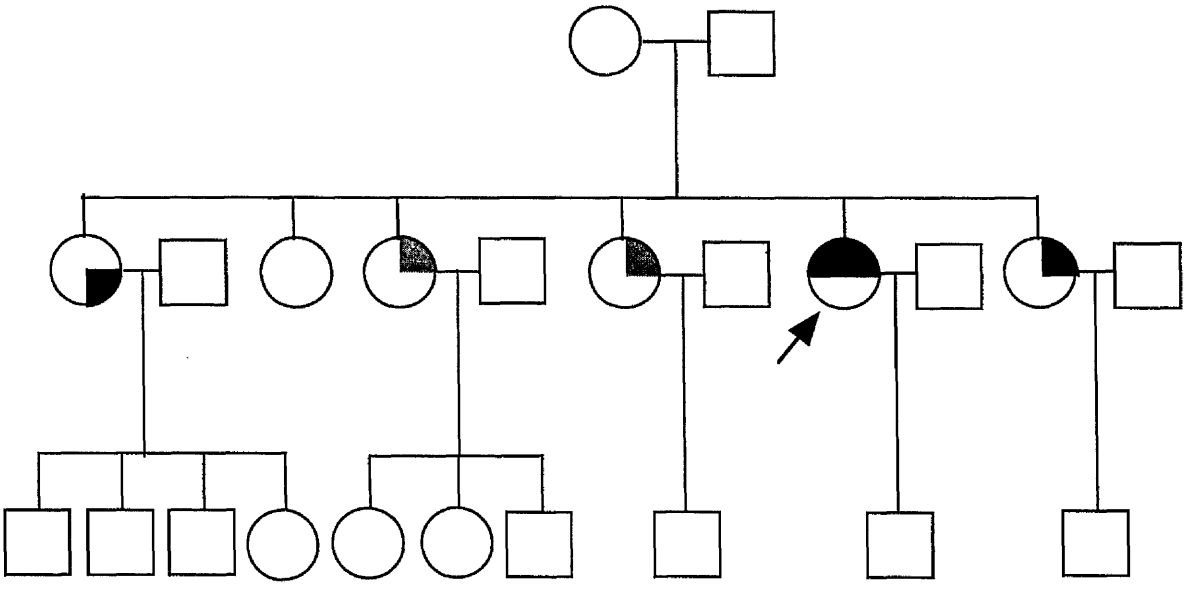


IUD

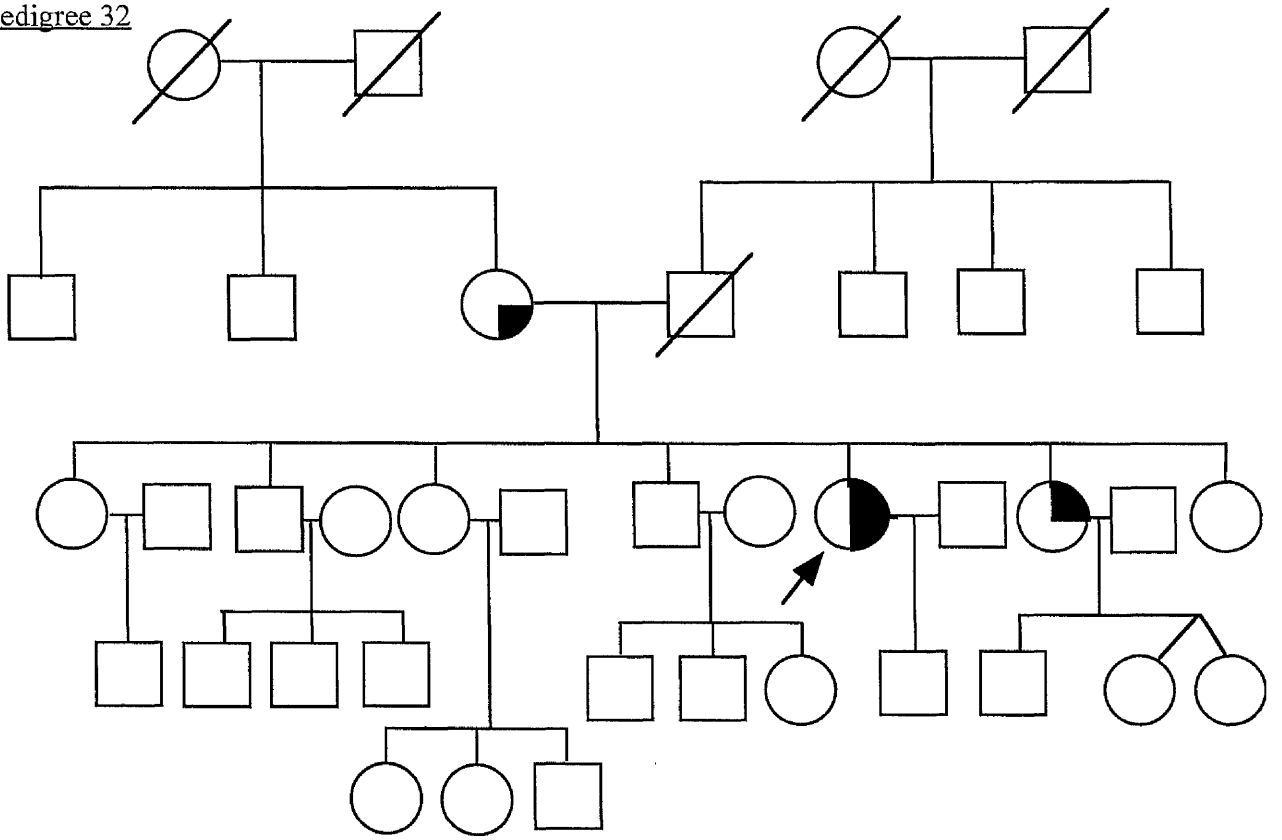
Pedigree 30



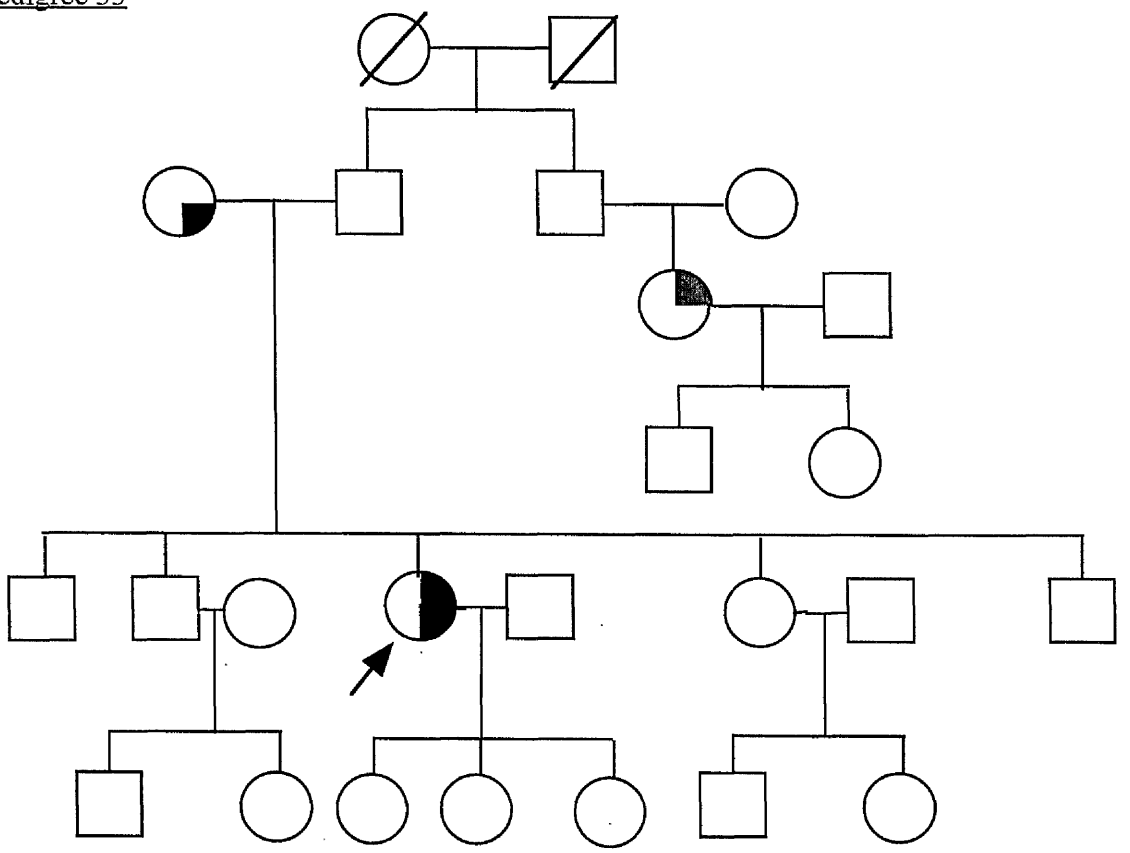
Pedigree 31



Pedigree 32



Pedigree 33



IV. Eperiments performed by Dr Kenneth Linton that were analysed by both Dr Linton and Dr Williamson and discussed in Chapter 3.

Site-directed mutagenesis

There are no established systems for quantification of PC translocation in mammalian systems making it currently not possible to study the function of the MDR3 protein. However, the transport of fluorescent substrates such as rhodamine by the multidrug transporter P-gp1, is relatively easy to assay in mammalian cell culture systems. The MDR3 protein shares 77% identity with P-gp1 and it has been shown that substitution of as few as three adjacent MDR3 protein residues in the first transmembrane domain by the equivalent residues from P-gp1 is sufficient to allow the chimeric molecule to transport substrates specific to P-gp1 (Zhou et al. 1999). The level of sequence identity between P-gp1 and the MDR3 protein increases to 87% in the NBDs. In addition, the NBDs of P-gp1 and the MDR3 protein, have been shown to be interchangeable (Buschman et al. 1991). We therefore introduced the equivalent A546D mutation into the first NBD of P-gp1 and studied the functional consequences of the change in transiently-transfected mammalian cells.

Alignment of the amino acid sequence of human P-gp1 with the MDR3 protein identified A544 as the P-gp1 equivalent of MDR3 protein A546. Site directed mutagenesis was used to alter codon 544 of *MDR1*, to encode an aspartic acid.

Site directed mutagenesis was used to alter codon 544 of *MDR1* to encode an aspartic acid, adopting the C to A tranversion of the 2nd position of the codon analogous to the A546D mutant of the MDR3 protein. The A544D mutation was introduced into NBD1 of P-gp1 by oligonucleotide-directed mutagenesis ('Altered sites' II; Promega) of pSBH. The mutagenic oligonucleotide (5'-CGCCATTGCACGAGACCTGGTT-

CGCAAC-3') introduced the desired codon change at the same time as removing the adjacent *Pml*I restriction site without further alteration to the amino acid sequence of P-gp1. The nucleotide sequence of the mutated DNA was verified by automated DNA sequencing as described above, prior to sub-cloning the mutated *Eco*RI-*Hin*dIII DNA fragment back into pMDR-wt to generate pMDR-A544D.

A further change, thymine (T) to adenine (A) was made in the wobble position of codon 543 of *MDR1* to remove, silently, the *Pml*I restriction site in order to follow the subsequent sub-cloning events. The mutated fragment was subcloned into the plasmid pMDR1-wt to generate pMDR1-A544D, and used to transiently-transfect human epithelial kidney (HEK293T) cells.

Transient transfection of mammalian HEK293T cells

Human epithelial kidney (HEK293T) cells (3.75×10^6, Imperial Cancer Research Fund, cell production unit) were seeded onto a $75cm^2$ tissue culture flask in 15ml Dulbecco's modified eagle media (DMEM), supplemented with 2mM L-glutamine and 10% (v/v) fetal calf serum (FCS) (Helena Biosciences). The cells were incubated under 5% CO_2 at 37°C for 18 hours to give approximately 70% confluency at which point the cells were transfected with polyethyleneimine (PEI)-DNA complexes: 60μg DNA in 30μl 5% glucose was mixed with 9μl 25kDa PEI solution (45mg PEI (Sigma Aldrich) dissolved in 8ml H_2O, pH to 7.2 with dilute HCl); this was diluted in 10ml supplemented DMEM and added directly to the cell monolayer. Twenty-four hours post-transfection the medium was replaced with fresh DMEM plus 2mM butyric acid (Sigma) to enhance expression of P-gp. The cells were harvested a further 24 hours later.

Fluorescent activated cell sorting analysis

Dual labelling of intact cells was used to assay for both P-gp function and localisation at the cell membrane. $1.25x10^6$ transiently-transfected HEK293T cells were harvested by incubation with versene (2mM EDTA in PBS) for 10 minutes at 37°C. The cells were washed twice with PBS and then incubated with 2μM rhodamine 123 (R123; Sigma), with or without cyclosporin A (CsA; 10μM), and incubated for a further 30 minutes at 37°C. The cells were washed three times with versene, then incubated with 20μg/ml anti-P-gp antibody (UIC2, phycoerythrin conjugate (UIC2-PE); Immunotech) or isotype control diluted in FACS buffer (1% FCS in PBS) for 15 minutes at 4°C. The cells were washed 3 times in FACS buffer before resuspension in 300μl FACS buffer. Samples were stored in the dark at 4°C until analysis. Flow cytometric analysis of the cells (10,000) was carried out using a FACS Vantage flow cytometer (Becton Dickinson) fitted with an argon ion laser. PE fluorescence was measured at 575nm and R123 fluorescence at 515nm. Fluorescence data was analyzed using CELLQuest (Becton Dickinson) software.

To detect intracellular antigen, $1.25x10^6$ cells, harvested and washed as before, were fixed by addition of formaldehyde to 3% and incubated at room temperature for 20 minutes. The cells were washed 3 times in FACS buffer and resuspended in 0.5% saponin (in FACS buffer) and incubated for 20min at 4°C. The cells were washed a further 3 times in FACS buffer before incubation with UIC2 and FACS as before.

Western analysis

Whole cell lysates were prepared from 5×10^5 transiently-transfected cells by resuspension of the washed cell pellet in 300μl lysis buffer (phosphate buffered saline

(PBS; pH 7.4), 1% SDS, 100μM phenylmethylsulfonyl fluoride, 1mM benzamidine, 4μg/ml pepstatin and 1mM ethylenediaminetetra-acetic acid). 10μg cell lysate was separated by SDS-PAGE and the proteins transferred electrophoretically to PVDF membrane (Immobilon-P^{TM}; Millipore). The membrane was probed with the anti-P-gp monoclonal antibody, C219 (Cis-Bio International), and developed using horse radish peroxidase conjugated goat anti-mouse antibody and enhanced chemiluminescence (Amersham).

BJOG: an International Journal of Obstetrics and Gynaecology
July 2004, Vol. 111, pp. 682–687
DOI: 10.1111/j.1471-0528.2004.00151.x

Spousal physical violence against women during pregnancy

Abraham Peedicayil,[a] Laura S. Sadowski,[b] Lakshman Jeyaseelan,[a] Viswanathan Shankar,[a] Dipty Jain,[c] Saradha Suresh,[d] Shrikant I. Bangdiwala,[e] the IndiaSAFE Group

Objective To determine the prevalence of physical violence during pregnancy and the factors associated with it.

Design A population-based, multicentre, cross sectional household survey.

Setting Rural, slum and urban non-slum areas of Bhopal, Chennai, Delhi, Lucknow, Nagpur, Trivandrum and Vellore, in India.

Participants A total of 9938 women who were 15 to 49 years of age and living with a child younger than 18 years old.

Methods Probability proportionate to size sampling of households was performed in three strata. Trained field workers administered a structured questionnaire. Women who reported domestic violence were asked about violence during pregnancy. Outcome variables included six violent behaviours: slap, hit, kick, beat, use of weapon and harm in any other way. Moderate to severe violence was defined as experience of any one or more of the following behaviours: hit, beat or kick. Odds ratios were calculated for risk and protective factors of violence during pregnancy using logistic regression.

Main outcome measures Physical spousal violence.

Results The lifetime experience, during pregnancy, of being slapped was 16%, hit 10%, beat 10%, kicked 9%, use of weapon 5% and harmed in any other way 6%. Eighteen percent of women experienced at least one of these behaviours and 3% experienced all six. The overall prevalence of moderate to severe violence during pregnancy was 13%. Logistic regression showed that the factors determining whether a woman experienced moderate to severe violence during pregnancy were: husband accusing wife of an affair (OR 7.1; 95% CI 5.1 to 9.8), dowry harassment (OR 4.1; 95% CI 2.8 to 6.1), husband having an affair (OR 3.7; 95% CI 2.8 to 4.8), husband being regularly drunk (OR 3.2; 95% CI 2.6 to 4.1), low education of husband (OR 2.8; 95% CI 1.4 to 5.6), substance abuse by husband (OR 2.6; 95% CI 1.3 to 5.5), no social support (OR 1.8; 95% CI 1.1 to 3.0), three or more children (OR 1.6; 95% CI 1.2 to 2.1) and household crowding (OR 1.1; 95% CI 1.0 to 1.2).

Conclusion In this study, 12.9% of women experienced moderate to severe physical violence during pregnancy. Suspicion of infidelity, dowry harassment, husband being regularly drunk and low education of husband were the main risk factors for violence during pregnancy.

INTRODUCTION

Recently, domestic violence in developing countries, such as India, has been acknowledged as a common health problem with a prevalence rate of 20% to 60%.[1]

Reasons for such varying reports are due to varying definitions of violence and non-uniform methodologies. One large population-based study of physical domestic violence in India reported a prevalence estimate of 17% but the information was collected from men.[2] Our study from seven sites in India found the prevalence of moderate to severe physical violence to be 41%.[3] In developed countries, the reported prevalence of violence against women by an intimate partner ranges from 9.7% to 30%.[4]

The reported prevalence of violence during pregnancy ranges from 0.9% to 20%.[5–9] Studies have shown that 40% to 60% of women who have experienced violence in their lifetime have also experienced violence during pregnancy.[9] Martin *et al.*[2] found that 5.4–13% of 1990 men from five districts in Uttar Pradesh (a northern state in India) had acknowledged physically assaulting their wives during pregnancy. In a study on women attending an antenatal clinic in Nagpur (central India), physical violence was reported in the index pregnancy by 22%.[10]

[a]*Clinical Epidemiology Unit, Christian Medical College and Hospital, Vellore, India*
[b]*Collaborative Research Unit, Cook County Hospital, Chicago, Illinois, USA*
[c]*Clinical Epidemiology Unit, Government Medical College, Nagpur, India*
[d]*Clinical Epidemiology Unit, Madras Medical College, Chennai, India*
[e]*Department of Biostatistics, University of North Carolina, Chapel Hill, North Carolina, USA*

Correspondence: Dr A. Peedicayil, Department of Obstetrics and Gynaecology, Christian Medical College and Hospital, Vellore, India.

10. Davies MH, da Silva RC, Jones SR, Weaver JB, Elias E. Fetal mortality associated with cholestasis of pregnancy and the potential benefit of therapy with ursodeoxycholic acid. *Gut* 1995;**37**(4):580–584.
11. Costoya AL, Leontic EA, Rosenberg HG, Delgado MA. Morphological study of placental terminal villi in intrahepatic cholestasis of pregnancy: histochemistry, light and electron microscopy. *Placenta* 1980;**1**(4):361–368.
12. Laatikainen T, Tulenheimo A. Maternal serum bile acid levels and fetal distress in cholestasis of pregnancy. *Int J Gynaecol Obstet* 1984; **22**(2):91–94.
13. Laatikainen TJ. Fetal bile acid levels in pregnancies complicated by maternal intrahepatic cholestasis. *Am J Obstet Gynecol* 1975;**122**(7): 852–856.
14. Alsulyman OM, Ouzounian JG, Ames-Castro M, Goodwin TM. Intrahepatic cholestasis of pregnancy: perinatal outcome associated with expectant management. *Am J Obstet Gynecol* 1996;**175**(4 Pt 1): 957–960.
15. Londero F, San Marco L. Intrahepatic cholestasis of pregnancy: are we really able to predict fetal outcome? *Am J Obstet Gynecol* 1997; **177**(5):1274.
16. Laatikainen T, Ikonen E. Serum bile acids in cholestasis of pregnancy. *Obstet Gynecol* 1977;**50**(3):313–318.
17. Shaw D, Frohlich J, Wittmann BA, Willms M. A prospective study of 18 patients with cholestasis of pregnancy. *Am J Obstet Gynecol* 1982; **142**(6 Pt 1):621–625.
18. Girling JC, Dow E, Smith JH. Liver function tests in pre-eclampsia: importance of comparison with a reference range derived for normal pregnancy. *Br J Obstet Gynaecol* 1997;**104**(2):246–250.
19. Morrison JJ, Rennie JM, Milton PJ. Neonatal respiratory morbidity and mode of delivery at term: influence of timing of elective caesarean section. *Br J Obstet Gynaecol* 1995;**102**(2):101–106.
20. Madar J, Richmond S, Hey E. Surfactant-deficient respiratory distress after elective delivery at 'term'. *Acta Paediatr* 1999;**88**(11): 1244–1248.
21. Mazzella G, Nicola R, Francesco A, et al. Ursodeoxycholic acid administration in patients with cholestasis of pregnancy: effects on primary bile acids in babies and mothers. *Hepatology* 2001;**33**(3): 504–508.
22. Palma J, Reyes H, Ribalta J, et al. Ursodeoxycholic acid in the treatment of cholestasis of pregnancy: a randomized, double-blind study controlled with placebo. *J Hepatol* 1997;**27**(6):1022–1028.
23. Hirvioja ML, Tuimala R, Vuori J. The treatment of intrahepatic cholestasis of pregnancy by dexamethasone. *Br J Obstet Gynaecol* 1992;**99**(2):109–111.
24. Williamson C, Gorelik J, Eaton BM, Lab M, de Swiet M, Korchev Y. The bile acid taurocholate impairs rat cardiomyocyte function: a proposed mechanism for intra-uterine fetal death in obstetric cholestasis. *Clin Sci (Colch)* 2001;**100**(4):363–369.
25. Campos GA, Guerra FA, Israel EJ. Effects of cholic acid infusion in fetal lambs. *Acta Obstet Gynecol Scand* 1986;**65**(1):23–26.
26. Israel EJ, Guzman ML, Campos GA. Maximal response to oxytocin of the isolated myometrium from pregnant patients with intrahepatic cholestasis. *Acta Obstet Gynecol Scand* 1986;**65**(6):581–582.
27. Bacq Y, Sapey T, Brechot MC, Pierre F, Fignon A, Dubois F. Intrahepatic cholestasis of pregnancy: a French prospective study. *Hepatology* 1997;**26**(2):358–364.

Accepted 27 February 2004

support the suggestion that bile acids cause the intrauterine death in obstetric cholestasis, it may be justifiable to defer delivery to a later gestation than 37 weeks in women who have responded to treatment.

Three of the five intrauterine deaths that occurred before 37 weeks of gestation were in twin pregnancies, suggesting that twin pregnancies may be at risk of intrauterine death at an earlier gestation than singleton pregnancies. There was a non-significant tendency towards female fetuses in pregnancies complicated by intrauterine death. The data on intrauterine deaths in twin pregnancies and in female fetuses also did not reach statistical significance, and a much larger series would be required to assess a possible gender difference. A recent initiative is the establishment of a Web-based international registry of all obstetric cholestasis-related intrauterine deaths (http://escuela.med.puc.cl/iris/welcome.html). This is called IRIS (International Registry of Intrahepatic Cholestasis of Pregnancy-related Stillbirth) and should permit prospective information from pregnancies complicated by intrauterine death to be collected.

The rate of spontaneous prematurity in women with obstetric cholestasis (16%) was lower than in most other studies (36–44%),[1,3] with the exception of one recent UK study.[6] This may be because 22% (77/352) of obstetric cholestasis pregnancies in this present study had received treatment with either UDCA alone or in addition to dexamethasone. While it has not been proved that treatment reduces the rate of spontaneous prematurity, studies in animals[25] and using myometrium from women with obstetric cholestasis[26] have implicated raised serum bile acids in the aetiology of prematurity.

This study has demonstrated that the onset of pruritus occurs at a significantly earlier gestation in obstetric cholestasis pregnancies complicated by spontaneous preterm delivery when compared with unaffected obstetric cholestasis pregnancies. A Chilean study has also found a positive correlation between prematurity and earlier gestation at the onset of pruritus during pregnancy.[2] If bile acids are the cause of the spontaneous prematurity, it is possible that the duration of exposure to bile acids may influence myometrial contractility or other processes that can initiate labour.

It was not possible to obtain accurate data about the rates of fetal distress in this study as most women did not know whether they had cardiotocograph abnormalities or fetal distress in their pregnancies. However, admission to the neonatal ICU was used as a surrogate marker of fetal distress, and the rates were high in obstetric cholestasis pregnancies. When the results were analysed further, the risk appears to be related more to preterm delivery than to obstetric cholestasis. It was beyond the scope of this study to assess the subsequent morbidity of the infants that were admitted to the neonatal ICU. However, given that elective delivery at 37 weeks is currently advocated as the best strategy for the prevention of intrauterine death, a large prospective study of the outcome of such obstetric cholestasis pregnancies would allow the consequences of both iatrogenic and spontaneous preterm delivery to be evaluated.

The prevalence of oral contraceptive pill-induced pruritus was higher than in a smaller French study of 24 women diagnosed with obstetric cholestasis during their pregnancies in which only one (4%) suffered pruritus and abnormal liver function tests after taking the oral contraceptive pill.[27] This may be because obstetric cholestasis in French women has a different aetiology, or because of the small number of cases in the study. These symptoms disappear once women stop taking the oral contraceptive pill. At present, there is no evidence that prolonged use of oral contraceptives in women with obstetric cholestasis can cause permanent liver damage. Given that 34% of women with cyclical pruritus also developed pruritus if they took oral contraceptives, these women may have a stronger genetic predisposition to obstetric cholestasis.

In summary, we have demonstrated that the majority of intrauterine deaths occur from 37 weeks of gestation in singleton pregnancies. Intrauterine deaths complicating twin pregnancies occur at an earlier date. The gestation at which the pruritus started did not allow prediction of pregnancies complicated by intrauterine death, but did occur at an earlier gestation in pregnancies complicated by spontaneous prematurity.

Acknowledgements

This work was supported by a grant from the Wellcome Trust.

References

1. Reid R, Ivey KJ, Rencoret RH, Storey B. Fetal complications of obstetric cholestasis. *BMJ* 1976;**1**(6014):870–872.
2. Reyes H. The enigma of intrahepatic cholestasis of pregnancy: lessons from Chile. *Hepatology* 1982;**2**(1):87–96.
3. Fisk NM, Storey GN. Fetal outcome in obstetric cholestasis. *Br J Obstet Gynaecol* 1988;**95**(11):1137–1143.
4. Rioseco AJ, Ivankovic MB, Manzur A, et al. Intrahepatic cholestasis of pregnancy: a retrospective case–control study of perinatal outcome. *Am J Obstet Gynecol* 1994;**170**(3):890–895.
5. Abedin P, Weaver JB, Egginton E. Intrahepatic cholestasis of pregnancy: prevalence and ethnic distribution. *Ethn Health* 1999;**4**(1–2): 35–37.
6. Kenyon AP, Nelson-Piercy C, Girling J, Williamson C, Tribe RM, Shennan AH. Obstetric cholestasis, outcome with active management: a series of 70 cases. *Br J Obstet Gynaecol* 2002;**109**:282–288.
7. Bacq Y, Myara A, Brechot MC, et al. Serum conjugated bile acid profile during intrahepatic cholestasis of pregnancy. *J Hepatol* 1995;**22**(1):66–70.
8. Heikkinen J, Maentausta O, Ylostalo P, Janne O. Changes in serum bile acid concentrations during normal pregnancy, in patients with intrahepatic cholestasis of pregnancy and in pregnant women with itching. *Br J Obstet Gynaecol* 1981;**88**(3):240–245.
9. Heinonen S, Kirkinen P. Pregnancy outcome with intrahepatic cholestasis. *Obstet Gynecol* 1999;**94**(2):189–193.

Table 2. Median gestational week that pruritus started in successive obstetric cholestasis pregnancies.

Order of affected pregnancy	Number of cases (total 352)	Gestational week median (IQR)
1st	182	30 (7)
2nd	113	28 (8)
3rd	43	28 (8)
4th	10	25 (6)
5th	4	21 (15)

Fig. 3. The median gestation of the spontaneous premature deliveries in the obstetric cholestasis group was 34 weeks (IQR 3.6). There were 33 (55%) female and 27 (45%) male fetuses in the obstetric cholestasis spontaneous prematurity group. The pruritus started earlier in pregnancies complicated by spontaneous prematurity (median 28.0 weeks [IQR 6.8]) when compared with other obstetric cholestasis pregnancies (30.0 [IQR 7.0] weeks) ($P < 0.001$) (Fig. 4). Also, when all obstetric cholestasis pregnancies were considered, those in which pruritus started earlier were more likely to be complicated by spontaneous prematurity (χ^2, $P = 0.04$) (Table 1).

There were 77 (22%) iatrogenic premature deliveries in obstetric cholestasis women (i.e. 46 induced, 25 elective caesarean sections and 6 pre-labour emergency caesarean sections).

When analysing the number of pregnancies in which the infant was admitted to the neonatal ICU for more than three days, the 23 pregnancies complicated by intrauterine death were not considered. Therefore, of 329 affected obstetric cholestasis pregnancies, 64 (20%) infants were admitted to the neonatal ICU. When only premature deliveries (spontaneous and iatrogenic) were considered, 41% (53/128) of infants were admitted to the ICU. The proportion for spontaneous prematurity was 27/54 (50%) and for iatrogenic prematurity was 26/74 (35%).

Of the 131 women, 80 (61%) had obstetric cholestasis in all pregnancies and 51 (39%) did not. If a woman had obstetric cholestasis in a previous pregnancy, her risk of having it in a subsequent pregnancy was 90%. The pruritus started at an earlier gestation in subsequent pregnancies (Kruskal–Wallis, $P = 0.006$) (Table 2).

Cyclical pruritus was present in 46 (20%) of women with obstetric cholestasis. Some women experienced pruritus at the time of ovulation (day 14), others just prior to or during the menses, supporting the role of oestrogen and progesterone in the pathogenesis of obstetric cholestasis.

Pruritus was experienced following the oral contraceptive pill in 30 (14%) of women with obstetric cholestasis. Cyclical pruritus occurred in combination with oral contraceptive-induced pruritus in 15 women. Therefore, 61 (27%) women with a history of obstetric cholestasis had either cyclical or oral contraceptive-induced pruritus.

In addition, 30 (13%) women with obstetric cholestasis had a history of gallstones; 21 had symptomatic gallstones and 9 had asymptomatic gallstones documented following ultrasound examination in pregnancy.

DISCUSSION AND CONCLUSION

The data in this paper represent the largest collection of affected obstetric cholestasis pregnancies in the world literature. Specific features were demonstrated that will be of use to clinicians managing the condition; in particular, the findings that in singleton pregnancies intrauterine death mainly occurs from 37 weeks (although the intrauterine deaths in multiple pregnancies occur earlier); also the finding that the earlier in gestation at which pruritus is first reported, the higher the incidence of spontaneous prematurity.

The high prevalence of complications in the obstetric cholestasis group in this study probably results from ascertainment bias, as the women had self-referred to the OCPO. Therefore, this study does not claim to predict the frequency of intrauterine death or prematurity in the total obstetric cholestasis population. It does however allow features of the pregnancy complications of the condition to be studied.

The demonstration that the majority of the intrauterine deaths occur from 37 weeks of gestation is consistent with the largest previous series[10] and should be of clinical value. Elective delivery at 37 weeks of gestation may prevent intrauterine death in affected singleton obstetric cholestasis pregnancies, particularly given that there have been no reliable methods reported for the prediction of fetuses at risk to date.

However, there are fetal risks from early delivery. A study of neonatal respiratory morbidity following elective caesarean section reported a higher incidence of respiratory distress syndrome and transient tachypnoea of the newborn in those that were delivered during the week 37^{+0}–37^{+6} compared with 38^{+0}–38^{+6}, and a similar difference was seen when this was compared with the following week.[19] Another study demonstrated that a markedly higher proportion of babies required ventilation for respiratory distress syndrome when delivered at an earlier gestation; that is, 1:73 (1.4%) at 35 weeks, compared with 1:557 (0.2%) at 37 weeks (and 1:1692 at 38 weeks).[20] These risks are lower than the published rates of intrauterine death in obstetric cholestasis pregnancies that are not delivered by 38 weeks (i.e. 9–11%).[1,2]

A diagnosis of obstetric cholestasis had only been made in two of the pregnancies complicated by intrauterine death in this study. If a diagnosis had been made in more cases, it is possible that treatment with ursodeoxycholic acid (UDCA) or dexamethasone, both of which improve symptoms and liver function tests including serum bile acid levels,[21–23] could have reduced the risk of intrauterine death in these pregnancies. Animal studies have implicated bile acids in the pathophysiology of intrauterine death[24] and spontaneous prematurity.[25] If future prospective studies

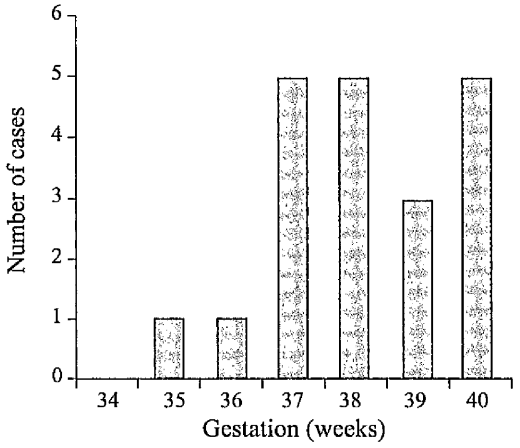


Fig. 2. Gestational week at which intrauterine death occurred in singleton pregnancies complicated by obstetric cholestasis.

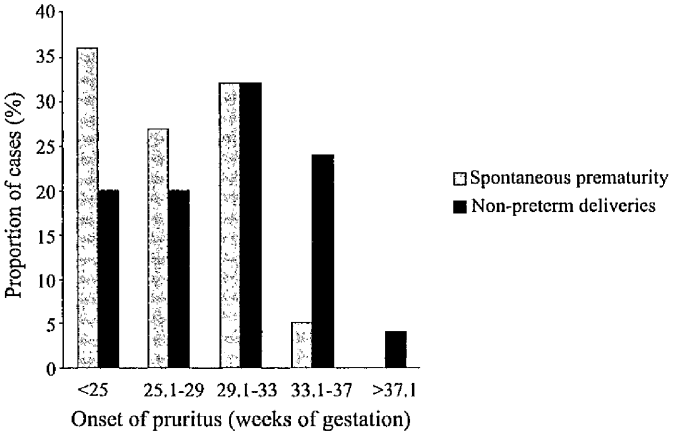


Fig. 4. Gestational week at which pruritus started in obstetric cholestasis pregnancies complicated by spontaneous prematurity and in those in which there was no prematurity.

female and 7 were male. There was an additional female intrauterine death delivered at 26 weeks of gestation, but this pregnancy was not complicated by pruritus (i.e. not defined as being affected by obstetric cholestasis).

This gender difference in singleton pregnancies complicated by intrauterine death (female preponderance) compared with the gender in other obstetric cholestasis pregnancies (χ^2, $P = 0.16$) was not statistically significant. The median gestational week at which intrauterine death occurred in singleton pregnancies was 38 for females and 39 for males.

Three of the intrauterine deaths occurred in twin pregnancies (i.e. 3 of the 17 [18%] twin pregnancies in women with obstetric cholestasis). Two female intrauterine deaths occurred in a male plus female twin and a female plus female twin at 35 weeks and 31 weeks of gestation, respectively. One male intrauterine death occurred in a male plus male twin pregnancy at 36 weeks of gestation.

If all intrauterine deaths are considered, 22% occurred before 37 weeks of gestation. However, only 10% (2/20) of singleton intrauterine deaths occurred before 37 weeks of gestation, and the fetus in both cases was female.

There were no significant differences in gestational week for the onset of pruritus in obstetric cholestasis

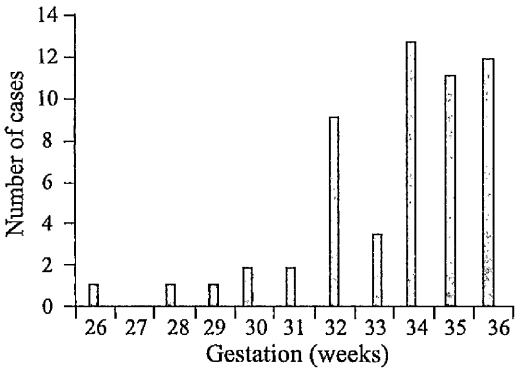


Fig. 3. Gestational week at which spontaneous prematurity occurred in pregnancies complicated by obstetric cholestasis.

pregnancies complicated by an intrauterine death when compared with other obstetric cholestasis pregnancies.

A clinical diagnosis of obstetric cholestasis had been made in two of the pregnancies before they were complicated by intrauterine death. One woman was told that the condition is not associated with intrauterine death and that treatment is not necessary. The other was treated with cholestyramine for cholestasis and the pregnancy was otherwise managed expectantly. In the other 21 cases, the diagnosis of obstetric cholestasis was confirmed by retrospective review of blood specimens taken in the affected pregnancy and/or was made in subsequent pregnancies. Overall, in 14 of the obstetric cholestasis pregnancies complicated by intrauterine death reported in this manuscript, the diagnosis was confirmed by retrospective study of liver function tests from the affected pregnancy and in another case the bile acids were measured using a stored TORCH specimen from the time of the intrauterine death. In the remaining eight cases, the diagnosis of obstetric cholestasis was not confirmed from laboratory tests in the pregnancy that was complicated by intrauterine death, but the diagnosis was made in a subsequent pregnancy.

One hundred and thirty-three (38%) obstetric cholestasis pregnancies were delivered prematurely. There were 56 (16%) spontaneous premature deliveries, and the gestation at which spontaneous prematurity occurred is shown in

Table 1. Relationship between gestational week at which pruritus started and the rate of spontaneous prematurity in obstetric cholestasis pregnancies.

Gestation pruritus started	Total no. cases	Spontaneous prematurity (%)
<25 weeks	91	20 (22%)
25.1–29 weeks	79	15 (19%)
29.1–33 weeks	111	18 (16%)
33.1–37 weeks	63	3 (5%)
>37.1 weeks	8	0 (0%)
Total	352	56

There have been no large studies of the clinical features of obstetric cholestasis pregnancies complicated by, for example, intrauterine death or spontaneous prematurity. The women with obstetric cholestasis in the present study were recruited through a patient organisation, and it is therefore likely that the series was 'enriched' with pregnancies where such complications occurred. However, this was the only practical way to obtain such a large series of obstetric cholestasis pregnancies complicated by intrauterine death and prematurity and it has allowed the features of these clinically important complications to be studied in a large number of cases. Previous studies have been of mixed, or of non-Caucasian, ethnic groups and this is the first study of solely Caucasian women with obstetric cholestasis.

METHODS

Women with obstetric cholestasis were identified via the UK Obstetric Cholestasis Patient Organisation (OCPO) and were invited to participate in a study of the genetic aetiology of obstetric cholestasis. Index cases were mailed a questionnaire to ascertain details of their symptoms, their pedigree and whether they were willing to participate. Returned questionnaires were reviewed to confirm the diagnosis, using the criteria of pruritus and raised serum bile acids and/or raised serum transaminases in at least one pregnancy. As hospitals have different normal ranges for liver transaminase levels, the upper end of the normal range in pregnancy was assumed to be 80% of the level quoted outside pregnancy at the local hospital for each case, consistent with published studies,[18] and any values above this were considered to be abnormal. For some women who fulfilled the diagnostic criteria in a later pregnancy, some previous pregnancies had been complicated by pruritus, but biochemical confirmation had not been obtained. These prior pregnancies were included as affected pregnancies providing the diagnosis had been confirmed in the subsequent pregnancy. Further details were obtained by telephone. Women were asked at what gestational week the different features of the condition occurred. In most cases, this was given as total weeks. If a woman also quoted the number of days (e.g. 35 weeks and 4 days gestation), the figure was rounded down to the number of completed weeks. Women were also asked whether they had experienced pruritus with exogenous oestrogens and whether they had cyclical pruritus or a history of gallstones.

As almost all of the women who had contacted the OCPO were white Caucasians, it was decided to limit the study to this ethnic group.

The gestation at delivery was recorded for all pregnancies that progressed past 24 weeks of gestation. Deliveries less than or equal to 36 weeks and 6 days gestation were divided into spontaneous and iatrogenic premature deliveries. The latter included those that were induced and those delivered by elective caesarean section. The gestational week at which any intrauterine deaths occurred, the sex of the fetus and any treatment given were recorded. Meconium-stained amniotic fluid or cardiotocograph abnormalities were not recorded because accurate information could not be obtained without access to the hospital notes, but information on infant admission to the neonatal intensive care unit (ICU) was collected as an indicator of the condition of the child at birth.

The number of twin pregnancies and the frequency of clinically apparent gallstones were also determined.

Local research ethics committee approval had been granted for this study, and all subjects who participated in the study did so voluntarily having given their informed consent.

The Mann–Whitney U test was used to identify differences in the values between two groups, whereas the Kruskal–Wallis H test was used for more than two groups. The χ^2 test 2×2 was used to compare proportions and replaced by Fischer's exact test when there were small sample sizes. Statistical analysis of the data was performed using SPPS for Windows, release 10.0, SPSS, Chicago, Illinois.

RESULTS

Details of the 227 women with obstetric cholestasis are given in Fig. 1.

Twenty-three (7%) obstetric cholestasis pregnancies were complicated by an intrauterine death; 20 were in singleton and 3 were in twin pregnancies. In the singleton pregnancies, the median gestation at which intrauterine death occurred was 38 weeks (IQR 2.5) (Fig. 2). Of the intrauterine deaths in this group, 13 of the fetuses were

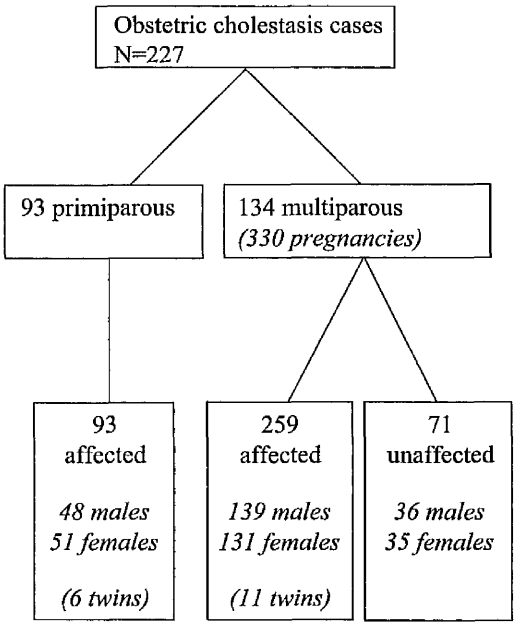


Fig. 1. Subject details.

BJOG: an International Journal of Obstetrics and Gynaecology
July 2004, Vol. 111, pp. 676–681
DOI: 10.1111/j.1471-0528.2004.00167.x

Clinical outcome in a series of cases of obstetric cholestasis identified via a patient support group

Catherine Williamson,[a,b] Laura M. Hems,[a] Dimitrios G. Goulis,[a] Ian Walker,[c] Jennifer Chambers,[a] Oscar Donaldson,[a] Michael de Swiet,[b] Desmond G. Johnston[a]

Objective To explore the clinical features of obstetric cholestasis pregnancies in UK white Caucasians.

Design A questionnaire survey.

Setting Study coordinated at Queen Charlotte's Hospital.

Population Clinical features of 352 affected pregnancies in 227 Caucasian women identified via a patient support group.

Methods Evaluation of the gestation at which prematurity and intrauterine death occur, and recording of additional clinical features in pregnancies complicated by obstetric cholestasis.

Main outcome measures The timing of pregnancies complicated by intrauterine death and prematurity.

Results Among the affected pregnancies, 23 (7%) were complicated by intrauterine death (20 singletons and 3 twins) and 133 (38%) were delivered prematurely (56 spontaneous and 77 iatrogenic). Eighteen of the 20 singleton intrauterine deaths occurred after 37 weeks. All three intrauterine deaths in twin pregnancies occurred before 37 weeks. Pruritus started earlier in pregnancies complicated by spontaneous prematurity, but not in those complicated by intrauterine death.

Conclusions Intrauterine death in singleton pregnancies complicated by obstetric cholestasis death mainly occurs after 37 weeks. The gestation at which pruritus is first reported may help to predict spontaneous prematurity.

INTRODUCTION

Obstetric cholestasis is a disease of pregnancy that causes maternal pruritus and liver impairment, and which can be complicated by fetal distress, spontaneous preterm labour and sudden intrauterine death.[1–4] It affects 0.6% of pregnancies in UK white Caucasians[5] and double this proportion of Indian and Pakistani Asians.[5,6] As there are approximately 700, 000 deliveries in the UK annually, it can be assumed that there will be 4000 cases of obstetric cholestasis. The diagnosis is confirmed by the demonstration of raised liver transaminase and serum bile acid levels.[7,8]

Reported perinatal mortality fell from 9.2–11% in older studies[1,2] to 2.0–3.5% in more recent series,[2–4] perhaps because most women were delivered by 38 weeks of gestation. There were no intrauterine deaths in two recent series, each of approximately 80 women.[6,9] However, in most old and recent series, approximately 50% of obstetric cholestasis pregnancies were complicated by fetal distress, defined as fetal heart rate abnormality or meconium-stained amniotic fluid,[2–4] and this has not changed despite early delivery. Spontaneous preterm delivery (<37 weeks of gestation) has been quoted as affecting 7% of pregnancies in a recent UK series,[6] and 12–44% in studies from Chile and Australia.[1,3,4]

The aetiology of the fetal complications is poorly understood. The intrauterine deaths are thought to occur suddenly, as there is no evidence of preceding intrauterine growth restriction or uteroplacental insufficiency and the fetal autopsy is normal.[3,10] Placental histology shows non-specific changes consistent with hypoxia, but it cannot be established whether the hypoxia is primary or secondary.[11]

At present, it is not possible to predict which pregnancies are at risk of the fetal complications of obstetric cholestasis. Some studies have demonstrated an abnormal fetal heart rate (≤100 or ≥180 bpm).[1,12,13] However, cardiotocograph monitoring is not always helpful and intrauterine deaths have been reported in pregnancies with normal cardiotocographs in the preceding 24 hours.[3,5,14,15] It has also not been established whether the absolute levels of maternal total serum bile acids or the severity of maternal symptoms can be used to predict an adverse pregnancy outcome in obstetric cholestasis[12,16,17] or whether treatment can reduce the risk of the fetal complications.

[a]*Division of Medicine, Imperial College London, Hammersmith Campus, UK*
[b]*Division of Paediatrics, Obstetrics and Gynaecology, Imperial College London, Hammersmith Campus, UK*
[c]*Department of Biochemistry, Wexham Park Hospital, Slough, Berkshire, UK*

Correspondence: Dr C. Williamson, Maternal and Fetal Disease Group, Institute of Reproductive and Developmental Biology, Imperial College London, Hammersmith Campus, Du Cane Road, London W12 0NN, UK.

Human Molecular Genetics, 2000, Vol. 9, No. 8 1209–1217

Heterozygous *MDR3* missense mutation associated with intrahepatic cholestasis of pregnancy: evidence for a defect in protein trafficking

P.H. Dixon[1,2,+], N. Weerasekera[1,2], K.J. Linton[2], O. Donaldson[1], J. Chambers[1], E. Egginton[3], J. Weaver[4], C. Nelson-Piercy[5], M. de Swiet[6], G. Warnes[2], E. Elias[3], C.F. Higgins[2], D.G. Johnston[1], M.I. McCarthy[1,2] and C. Williamson[1,26,§]

[1]Division of Medicine, Imperial College School of Medicine and [2]MRC Clinical Sciences Centre, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK, [3]Department of Gastroenterology, Queen Elizabeth Hospital, Birmingham B15 2TH, UK, [4]Department of Obstetrics and Gynaecology, Birmingham Women's Hospital, Birmingham, B15 2TG, UK, [5]Department of Obstetrics, St Thomas' Hospital, Lambeth Palace Road, London SE1 7EH, UK and [6]Institute of Obstetrics and Gynaecology, Queen Charlottes' Hospital, Goldhawk Road, London W5 0XG, UK

Received 14 January 2000; Revised and Accepted 7 March 2000

Intrahepatic cholestasis of pregnancy (ICP) is a liver disease of pregnancy with serious consequences for the mother and fetus. Two pedigrees have been reported with ICP in the mothers of children with a subtype of autosomal recessive progressive familial intrahepatic cholestasis (PFIC) with raised serum γ-glutamyl transpeptidase (γ-GT). Affected children have homozygous mutations in the *MDR3* gene (also called *ABCB4*), and heterozygous mothers have ICP. More frequently, however, ICP occurs in women with no known family history of PFIC and the genetic basis of this disorder is unknown. We investigated eight women with ICP and raised serum γ-GT, but with no known family history of PFIC. DNA sequence analysis revealed a C to A transversion in codon 546 in exon 14 of *MDR3* in one patient, which results in the missense substitution of the wild-type alanine with an aspartic acid. We performed functional studies of this mutation introduced into *MDR1*, a closely related homologue of *MDR3*. Fluorescence activated cell sorting (FACS) and western analysis indicated that this missense mutation causes disruption of protein trafficking with a subsequent lack of functional protein at the cell surface. The demonstration of a heterozygous missense mutation in the *MDR3* gene in a patient with ICP with no known family history of PFIC, analysed by functional studies, is a novel finding. This shows that *MDR3* mutations are responsible for the additional phenotype of ICP in a subgroup of women with raised γ-GT.

INTRODUCTION

Intrahepatic cholestasis of pregnancy (ICP), also known as obstetric cholestasis, is a liver disorder of pregnancy with serious consequences for the mother and fetus (1–4). Cholestasis results from abnormal biliary transport from the liver into the small intestine, and ICP is characterized by the occurrence of cholestasis in pregnancy in women with an otherwise normal medical history. The classical maternal feature is generalized pruritus, becoming more severe with advancing gestation. ICP causes fetal distress, spontaneous premature delivery and unexplained third trimester intrauterine death (1–4). Delivery by 38 weeks gestation has reduced the perinatal mortality rate from 10–15% (1–2) to 2.0–3.5% (2–4). Abnormal maternal liver function tests (LFTs) are necessary to make the diagnosis. In particular, the serum total bile acid (BA) concentration is raised compared with normal pregnancy (5–8) and this is thought to be due to abnormal biliary transport across the hepatic canalicular membrane (9,10). All LFTs return to normal after delivery. In a subgroup of women the serum γ-glutamyl transpeptidase (γ-GT) level is also increased.

Several human autosomal recessive disorders are recognized to cause abnormal biliary transport. Progressive familial intrahepatic cholestasis (PFIC) is characterized by the onset of cholestasis in early childhood which can progress to cirrhosis and liver failure before adulthood (11), and can be classified into three subtypes (PFIC1–3). PFIC1 and 2 have low concentrations of biliary bile acids and low to normal γ-GT in the serum. PFIC1 (also called Byler disease) is clinically characterized by features of hepatic failure including jaundice, steatorrhoea and reduced growth, and is caused by mutations in the familial intrahepatic cholestasis 1 (*FIC1*) gene (12). Mutations in this gene have also been reported in benign recurrent intrahepatic cholestasis (BRIC), a separate condition in which affected individuals have

+Present address: Neurogenetics Unit, Institute of Neurology, Queen Square, London WC1N 3BG, UK
§To whom correspondence should be addressed at: ICSM Maternal and Fetal Disease Group, MRC Clinical Sciences Centre, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK. Tel: +44 208 383 3014; Fax: +44 208 383 8306; Email: catherine.williamson@ic.ac.uk

Table 1. Patient details

Patient no.	Parity[a]	Start of pruritus (gestational week)	Fetal distress[b]	Gestation at delivery (weeks + days)	Max. ALT[c] (<28 U/l)	Max. γ-GT[d] (<30 U/l)	Max. BA[e] (<6 mM)
1	1 + 0 (twins)	34	No	34 + 2	260	56	233
2	2 + 0	20	No	38	54	85	56
3	2 + 2	33	No	37 + 3	323	34	NT
4	4 + 4	36	Yes	38	163	36	45
5	3 + 4	31	Yes	36 + 2	691	74	34
6	1 + 0	32	No	36	NT	46	100
7	1 + 0	28	No	35 + 4	56[f]	134	236
8	2 + 2	15	Yes	35 + 6	163	50	237

NT not tested.
[a]Expressed as the number of live births + previous pregnancies that have not resulted in a live birth.
[b]Diagnosed if there was meconium staining of the amniotic fluid or fetal heart rate abnormality.
[c]Maximum alanine aminotransferase level (ALT). Normal range is given in parentheses.
[d]Maximum γ-glutamyl transpeptidase. Normal range is given in parentheses.
[e]Maximum bile acids (BA) level. Normal range is given in parentheses.
[f]Alanine aminotransferase level (ALT) was not available for patient 7, so the aspartate aminotransferase level (AST) is shown (normal range in the 3rd trimester <29 U/l).

transient episodes of cholestasis but do not develop hepatic failure (12). The *FIC1* gene encodes a P-type ATPase, which is believed to play a role in the enterohepatic circulation of bile acids. PFIC2 is caused by mutations in the bile salt export pump (*BSEP*) gene (13) (also called *ABCB11*). The clinical features of PFIC2 are similar to those of patients with PFIC1, although the appearance of the bile by electron microscopy is different (11), and the clinical outcome following orthotopic liver transplantation is better than for some PFIC1 patients (11,13).

PFIC3 patients are distinguished by high serum levels of γ-GT and bile which lacks phospholipid but has a normal biliary bile acid concentration (14), together with distinctive liver histology that shows portal duct inflammation and ductular proliferation (15). The raised serum γ-GT is a result of the detergent effect of the relatively high level of bile acid compared with phospholipid in the bile causing release of γ-GT from the biliary epithelium. Homozygous mutations of the multi-drug resistance 3 (*MDR3*) gene have been described in three pedigrees with PFIC3 (10,14). The heterozygote mothers of two affected children with PFIC3 had symptoms consistent with ICP (10,14). In one of these pedigrees in which a large consanguineous family had co-existing PFIC and ICP, three of the six mothers with ICP had pregnancies complicated by unexplained intrauterine death (10). Four of the six women were investigated and shown to be heterozygous for the *MDR3* mutation for which the proband was homozygous (10).

The *MDR3* gene [also known as *ABCB4* and *MDR2*, the homologue of murine *mdr2* (16,17)] encodes the MDR3 protein which is a member of the superfamily of ATP-binding cassette (ABC) transporters (18,19). The MDR3 protein has four domains: two homologous nucleotide-binding domains (NBDs) which bind and hydrolyse ATP and whose sequence is highly conserved throughout the ABC transporter family, and two hydrophobic transmembrane domains (TMDs) which span the membrane multiple times. The MDR3 protein has been shown to be localized to the hepatocyte canilicular membrane and demonstrated to be a phosphatidyl choline (PC) flippase (20–22).

It is possible that mutations of the *MDR3* gene cause the more prevalent disorder, ICP without a family history of PFIC. We investigated this hypothesis by screening the *MDR3* gene in eight individuals with ICP and a raised γ-GT, but no known family history of PFIC. We describe here the identification of a heterozygote *MDR3* mutation in a patient with ICP. We report the functional characterization of the equivalent mutation in a closely related homologue of the gene (*MDR1*), which strongly suggests that the mutant membrane protein misfolds and fails to traffic properly from the endoplasmic reticulum (ER) to the cell membrane.

RESULTS

Mutation detection

DNA sequence analysis of the 27 coding exons of the *MDR3* gene, together with the respective exon–intron boundaries, was performed in eight ICP patients with raised γ-GT levels but no PFIC, together with a normal individual as a control. Patient details are given in Table 1.

In patient 8, a heterozygous DNA base change was identified in exon 14, at the second nucleotide of codon 546 (Fig. 1). In addition to the normal cytosine at this position, an adenine was also detected; this results in the substitution of the wild-type alanine with a mutant aspartic acid (A546D). This mutation is in the highly conserved first NBD of the MDR3 protein. The DNA base change introduces a *Sex*AI restriction enzyme site, which facilitated confirmation of the mutation (Fig. 1). This restriction enzyme change was used to exclude the presence of this mutation in two control panels consisting of 50 parous women without ICP and 40 ICP women without a known raised γ-GT during pregnancy. No other *MDR3* mutations were identified in the other seven individuals with ICP.

A546 is highly conserved in the NBDs of ABC transporters

A546 is a highly conserved residue, not only in the NBDs of proteins orthologous to the MDR3 protein in rat and mouse (16,23), but also in other members of the superfamily of ABC

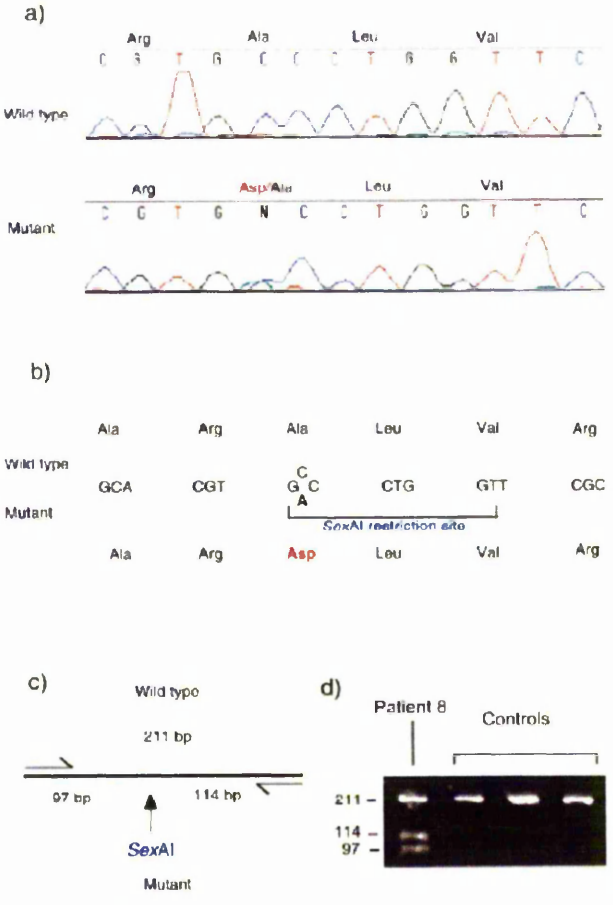


Figure 1. (**a**) The DNA sequence from patient 8 and a control patient. The C→A transversion results in the substitution of the wild-type alanine with an aspartic acid. The transversion also generates a *Sex*AI restriction site (**b**). Amplification of exon 14 by PCR gives a 211 bp product. If the C→A mutation is present, digestion of this product with *Sex*AI produces two smaller fragments of 97 and 114 bp, respectively (**c**). Thus, digestion of DNA from patient 8 with *Sex*AI reveals three products: the larger wild type product, and the two smaller fragments. In control subjects the 211 bp product is insensitive to cleavage by *Sex*AI (**d**).

transporters, including human P-glycoprotein (P-gp1) (24) and the NBD of the histidine permease (HisP) of *Salmonella typhimurium*. Whereas little is known about the structure of mammalian ABC transporters, the high resolution structure of HisP has been reported (25). From the crystal structure of HisP (25), A167 (the equivalent of the MDR3 protein A546) is located towards the C-terminus of α-helix 5 and, spatially, is in close proximity to the hydrophobic residues preceding the Walker B motif (Fig. 2).

Mature A544D-P-gp1 at the cell surface is functional

There are no established systems for quantification of PC translocation in mammalian systems making it currently not possible to study the function of the MDR3 protein. However, the transport of fluorescent substrates such as rhodamine by the multi-drug transporter P-gp1 is relatively easy to assay in mammalian cell culture systems. The MDR3 protein shares

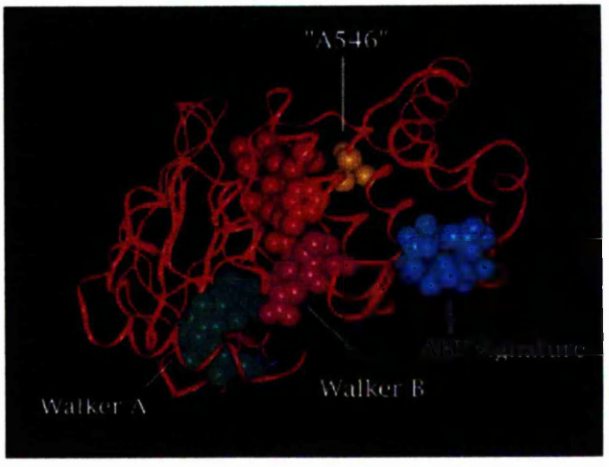


Figure 2. Ribbon representation of the α-carbon backbone from the crystal structure of HisP (25), the NBD from the histidine permease of *Salmonella typhimurium*. The side chains of A167 (the equivalent of A546 in MDR3 protein), the Walker A motif, the Walker B motif and the ABC signature are shown space filled. The four hydrophobic residues at the N-terminal end of the Walker B motif are shown in red and the C-terminal residues are shown in purple. The bound ATP is shown as ball and stick.

77% identity with P-gp1 and it has been shown that substitution of as few as three adjacent MDR3 protein residues in the first TMD by the equivalent residues from P-gp1 is sufficient to allow the chimeric molecule to transport substrates specific to P-gp1 (26). The level of sequence identity between P-gp1 and the MDR3 protein increases to 87% in the NBDs. In addition, the NBDs of P-gp1 and the MDR3 protein have been shown to be interchangeable (27). We therefore introduced the equivalent A546D mutation into the first NBD of P-gp1 and studied the functional consequences of the change in transiently transfected mammalian cells.

Alignment of the amino acid sequence of human P-gp1 with the MDR3 protein identified A544 as the P-gp1 equivalent of MDR3 protein A546. Site-directed mutagenesis was used to alter codon 544 of *MDR1*, to encode an aspartic acid. The mutated fragment was subcloned into the plasmid pMDR1-wt to generate pMDR1-A544D, and used to transiently transfect human epithelial kidney (HEK293T) cells.

Fluorescence activated cell sorting (FACS) of live cells was used to correlate the ability of transiently transfected cells to extrude a fluorescent substrate of P-gp1, rhodamine 123 (R123). The presence of P-gp1 at the cell surface in the same batch of transfected cells was determined by the P-gp1-specific monoclonal antibody (mAb) UIC2 conjugated to phycoerythrin (UIC2-PE).

Cells transiently transfected with pCIneo-βgal which does not encode P-gp1 all behaved in a similar way and accumulated R123 and failed to label with UIC2-PE (Fig. 3a). In contrast, cells transiently transfected with pMDR1-A544D formed two distinct populations. The first population behaved similarly to the control cells with no UIC2-PE fluorescence and high R123 fluorescence (Fig. 3b, lower right quadrant), consistent with failure to express A544D-P-gp1 at the cell surface (and thus accumulate R123). The second cell population was characterized by high UIC2-PE fluorescence and no

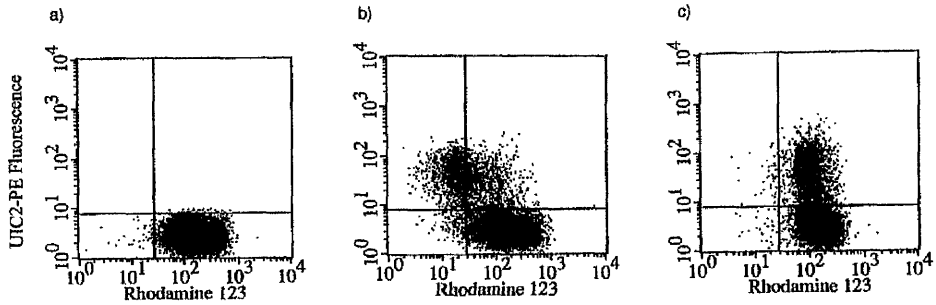


Figure 3. FACS analysis of HEK293T cells transiently transfected with pCIneo-βgal (a), or pMDR1-A544D (b and c). Cells were incubated first with the P-gp1 substrate R123 in the presence (c) or absence (a and b) of the P-gp inhibitor CsA. The cells were then incubated with the P-gp1-specific mAb (UIC2-PE) before FACS.

Table 2. Ratio of intracellular to cell surface P-glycoprotein

	Mean fluorescence (relative arbitrary fluorescence units)		
	A544D-P-gp1	Wt-P-gp1	HEK293T
Intact cells	40	171	3
F&P then labelled with UIC2-PE	197	497	13
Labelled with UIC2-PE then F&P	32	144	3
Intracellular P-gp	*155*	*343*	–
Cell surface P-gp	*37*	*168*	–
Total P-gp	*192*	*511*	–

Summary of the mean observed fluorescence of intact cells (Fig. 4), and of the mean fluorescence following fixation and permeabilization (F&P) either before or after P-gp1-specific mAb UIC2-PE labelling (Fig. 6). This allowed the intracellular, cell surface and total P-gp of wt-P-gp1 and A544D to be calculated (calculated values are shown in italics).

R123 fluorescence (Fig. 3b, upper left quadrant) consistent with cell surface expression of A544D-P-gp1 and extrusion of R123. Further confirmation that the extrusion of R123 in the A544D-P-gp1-expressing cells is due to the function of the mutant P-gp was obtained by incubating the cells with the P-gp1-inhibitor cyclosporin A. Under these conditions the cells with a high UIC2-PE fluorescence accumulated high levels of R123 fluorescence (Fig. 3c, upper right quadrant). These data are typical of cells expressing functional P-gp1 (28) and indicate that the A544D-P-gp1 mutant is functional if it reaches the cell membrane.

Evidence that A544D-P-gp1 is a trafficking mutant

The finding that substitution of an aspartic acid for an alanine in a highly conserved region of NBD1 did not alter the ability of the mature protein to transport R123 was unexpected and suggested that the equivalent A546D-MDR3 protein mutant would also be functional. However, the FACS analysis provided evidence that the mutant protein is inefficiently expressed compared with the wild-type protein (Table 2).

When transiently transfected with pMDR1-wt, 97% of HEK293T cells expressed wild-type P-gp1 at the cell surface [compare the UIC2-PE labelling of intact cells transfected with pCIneo-βgal or pMDR1-wt (Fig. 4)]. There were two populations of pMDR1-wt-transfected cells which differed by the amount of wild-type P-gp1 at the cell surface (Fig. 4). The first population (M1) had a mean UIC2-PE fluorescence of 38 relatively arbitrary fluorescence units and the second population (M2) had a mean of 200. The overall mean fluorescence was 171, compared with a control value of 3. We suspect that those cells that have a lower level of P-gp1 at the cell surface (M1) took up the DNA shortly after mitosis and that this resulted in a lag phase of one cell cycle until the nuclear membrane dissolved to permit entry into the nucleus and expression of the *MDR1* gene. Effectively, these cells have had a delayed transfection time and so may have only had 24 h for gene expression from the introduced plasmid, which is insufficient time to accumulate maximal levels of wild-type P-gp1 at the cell surface. The population of cells that express high levels of P-gp1 at the cell surface (M2) probably entered mitosis soon after the introduction of the pMDR1-wt DNA and so have effectively had double the expression time.

Cells transfected with pMDR1-A544D also formed two populations (Fig. 4); the first population (M3) had a mean UIC2-PE fluorescence of 10 [barely above the mean (= 3) of the negative control cells], and the second population (M4) had a mean of 50. The overall mean fluorescence was 40. These data indicate that the mutant protein is expressed at the cell surface at much lower levels than the wild-type protein (Table 2).

Western blot analysis of A544D-P-gp1

Transiently transfected cells normally express two forms of P-gp1, discernible by polyacrylamide gel electrophoresis (PAGE) and western analysis: immature, non- or core-glyco-

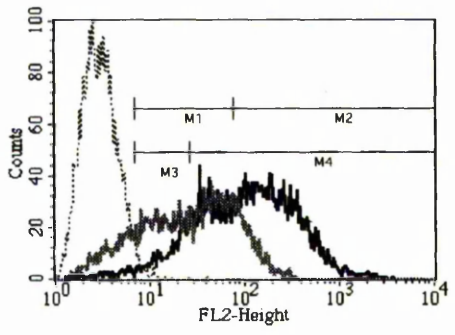


Figure 4. UIC2-PE antibody labelling of intact HEK293T cells transiently transfected with pCIneo-βgal (grey intermittent line), pMDR1-wt (black) or pMDR1-A544D (grey). The counts represent mean fluorescence (relative arbitrary fluorescence units), and this is summarised for intact cells in Table 2. The transfected populations were observed to consist of two separate populations (M1 and M2 for pMDR1-wt, M3 and M4 for pMDR1-A544D) with different amounts of protein at the cell surface.

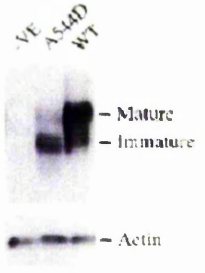


Figure 5. Western analysis of HEK293T cells transiently transfected with pCIneo-βgal (–VE), pMDR1-A544D (A544D) and pMDR1-wt (WT). The blot was probed with anti-P-gp mAb, C219, and the 170 kDa mature, glycosylated form and the 140 kDa immature form of the protein are indicated. The blot was reprobed with anti-actin mAb to confirm that the same amount of protein (20 mg) was added to each lane.

sylated P-gp1 and mature glycosylated P-gp1. All of the immature P-gp1 is found in intracellular compartments (29), and probably represents nascent polypeptide in the ER. The glycosylation of the first extracellular loop of P-gp1 is completed in the distal cisternae of the Golgi apparatus. If the A544D mutation impairs the trafficking of the protein from the ER to the cell membrane then it might be expected to alter the ratio of immature to mature protein found in the cell. PAGE and western analysis (Fig. 5) showed that wild-type P-gp1 was expressed at higher levels than A544D-P-gp1 and was predominantly present as the 170 kDa mature, glycosylated form. In contrast, the A544D-P-gp1 was predominantly found in the 140 kDa immature form. Reprobing of the blot with anti-actin mAb indicated that the same amount of cell protein was added to each lane (Fig. 5).

The ratio of cell surface to intracellular P-gp1 is also altered in the A544D mutant

Additional evidence that A544D-P-gp1 is a trafficking mutant was provided by calculating the ratio of cell surface to intracellular P-gp1 by FACS analysis (Fig. 6 and Table 2). Cells that were fixed with paraformaldehyde and permeabilized with saponin prior to incubation with antibody, provided an indication of the total P-gp in the cell (Fig. 6, black traces). The intracellular P-gp fraction was then determined by subtraction from this figure of the cell surface P-gp determined by incubation of live cells with antibody before fixation and permeabilization and FACS (Fig. 6, grey traces); this controlled for the possible loss of cell surface antibody–antigen conjugate during the fixation and permeabilization. It was also necessary to subtract the antibody bound non-specifically to cells as this fraction changes when intracellular antigens are exposed (Fig. 6c compare black and grey traces). These data, obtained for cells expressing wild-type P-gp1 and for cells expressing the A544D mutant, are summarized along with the background levels of fluorescence in Table 2. Calculation of total P-gp1 level and the ratio of intracellular to cell surface P-gp clearly showed that cells transfected with pMDR1-A544D expressed a lower level of P-gp1 than cells transfected with pMDR1-wt (total fluorescence 192 and 511, respectively), and that much less of it got to the cell surface (only 19% of the total produced, compared with 33% of wild-type protein). The western analysis of cells expressing wild-type P-gp1 demonstrated that there is more of the mature, glycosylated form than of the immature form of the protein. When considered together, these results demonstrate that not all of the mature protein is at the cell surface. These results show that the MDR3 mutation A546D causes abnormal protein glycosylation and trafficking when its equivalent is expressed in P-gp1.

DISCUSSION

Our analysis of the sequence of the coding region of the *MDR3* gene in eight patients with ICP and raised γ-GT has revealed the presence of a mutation in exon 14 (A546D) in one individual. This mutation was not found in 50 parous controls without ICP, nor in 40 ICP women without a raised γ-GT, indicating that it is not a common polymorphism. The location of this mutation within the highly conserved first NBD indicates that this is not likely to be a benign alteration, and we provide data showing that it interferes with protein trafficking. Thus, ICP without PFIC can be caused by mutations in *MDR3*. We only found a *MDR3* mutation in one of eight women; this could be because other mutations are in untranslated regions, or in regulatory regions of the gene that were not screened in this study.

To date, only three other mutations of the *MDR3* gene have been published, all in individuals with PFIC3 (10,14); in two of the pedigrees, the heterozygous mothers had symptoms consistent with ICP. These mutations are believed to cause loss of function, as they all introduce premature stop codons. The occurrence of the A546D mutation, the substitution of a hydrophobic amino acid with a charged polar amino acid, in the highly conserved first NBD, suggested that this mutation would also result in loss-of-function of the MDR3 protein. In order to gain insight into the functional consequences of this mutation, we tested the effects of the equivalent mutation to A546D in P-gp1 (A544D). Transient expression of A544D-P-gp1 in HEK293T cells, and subsequent FACS and western analysis suggested that this mutation reduces the abundance of the protein and impairs protein trafficking to the cell

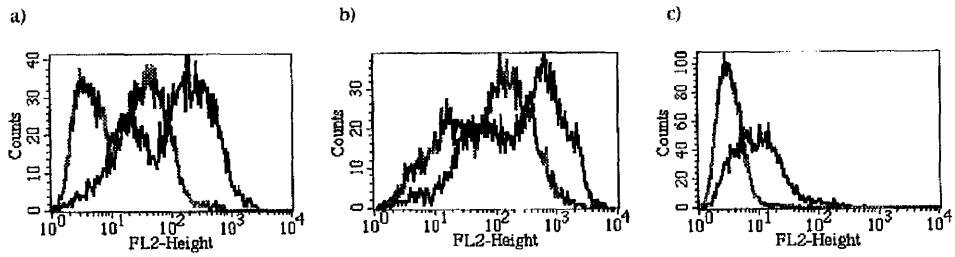


Figure 6. FACS analysis of cells transiently transfected with pMDR1-A544D (a), pMDR1-wt (b) or pCIneo-βgal (c). Fluorescence from cells which have been fixed with paraformaldehyde and permeabilized with saponin, prior to incubation with antibody (UIC2-PE) is shown in black, and from cells incubated with antibody before fixation and permeabilization in grey. The mean fluorescence for each population of cells is given in Table 2.

membrane. This probably arises as a consequence of misfolding of the first NBD leading to retention of the protein in the ER and an increase in the rate of degradation. The A546D mutation in the MDR3 protein is likely to have a similar phenotype as there is considerable sequence identity between the proteins in this domain (87%), and because the NBDs of P-gp1 and the MDR3 protein have been shown to be functionally interchangeable (27).

A number of missense mutations have been identified in other genes that result in trafficking defects, including the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (30,31), which encodes the CFTR protein, another member of the ABC transporter superfamily, and genes encoding the Wilson disease and Menkes disease proteins, both of which are P-type ATPases (32,33). Such mutations in membrane transport proteins commonly result in reduced function in addition to abnormal protein trafficking (32–34). However, a disease-causing mutation has been reported in *CFTR* which is associated with protein mislocalization, but normal CFTR protein function (31). The *MDR3* mutation we have identified may be similar, resulting in an increased proportion of mislocalized protein, but normal MDR3 protein function if it reaches the cell membrane. The results of our functional studies in P-gp1 support this hypothesis.

Western and FACS analysis suggest that cells transiently transfected with pMDR1-wt produce more of the mature, glycosylated form of wild-type P-gp1, but that the majority of this is not localized at the cell surface. There is *in vitro* evidence for the existence of cytoplasmic reservoirs of bile acid transporters in intracellular vesicles which are recruited to the hepatocyte canalicular membrane, resulting in increased bile acid transport (35). This mechanism has not been specifically investigated in P-gp1, but it is possible that mature P-gp1 also resides in the Golgi apparatus and recycles to the cell surface. It has also been demonstrated that the mature Wilson disease protein and the Menkes disease protein reside in the *trans*-Golgi apparatus and recycle to the cell surface when copper transport is required (32,33).

It is not known why women with ICP only develop symptoms of cholestasis in pregnancy. The short-term administration of oral oestrogens to women with a history of ICP causes pruritus and abnormal LFTs (36,37), suggesting that predisposed women develop ICP due to the cholestatic effect of raised oestrogens in pregnancy. Progestogens may also play a role (6,38). Progestogens and oestrogens can inhibit MDR-catalysed drug efflux and interact with P-gp (39). It is therefore possible that raised serum oestrogens and progestogens in normal pregnancy cause reduced function of the MDR3 protein, and that women who are heterozygotes for a trafficking mutation in the MDR3 protein have a reduced ability to compensate for this.

Although the cause of fetal distress and intrauterine death in ICP is not fully understood, some studies have reported a more frequent occurrence of fetal distress in cases with high maternal (8) or fetal (40) bile acid levels. Loss of function of the MDR3 protein results in raised serum bile acids as a secondary effect, as is seen in children with PFIC3 (14) and the heterozygote mothers with ICP (10). Thus, *MDR3* mutations which result in raised serum bile acids, such as the one reported in this study, may predispose to fetal distress and subsequent unexplained intrauterine death.

Of the 87 patients with confirmed diagnosis of ICP and raised serum bile acids, 20 had a raised γ-GT. The finding that 23% of women with ICP had a raised γ-GT was of interest as the frequency of high γ-GT PFIC in non-consanguineous families is extremely low. However, there are several non-hereditary causes of cholestasis in pregnancy that can result in raised serum bile acids with a raised γ-GT, including viral hepatitis, autoimmune hepatitis, drug-induced cholestasis, cholelithiasis and other causes of biliary obstruction, e.g. malignancy. Although these diagnoses are rare and will have been excluded in the majority of women in this study, we did not have access to the results of these investigations in all of the women in whom the retrospective measurement of γ-GT was performed.

Therapeutic interventions to improve protein trafficking are under investigation for the ΔF508 mutation in CFTR (41), and this approach may be useful for the treatment of protein trafficking mutations in ICP.

In conclusion, we report a heterozygous missense mutation in the *MDR3* gene in a patient with ICP. Our analysis of this mutation in P-gp1 suggests that it results in abnormal protein trafficking and a subsequent lack of functional protein at the cell surface. These results confirm that *MDR3* mutations are responsible for ICP in some women with raised γ-GT. Identification and characterization of further mutations in this gene will greatly increase our understanding of the role of *MDR3* in this subgroup of women with ICP.

MATERIALS AND METHODS

Patients

For mutational analysis of the *MDR3* gene, eight patients with raised γ-GT were analysed together with a normal control individual. Of these eight individuals, two were recruited prospectively on detection of raised γ-GT and the remaining six were added following retrospective measurement of γ-GT levels from stored serum in 87 patients. Of the 87 patients with a confirmed diagnosis of ICP and raised serum bile acids, 20 had raised γ-GT. As different hospitals have different normal ranges for liver function tests, the upper end of the normal range in pregnancy was assumed to be 80% of the level quoted outside pregnancy for each hospital, consistent with published studies (42). Full clinical details of the patients are given in Table 1.

Having identified a mutation in patient 8, we had wanted to obtain blood samples from her relatives for DNA extraction to establish whether the mutation was maternally or paternally acquired, or sporadic. However, this was not possible because she was adopted and has no contact with her biological parents.

To check for the presence of sequence variants in normal individuals we utilized a panel of 50 women with gestational diabetes but with otherwise normal pregnancies who had no history of cholestasis. We also analysed 40 women with obstetric cholestasis without known raised γ-GT (the γ-GT was not tested in all cases).

Sequence analysis and mutation detection

Long-range PCR products covering parts of the *MRD3* gene were generated using primers designed from the published sequence (17). Products varying in size from 4 to 12.3 kb were amplified using the Expand long-range PCR kit (Boehringer Mannheim, East Sussex, UK), and subcloned using the TOPO XL kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Multiple independent positive colonies were picked and DNA prepared using the Wizard miniprep kit (Promega, Maddison, WI). Inserts were sequenced with vector-specific and *MDR3*-specific primers using the FS$^+$ Dye Terminator sequencing kit (PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Products were resolved and the sequence determined using a 373XL semi-automated sequencer (PE Applied Biosystems), and the sequence analysed utilising the Sequence Analysis, Sequence Navigator and AutoAssembler software packages (PE Applied Biosystems).

Sequence information from these experiments was combined with previously published information (17) and with sequence information from the Washington University chromosome 7 sequencing project (http://genome.wustl.edu/gsc) to design 27 pairs of exon-specific primers for the *MDR3* gene (available from the authors on request). These primers were utilized to amplify the 27 coding exons of the *MDR3* gene together with the respective exon–intron boundaries. Amplification of genomic DNA by PCR was carried out using an automated DNA thermal cycler (MJ Research Tetrad, Genetics Research Instrumentation Ltd, Felstead, Essex) in a total volume of 50 µl containing 50–100 ng of DNA template, 100 ng of each oligonucleotide primer, 50 mM KCl, 10 mM Tris–HCl pH 8.3, 1–3 mM $MgCl_2$, 0.01% gelatin, 100 mM dNTP and 2 U *Taq* polymerase (Bioline, London, UK). Reaction conditions were as follows: 96°C for 3 min, 35 cycles of 30 s at 94°C, 30 s at 55–62°C and 1 min at 72°C, followed by a final extension for 10 min at 72°C. Following amplification, products were gel-purified and subject to DNA sequencing reactions as described above to obtain sequence for both strands of the PCR product. Sequences were compared with the Sequence Navigator software package (PE Applied Biosystems).

Digestion of the PCR products with the restriction enzyme *Sex*AI (New England Biolabs, Beverley, MA), to assay for the presence of the mutated sequence, was carried out in a 30 µl volume using the conditions recommended by the manufacturer. Restriction fragments were resolved on 2% agarose gels.

Study of the MDR3 protein A546D mutation in P-gp1

Plasmid pMDR-wt encodes wild-type P-gp1 with a hexa-histidine tag at the C-terminus and has been described previously (28). This protein shows normal P-gp1 activity. Plasmid pCIK-βgal was kindly provided by D. Gill, University of Oxford, UK, and is based on pCI (Promega) which was engineered to express the *lacZ* sequence. Plasmid pSBH is based on the mutagenesis vector pAlter (Promega) and contains the first half of wild-type *MDR1* from the *Bam*HI site 5′ to the coding sequence, to the *Hin*dIII site spanning codons 681 and 682.

Site-directed mutagenesis

Alignment of the amino acid sequence of human P-gp1 with the MDR3 protein identified A544 as the P-gp1 equivalent of MDR3 protein A546. Site-directed mutagenesis was used to alter codon 544 of *MDR1* to encode an aspartic acid, adopting the C→A tranversion of the second position of the codon analogous to the A546D mutant of the MDR3 protein. The A544D mutation was introduced into NBD1 of P-gp1 by oligonucleotide-directed mutagenesis ('Altered sites' II; Promega) of pSBH. The mutagenic oligonucleotide (5′-CGCCATTGCAC-GAGACCTGGTTCGCAAC-3′) introduced the desired codon change at the same time as removing the adjacent *Pml*I restriction site without further alteration to the amino acid sequence of P-gp1. The nucleotide sequence of the mutated DNA was verified by automated DNA sequencing as described above, prior to subcloning the mutated *Eco*RI–*Hin*dIII DNA fragment back into pMDR-wt to generate pMDR-A544D.

A further change, thymine (T) to adenine (A) was made in the wobble position of codon 543 of *MDR1* to remove, silently, the *Pml*I restriction site in order to follow the subsequent subcloning events. The mutated fragment was subcloned into pMDR1-wt to generate pMDR1-A544D for expression studies in mammalian cells.

Transient transfection of mammalian HEK293T cells

HEK293T human epithelial kidney cells (3.75×10^6; Imperial Cancer Research Fund, cell production unit, Clare Hall, London, UK) were seeded on to a 75 cm^2 tissue culture flask in 15 ml of Dulbecco's modified Eagle's medium (DMEM), supplemented with 2 mM L-glutamine and 10% (v/v) fetal calf serum (FCS; Helena Biosciences, Sunderland, UK). The cells were incubated under 5% CO_2 at 37°C for 18 h to give ~70% confluency at which point the cells were transfected with polyethyleneimine (PEI)–DNA complexes: 60 µg of DNA in 30 µl

5% glucose was mixed with 9 µl of 25 kDa PEI solution [45mg PEI (Sigma Aldrich, Gillingham, UK) dissolved in 8 ml H_2O, corrected to pH 7.2 with dilute HCl]; this was diluted in 10 ml of supplemented DMEM and added directly to the cell monolayer. Twenty-four hours post-transfection the medium was replaced with fresh DMEM plus 2 mM butyric acid (Sigma Aldrich) to enhance expression of P-gp. The cells were harvested a further 24 h later.

Western analysis

Whole cell lysates were prepared from 5×10^5 transiently transfected cells by resuspension of the washed cell pellet in 300 µl lysis buffer [phosphate-buffered saline (PBS; pH 7.4), 1% SDS, 100 µM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 4 µg/ml pepstatin and 1 mM ethylenediaminetetraacetic acid]. Cell lysate (10 µg) was separated by SDS–PAGE and the proteins transferred electrophoretically to PVDF membrane (Immobilon-P; Millipore, Bedford, MA). The membrane was probed with the anti-P-gp monoclonal antibody, C219 (Cis-Bio International, Gif-sur-Yvette, France), and developed using horseradish peroxidase-conjugated goat anti-mouse antibody and enhanced chemiluminescence (Amersham, Uppsala, Sweden).

FACS analysis

Dual labelling of intact cells was used to assay for both P-gp function and localization at the cell membrane. Transiently transfected HEK293T (1.25×10^6) cells were harvested by incubation with versene (2 mM EDTA in PBS) for 10 min at 37°C. The cells were washed twice with PBS and then incubated with 2 µM rhodamine 123 (R123; Sigma Aldrich), with or without cyclosporin A (CsA; 10 µM), and incubated for a further 30 min at 37°C. The cells were washed three times with versene, then incubated with 20 µg/ml anti-P-gp antibody [UIC2, phycoerythrin conjugate (UIC2-PE); Immunotech, Marseille, France] or isotype control diluted in FACS buffer (1% FCS in PBS) for 15 min at 4°C. The cells were washed three times in FACS buffer before resuspension in 300 µl of FACS buffer. Samples were stored in the dark at 4°C until analysis. Flow cytometric analysis of the cells (10 000) was carried out using a FACS Vantage flow cytometer (Becton Dickinson, San Diego, CA) fitted with an argon ion laser. PE fluorescence was measured at 575 nm and R123 fluorescence at 515 nm. Fluorescence data was analysed using CELLQuest (Becton Dickinson) software.

To detect intracellular antigen, 1.25×10^6 cells, harvested and washed as before, were fixed by addition of formaldehyde to 3% and incubated at room temperature for 20 min. The cells were washed three times in FACS buffer and resuspended in 0.5% saponin (in FACS buffer) and incubated for 20 min at 4°C. The cells were washed a further three times in FACS buffer before incubation with UIC2 and FACS as before.

ACKNOWLEDGEMENTS

Retrospective measurement of γ-GT was kindly performed by Ruth Gallagher, Deborah Fern and Rosanna Penn at Selly Oak Hospital (Birmingham, UK). This work was supported by the Wellcome Trust and the Medical Research Council. C.W. is a Wellcome Advanced Fellow.

REFERENCES

1. Reid, R., Ivey, K.J., Rencoret, R.H. and Storey, B. (1976) Fetal complications of obstetric cholestasis. *Br. Med. J.*, **1**, 870–872.
2. Reyes, H. (1982) The enigma of intrahepatic cholestasis of pregnancy: lessons from Chile. *Hepatology*, **2**, 87–96.
3. Fisk, N.M. and Storey, G.N.B. (1988) Fetal outcome in obstetric cholestasis. *Br. J. Obstet. Gynecol.*, **95**, 1137–1143.
4. Rioseco, A.J., Ivankovic, M.B., Manzur, A., Hamed, F., Kato, S.R., Parer, J.T. and Germain, A.M. (1994) Intrahepatic cholestasis of pregnancy: a retrospective case–control study of perinatal outcome. *Am. J. Obstet. Gynecol.*, **170**, 890–895.
5. Sjovall, K. and Sjovall, J. (1966) Serum bile acid levels in pregnancy with pruritus. *Clin. Chem. Acta*, **13**, 207–211.
6. Bacq, Y., Myara, A., Brechot, M.C., Hamon, C., Studer, E., Trivin, F. and Metman, E.H. (1995) Serum conjugated bile acid profile during intrahepatic cholestasis of pregnancy. *J. Hepatol.*, **22**, 66–70.
7. Laatikainen, T.J. and Ikonen, E. (1977) Serum bile acids in cholestasis of pregnancy. *Obstet. Gynecol.*, **50**, 313–318.
8. Laatikainen, T.J. and Tulenheimo, A. (1984) Maternal serum bile acid levels and fetal distress in cholestasis of pregnancy. *Int. J. Gynaecol. Obstet.*, **22**, 91–94.
9. Meng, L.J., Reyes, H., Palma, J., Hernandez, I., Ribalta, J. and Sjovall, J. (1997) Profiles of bile acids and progesterone metabolites in the urine and serum of women with intrahepatic cholestasis of pregnancy. *J. Hepatol.*, **27**, 346–357.
10. Jacquemin, E., Crestil, D., Manouvier, S., Boute, O. and Hadchouel, M. (1999) Heterozygous nonsense mutation in the MDR3 gene in familial intrahepatic cholestasis of pregnancy. *Lancet*, **353**, 210–211
11. Bull, L.N., Carlton, V.E., Stricker, N.L., Baharloo, S., DeYoung, J.A., Freimer, N.B., Magid, M.S., Kahn, E., Markowitz, J., DiCarlo, F.J. *et al.* (1997) Genetic and morphological findings in progressive familial intrahepatic cholestasis (Byler disease [PFIC-1] and Byler syndrome): evidence for heterogeneity. *Hepatology*, **26**, 155–164.
12. Bull, L.N., van Eijk, M.J.T., Pawlikowska, L., DeYoung, J.A., Juijn, J.A., Liao, M., Klomp, L.W.J., Lomri, N., Berger, R., Scharschmidt, B.F. *et al.* (1998) A gene encoding P-type ATPase mutated in two forms of hereditary cholestasis. *Nature Genet.*, **18**, 219–224.
13. Strautnieks, S.S., Bull, L.N., Knisely, A.S., Kocoshis, S.A., Dahl, N., Arnell, H., Sokal, E., Dahan, K., Childs, S., Ling, V. *et al.* (1998) A gene encoding a liver-specific ABC transporter is mutated in progressive familial intrahepatic cholestasis. *Nature Genet.*, **20**, 233–238.
14. de Vree, J.M.L., Jacquemin, E., Sturm, E., Cresteil, D., Bosma, P.J., Aten, J., Deleuze, J.-F., Desrochers, M., Burdelski, M., Bernard, O. *et al.* (1998) Mutations in the *MDR3* gene cause progressive familial intrahepatic cholestasis. *Proc. Natl Acad. Sci. USA*, **95**, 282–287.
15. Maggiore, G., Bernard, O., Riely, C.A., Hadchouel, M., Lemonnier, A. and Alagille, D. (1991) Diagnostic value of serum gamma-glutamyl transpeptidase activity in liver diseases in children. *J. Pediatr. Gastroenterol. Nutr.*, **12**, 21–26.
16. Gros, P., Ben Neriah, Y.B., Croop, J.M. and Housman, D.E. (1986) Isolation and expression of a complementary DNA that confers multidrug resistance. *Nature*, **323**, 728–731.
17. Linke, C.R., Smit, J.J.M., van der Velde-Koerts, T. and Borst, P. (1991) Structure of the human MDR3 gene and physical mapping of the human MDR3 locus. *J. Biol. Chem.*, **266**, 5303–5310.
18. Higgins, C.F., Hiles, I.D., Salmond, G.P., Gill, D.R., Downie, J.A., Evans, I.J., Holland, I.B., Gray, L., Buckel, S.D., Bell, A.W. *et al.* (1986) A family of related ATP-binding subunits coupled to many distinct biological processes in bacteria. *Nature*, **323**, 448–450.
19. Higgins, C.F. (1992) ABC transporters: from microorganisms to man. *Annu. Rev. Cell. Biol.*, **6**, 67–113.
20. Ruetz, S. and Gros, P. (1994) Phosphatidylcholine translocase: a physiological role for the mdr2 gene. *Cell*, **77**, 1071–1081.
21. Smith, A.J., Timmermans-Hereijgers, J.L., Roelofsen, B., Wirtz, K.W., van Blitterswijk, W.J., Smit, J.J., Schinkel, A.H. and Borst, P. (1994) The human MDR3 P-glycoprotein promotes translocation of phosphatidylcholine through the plasma membrane of fibroblasts from transgenic mice. *FEBS Lett.*, **354**, 263–266.
22. van Helvoort, A., Smith, A.J., Sprong, H., Fritzsche, I., Schinkel, A.H., Borst, P. and van Meer, G. (1996) MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. *Cell*, **87**, 507–517.

23. Brown, P.C., Thorgeirsson, S.S. and Silverman, J.A. (1993) Cloning and regulation of the rat mdr2 gene. *Nucleic Acids Res.*, **21**, 3885–3891.
24. Chen, C., Chin, J.E., Ueda, K., Clark, D.P., Pastan, I., Gottesman, M.M. and Roninson, I.B. (1986) Internal duplication and homology with bacterial transport proteins in the mdr1/P-glycoprotein gene from multidrug resistant human cells. *Cell*, **47**, 381–389.
25. Hung, L.-W., Wang, I.X., Nikaido, K., Liu, P.-Q., Ames, G.F.-L. and Kim, S.-H. (1998) Crystal structure of the ATP-binding subunit of an ABC transporter. *Nature*, **396**, 703–707.
26. Zhou, Y., Gottesman, M.M. and Pastan, I. (1999) Studies of human MDR1-MDR2 chimeras demonstrate the functional exchangeability of a major transmembrane segment of the multidrug transporter and phosphatidylcholine flippase. *Mol. Cell. Biol.*, **19**, 1450–1459.
27. Buschman, E. and Gros, P. (1991) Functional analysis of chimeric genes obtained by exchanging homologous domains of the mouse mdr1 and mdr2 genes. *Mol. Cell. Biol.*, **11**, 595–603.
28. Blott, E.J., Higgins, C.F. and Linton, K.J. (1999) Cysteine scanning mutagenesis provides no evidence for the extracellular accessibility of the nucleotide-binding domains of the multidrug resistance transporter P-glycoprotein. *EMBO J.*, **18**, 6800–6808.
29. Loo T.W. and Clarke, D.M. (1995) Membrane topology of a cysteine-less mutant of human P-glycoprotein. *J. Biol. Chem.*, **270**, 838–848.
30. Cheng, S.H., Gregory, R.J., Marshall, J., Paul, S., Souza, D.W., White, G.A., O'Riordan, C.R. and Smith, A.E. (1990) Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell*, **63**, 827–834.
31. Smit, L.S., Strong, T.V., Wilkinson, D.J., Macek Jnr, M., Mansoura, M.K., Wood, D.L., Cole, J.L., Cutting, G.R., Cohn, J.A., Dawson, D.C. *et al.* (1995) Missense mutation (G480C) in the CFTR gene associated with protein mislocalisation but normal chloride channel activity. *Hum. Mol. Genet.*, **4**, 269–273.
32. Ambrosini, L. and Mercer, J.F.B. (1999) Defective copper-induced trafficking and localization of the Menkes protein in patients with mild and copper-treated classical Menkes disease. *Hum. Mol. Genet.*, **8**, 1547–1555.
33. Payne, A.S., Kelly, E.J. and Gitlin, J.D. (1998) Functional expression of the Wilson disease protein reveals mislocalisation and impaired copper-dependent trafficking of the common H1069Q mutation. *Proc. Natl Acad. Sci. USA*, **95**, 10854–10859.
34. Drumm, M.L., Wilkinson, D.J., Smit, L.S., Worrell, R.T., Strong, T.V., Frizzell, R.A., Dawson, D.C. and Collins, F.S. (1991) Chloride conductance expressed by delta F508 and other mutant CFTRs in Xenopus oocytes. *Science*, **254**, 1797–1799.
35. Boyer, J.L. and Soroka, C.J. (1995) Vesicle targeting to the apical domain regulates bile excretory function in isolated rat hepatocyte couplets. *Gastroenterology*, **109**, 1600–1611.
36. Kreek, M.J., Weser, E., Sleisenger, M.H. and Jeffries, G.H. (1967) Idiopathic cholestasis of pregnancy: the response to challenge with the synthetic estrogen, ethinyl estradiol. *N. Engl. J. Med.*, **277**, 1391–1395.
37. Holzbach, R.T., Sivak, D.A. and Braun, W.E. (1983) Familial recurrent intrahepatic cholestasis of pregnancy: a genetic study providing evidence for transmission of a sex-limited, dominant trait. *Gastroenterology*, **85**, 175–179.
38. Bacq, Y., Sapey, T., Brechot, M.C., Pierre, F., Fignon, A. and Dubois, F. (1997) Intrahepatic cholestasis of pregnancy: a French prospective study. *Hepatology*, **26**, 358–364.
39. Taylor, J.C., Ferry, D.R., Higgins, C.F. and Callaghan, R. (1999) The equilibrium and kinetic drug binding properties of the mouse P-gp1a and P-gp1b P-glycoproteins are similar. *Br. J. Cancer*, **81**, 783–789.
40. Laatikainen, T.J. (1975) Fetal bile acid levels in pregnancies complicated by maternal intrahepatic cholestasis. *Am. J. Obstet. Gynecol.*, **122**, 852–856.
41. Brown, C.R., Hong-Brown, L.Q., and Welch, W.J. (1997) Strategies for correcting the delta F508 CFTR protein-folding defect. *J. Bioenerg. Biomembr.*, **29**, 491–502.
42. Girling, J.C., Dow, E. and Smith, J.H. (1997) Liver function tests in pre-eclampsia: importance of comparison with a reference range derived for normal pregnancy. *Br. J. Obstet. Gynecol*, **104**, 246–250.

The bile acid taurocholate impairs rat cardiomyocyte function: a proposed mechanism for intra-uterine fetal death in obstetric cholestasis

Catherine WILLIAMSON*†, Julia GORELIK*, Bryan M. EATON‡, Max LAB§, Michael de SWIET† and Yuri KORCHEV*

*Division of Medicine, Imperial College School of Medicine, MRC Clinical Sciences Centre, Hammersmith Campus, Du Cane Road, London W12 0NN, U.K., †Division of Paediatrics, Obstetrics and Gynaecology, Imperial College School of Medicine, Queen Charlotte's Hospital, Du Cane Road, London W12 0NN, U.K., ‡Division of Paediatrics, Obstetrics and Gynaecology, Imperial College School of Medicine, Chelsea and Westminster Campus, Fulham Road, London SW10 9NH, U.K., and §National Heart and Lung Institute, Imperial College School of Medicine, Charing Cross Campus, Fulham Palace Road, London W6 8RF, U.K.

ABSTRACT

Obstetric cholestasis is a liver disease of pregnancy that can be complicated by sudden, hitherto unexplained, intra-uterine fetal death. Because intra-uterine death occurs suddenly, and because fetal heart rate abnormalities have been reported in obstetric cholestasis, we hypothesized that intra-uterine death is caused by impaired fetal cardiomyocyte function, resulting in fetal cardiac arrest. Obstetric cholestasis is associated with raised levels of maternal and fetal serum bile acids, and we propose that these may alter cardiomyocyte function. It was not possible to investigate the effects of bile acids on the intact human fetal heart at a cellular level. Therefore we used the closest available model of fetal myocardium at term: a primary culture of neonatal rat cardiomyocytes in which cells beat synchronously and develop pacemaker activity. The effect of the primary bile acid taurocholate (0.3 mM and 3 mM) on cultures of single cardiomyocytes, each with its own independent rate of contraction, was a reversible decrease in the rate of contraction and in the proportion of beating cells ($P < 0.001$). Addition of taurocholate to a network of synchronously beating cells caused a similar decrease in the rate of contraction. Furthermore, the integrity of the network was destroyed, and cells ceased to beat synchronously. Taurocholate also resulted in altered calcium dynamics and loss of synchronous beating. These data suggest that raised levels of the bile acid taurocholate in the fetal serum in obstetric cholestasis may result in the development of a fetal dysrhythmia and in sudden intra-uterine death.

INTRODUCTION

Obstetric cholestasis (OC) is a liver disorder of pregnancy with serious consequences for the mother and fetus [1–4]. The most characteristic maternal feature is generalized pruritus, which becomes more severe with advancing gestation. OC can cause fetal distress, spontaneous premature delivery and unexplained third-trimester intra-uterine death [1–4], which usually occurs after 37 weeks' gestation. Abnormal maternal liver function tests are necessary to make the diagnosis. In particular, the serum total bile acid concentration is raised compared with normal pregnancy [5–8]. In OC the serum bile acid concentrations can increase 100-fold, but

Key words: bile, calcium, cardiomyocyte, intra-uterine death, obstetric cholestasis, taurocholate.

Abbreviations: OC, obstetric cholestasis; TC, taurocholate.

Correspondence: Dr C. Williamson (e-mail catherine.williamson@ic.ac.uk).

return to normal within 1 week of delivery. In addition, the increase in cholic acid is much more marked than that in chenodeoxycholic acid and deoxycholic acid [5,6,9]. Raised maternal bile acid levels have been shown to be associated with fetal distress [7,8], and several authors have postulated that raised maternal and fetal bile acid levels are the cause of the adverse fetal consequences of OC [6,7,9,12].

The mechanism of intra-uterine death in OC pregnancies is poorly understood. It is thought to occur suddenly, as there is no evidence of preceding intra-uterine growth restriction or utero–placental insufficiency, and the fetal autopsy is normal. Some studies have demonstrated an abnormal fetal heart rate ($\leqslant$ 100 or $\geqslant$ 180 beats/min) in OC pregnancies [8,12], and severe fetal bradycardia ($<$ 100 beats/min) was noted in 16% of neonates in one study [1].

Because intra-uterine death in OC occurs suddenly, and because an abnormal fetal heart rate has been demonstrated, we hypothesized that raised levels of bile acids cause impaired fetal cardiomyocyte function, resulting in fetal cardiac arrest. It is not possible to investigate the effects of bile acids on the intact human fetal heart at a cellular level. Therefore we used the closest available model of fetal myocardium at term: a primary culture of neonatal rat cardiomyocytes in which cells beat synchronously [13] and develop pacemaker activity [14]. Neonatal rat cardiomyocytes provide a unique *in vitro* model for studying the rhythm, intracellular calcium dynamics and cell-to-cell interactions of cardiomyocytes [15–17]. Cardiomyocytes can be grown as primary cultures of single cells, each with its own independent rate of contraction, or as a network of cells that beat synchronously. When cultured as a network, the early culture consists of single myocytes which exhibit an unsynchronized beating pattern. When any two spontaneously beating neonatal myocytes establish contact, they synchronize. Intercellular coupling via gap junction formation is crucial for this synchronization [18]. Subsequently the cells proliferate, migrate and assemble into a monolayer network that beats synchronously [14].

The aims of the present study were to investigate whether the tauroconjugated primary bile acid taurocholate (TC) alters the rate of contraction, the synchronization of contraction or the Ca^{2+} dynamics of rat neonatal cardiomyocytes.

METHODS

Preparation of a primary culture of neonatal rat cardiomyocytes

Heart cells were obtained as described by Iwaki et al. [19]. Cells were kept in Dulbecco's modified Eagle's minimum essential medium containing 5% (v/v) fetal calf serum (Gibco), 200 μg/ml streptomycin, 200 units/ml penicillin and non-essential amino acids (all from Gibco, Paisley, Scotland, U.K.). Geniticin (G418; Gibco) at 100 μg/ml was added to inhibit fibroblast growth. Cells were maintained at 37 °C, in an atmosphere of humidified air plus 5% CO_2. Cells were used 2 days (discrete cells) and 5 days (network of cells) after plating. The cardiomyocytes were cultured on Petri dishes or glass coverslips. The study protocol is summarized in Figure 1.

Investigation of the effects of TC on the rate of contraction and on the proportion of contracting cardiomyocytes

TC was added to give a final concentration of 0.3 mM or 3.0 mM in the culture medium of single cardiomyocytes and of the network of synchronously beating cells.

For cultures of single cells, the rate of contraction was calculated for 15 s in individual cells, and expressed as beats/min. All measurements were made by a single observer, to avoid operator bias. For the network of cardiomyocytes, the rate of contraction was measured in different parts of the network in the same way as for single cardiomyocytes. The rate of contraction in the cells exposed to TC was compared with that in a control group where no TC was added to the culture medium.

For cultures of single cells, the percentage of contracting cardiomyocytes was calculated using separate groups of 25 individual cells. All measurements were made by a single observer. The proportion of cells that remained beating following exposure to TC was compared with that in a control group where no TC was added to the culture medium.

Investigation of the effects of TC on cardiomyocyte cell death

To establish whether cells were living or dead, fluorescein diacetate/propidium bromide stain (Sigma, Poole, Dorset, U.K.) at a final concentration of 100 ng/ml was added to the cardiomyocyte cultures 3 min after TC administration [20]. The fluorescence of the cells was measured to determine whether cells were alive (indicated by a green colour) or dead (indicated by the nucleus staining red).

Calcium wave dynamics

The cardiomyocytes were loaded with the visible-wavelength fluo-3 Ca^{2+} indicator by cell incubation with the esterified derivative of fluo-3 (fluo-3 acetoxymethyl ester; Molecular Probes, Leiden, Netherlands) in a medium containing equal volumes of Leibovitz's L-15 medium (Gibco) and Hanks balanced salt solution buffer (Gibco) at room temperature for 15 min. Then cells were re-washed five times with the medium, followed by a

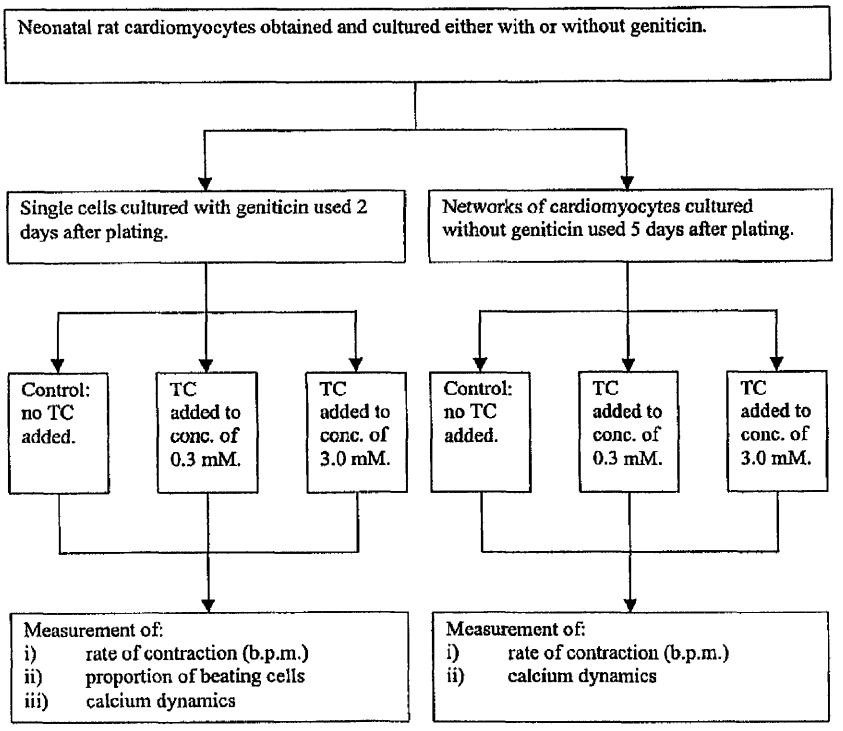


Figure 1 Study protocol
Abbreviation: b.p.m., beats/min.

post-incubation period of 20 min to allow for complete intracellular dye cleavage [21,22].

Scanning laser confocal microscopy was used to study Ca^{2+} dynamics in the cardiac myocyte network. Scanning was performed so that several elevations in Ca^{2+} were visible in the adjacent cells in a single scan, allowing monitoring of cell synchronization and the rhythm of Ca^{2+} wave propagation. We use a relatively unusual way of observing Ca^{2+} transients. This is because we wanted to gain maximum information on cell rhythm and synchronization while introducing minimum laser irradiation to the sample, so we will expand on this method briefly. The low-power (< 0.05 mW) laser beam rapidly scanned the conglomerate of cells, chronologically, from the bottom up. In this case the Ca^{2+} transients are recorded as bright horizontal bands. If the bands traverse all the cells in the field and the time distances between horizontal bands are equal, this indicates synchronous rhythmic beating of cells and adequate cell-to-cell conduction in the network (e.g. Figure 2A). The scanning laser confocal microscopy protocol has been described by Korchev et al. [23].

Statistics

Results are expressed as means $\pm$ S.D. Comparisons were made using one-way ANOVA. The analysis was performed using the InStat statistics package (GraphPad Software).

RESULTS

Effects of TC on the rate of contraction and on the proportion of beating cardiomyocytes when cultured as single cells

Addition of TC to give a concentration of 0.3 mM in the culture medium of single cardiomyocytes caused a decrease in the mean rate of contraction after 1 h, from 68.7 ± 5.7 beats/min (mean $\pm$ S.D.) to 31.4 ± 5.3 beats/min ($P < 0.001$). The rate had recovered to 58.0 ± 8.6 beats/min at 1 h after transfer to TC-free medium. A similar decrease in the rate of contraction was seen following the addition of TC to give a concentration of 3 mM, i.e. from 69.8 ± 8.9 to 25.4 ± 7.6 beats/min ($P < 0.001$), followed by recovery to 59.2 ± 7.0 beats/min (Figure 3A).

TC also affected the proportion of cells that demonstrated contractile activity. A concentration of 0.3 mM TC in the culture medium of single cardiomyocytes

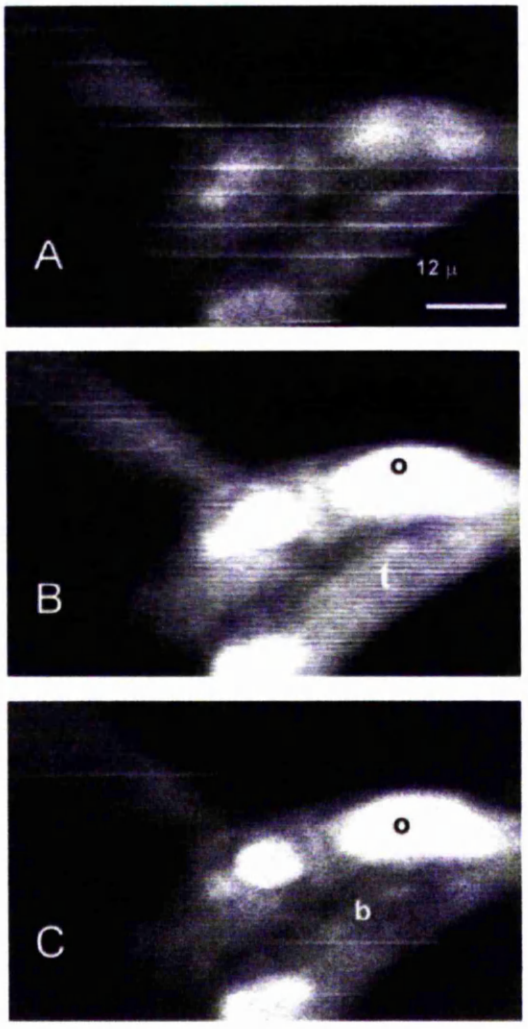


Figure 2 Effects of TC on calcium dynamics in cardiomyocytes

(A) Network of cardiomyocytes before the addition of TC to the culture medium. Horizontal lines represent calcium waves which extend across adjacent cells, consistent with synchronous beating. The spacing between the lines provides an indication of frequency. (B) The addition of 0.3 mM TC alters calcium wave dynamics, consistent with tachycardia (closely spaced lines; t), and with calcium overload with loss of contraction (o). The network of cells is no longer beating synchronously. (C) At 6 min after tachycardia, bradycardia (b) and overload (o) are seen. There is no synchronous contraction. Each scan took 20 s to acquire.

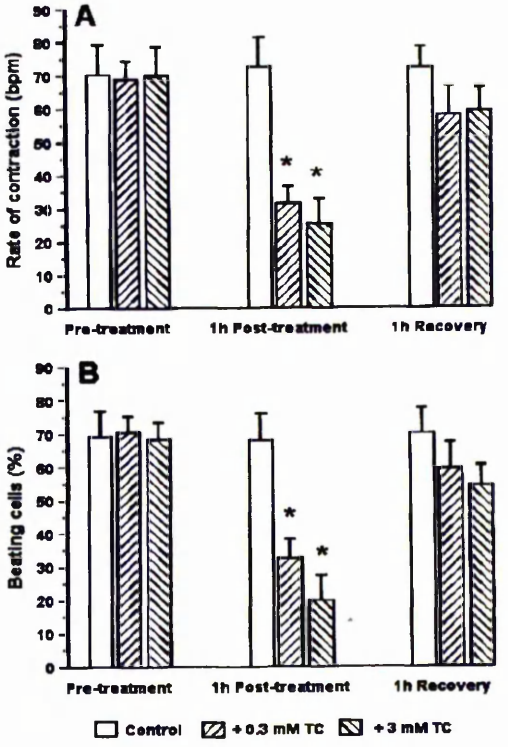


Figure 3 Effects of TC on (A) rate of contraction and (B) the proportion of actively beating cardiomyocytes when cultured as single cells

Abbreviation: bpm, beats/min. Results are expressed as means and S.D. Comparisons were made using one-way ANOVA (* $P < 0.001$).

resulted in a decrease in the percentage of beating cells from 70.5% to 32.6% ($P < 0.001$). This returned to 59.2% following transfer of the cells to TC-free medium. A similar, but greater, decrease was seen with 3 mM TC, i.e. from 68.3% to 19.8% ($P < 0.001$), followed by recovery to 54.2% (Figure 3B).

Effects of TC on the rate of contraction and integrity of networks of cardiomyocytes

A concentration of 0.3 mM TC in the culture medium of networks of cardiomyocytes caused a similar decrease in the rate of contraction to that seen with single cells. These changes were not reversible after transfer to TC-free medium. The higher concentration (3 mM TC) resulted in destruction of the integrity of the network, and cells ceased to beat synchronously. Transfer to TC-free medium resulted in recovery of the integrity of the network, but cells had a reduced rate of contraction. These changes are summarized in Table 1.

The effects of TC on cardiomyocytes do not cause cell death

The addition of TC to the culture medium of cardiomyocytes when cultured either as single cells or as a network did not cause cell death. This was indicated by the reversibility of the cessation of beating that was seen in the majority of cardiomyocytes following transfer of cells to TC-free medium (Figure 3B). Cells that did not

Table 1 **Effects of TC on the rate of contraction of cardiomyocytes cultured as a network**

Values are means ± S.D. Significance of differences: $^*P < 0.001$ compared with control. L.I., loss of integrity of the network.

	Rate of contraction (beats/min)		
TC conc. (mM)	Before adding TC	1 h exposure to TC	1–2 h TC-free
0 (control)	102.9 ± 6.7	104 ± 7.2	102.5 ± 8.0
0.3	103.2 ± 13	55.3 ± 3.5*	54.7 ± 2.0*
3.0	105.3 ± 11.8	L.I.	54.2 ± 5.5*

recover contractile function were shown to be alive by the administration of fluorescein diacetate/propidium bromide stain. No red staining was observed, consistent with TC not causing cardiomyocyte death.

Calcium wave experiments

Addition of TC to single cells initially caused an increase in the frequency of Ca^{2+} waves. After approx. 6 min this was followed either by a decrease in wave frequency or by Ca^{2+} overload. These changes are consistent with a decreased frequency of Ca^{2+} transients, and were associated with a decrease in the rate of contraction and cessation of beating, as was observed 1 h after addition of TC (Figure 3).

When TC was added to the network of cells, the altered Ca^{2+} dynamics were different in adjacent cells, resulting in asynchronous beating. The intracellular Ca^{2+} dynamics of a 5-day-old culture of newborn-rat cardiomyocytes, after cell loading with fluo-3, are shown in Figure 2(A). Standard bright-field microscopy revealed synchronous rhythmic contraction of the network of cells, and this was confirmed by the synchronous Ca^{2+} transients throughout the cluster of cardiomyocytes. A concentration of 0.3 mM TC in the culture medium resulted in altered Ca^{2+} wave dynamics, with an increased Ca^{2+} transient rate, and with Ca^{2+} overload and loss of contraction (Figure 2B). The network of cells is no longer beating synchronously. At 6 min after the changes shown in Figure 2(B), bradycardia and overload were seen (Figure 2C). Thus a concentration of 0.3 mM TC in the culture medium resulted in altered intracellular Ca^{2+} dynamics and loss of synchronous contraction within this small cluster of cardiomyocytes. Similar changes were seen following the addition of TC to give a concentration of 3 mM.

DISCUSSION

We have demonstrated that the primary bile acid, TC, can alter the rate and rhythm of cardiomyocyte contraction and cause abnormal Ca^{2+} dynamics in this *in vitro* system. These data suggest that raised fetal serum bile acids in OC may result in the development of a fatal dysrhythmia and sudden intra-uterine death.

The mechanisms by which TC could cause a fatal fetal dysrhythmia in the intact heart include altered cardiomyocyte Ca^{2+} dynamics and impaired gap junction function, resulting in impaired propagation of conduction and subsequent loss of synchronous contraction. Taurine-conjugated bile acids have been shown to act as Ca^{2+} ionophores, causing a dose-dependent increase in the intracellular Ca^{2+} concentration [24,25]. This is a result of transient permeabilization of the endoplasmic reticulum to Ca^{2+}. To date, this effect has only been demonstrated in hepatocytes, and was not seen in platelets or in a neuroblastoma cell line, neither of which have specific bile acid transporters in the cell membrane [25,26]. This is the first investigation of the effects of TC on Ca^{2+} concentration in cardiomyocytes. Increased Ca^{2+} cycling, coupled with the decrease in pH which would accompany the rapid beating [27], may also affect the function of intercellular gap junctions that provide low-resistance pathways for current flow between heart cells [18,28] and are thought to be crucial for beat synchronization [29,30] and for arrhythmia generation [20,21]. Intracellular calcium, through altered dynamics and overload, also has a pivotal role in the generation of arrhythmia [31,32].

Sudden cardiac death is usually attributed to tachyarrhythmias, although asystole and severe bradycardia are also distinct possibilities. In the latter two cases one would have to postulate substantial sino-atrial slowing, or partial to complete atrioventricular conduction block. We cannot, from our model, comment reliably on this type of conduction disturbance. We do see evidence of conduction block within the network. This cell-to-cell block could contribute to re-entry, an electrophysiological promoter of sustained tachyarrhythmias. Although our model is far from the clinical situation, our results would suggest ventricular tachycardia or fibrillation as the major contributors to cholestatically induced sudden death *in utero*.

Clinical studies of the fetal heart rate in OC pregnancies have demonstrated both tachycardia and bradycardia [1,8,12], consistent with the findings in the present study. However, intra-uterine death in OC is a sudden event, and fetal cardiotochography is not a reliable way of predicting the at-risk fetus [3,4]. This may be because there is a threshold above which the influence of bile

acids, such as TC, is observed. In addition, there may be changes in cardiac function from 37 weeks' gestation that result in an increased susceptibility of fetal cardiomyocytes to the effects of bile acids. This suggestion is supported by clinical studies that have demonstrated that delivery by 37–38 weeks' gestation has reduced the perinatal mortality rate in OC from 10–15% [1,2] to 2.0–3.5% [2–4].

Severe bradycardia has been reported in neonates following exchange transfusion [33], and bile duct ligation in adult male rats can cause bradycardia, increases in PR and QT intervals, and arrhythmia in association with raised serum bilirubin levels, accumulation of bilirubin in the myocardium and depletion of cardiac glycogen [34]. However, there are no *in vivo* studies that demonstrate bradycardia in association with raised bile acids.

The concentration of 0.3 mM TC was chosen because this is within the range of concentrations of total bile acids that we have observed in the serum of women with OC (C. Williamson, unpublished work). A concentration of 3.0 mM was used for comparison. The concentration of TC or of other bile acids to which the fetal cardiomyocytes are exposed *in vivo* is difficult to quantify, and may be greater or smaller than the concentrations used in the present study.

We have investigated the effects of the same doses of TC on adult rat cardiomyocytes, and have found that adult myocytes are less susceptible to alterations in the rate of contraction than are neonatal myocytes (S. Harding, J. Gorelik, M. Lab, Y. Korchev and C. Williamson, unpublished work). This is consistent with the fact that mothers with OC have not been reported to develop dysrhythmias. This suggests that the fetal heart is more susceptible to the development of dysrhythmias *in vivo*. The normal fetal heart beats faster than the maternal heart, and this is consistent with more Ca^{2+} transients per unit time. Therefore a small increase in the Ca^{2+}-wave frequency could result in the breakdown of synchronized contraction via the cellular mechanisms alluded to above.

The advantage of the experimental model used in the present study is that it is possible to observe the effects of bile acids on single cells and on adjacent cells within the network. This is useful as a model of the cell-to-cell interactions that occur in the intact human heart. It will be of interest to extend the observations reported in the present study, in order to investigate the effects of bile acids on the intact fetal and maternal heart in animals and humans. An advantage of *in vitro* experiments such as those reported in this study is that they provide a better understanding of the mechanisms by which bile acids may impair cardiomyocyte function, prior to the commencement of *in vivo* experiments.

The pharmacological agents ursodeoxycholic acid and dexamethasone both improve maternal serum bile acid levels and the clinical features of OC [35–38], but it is currently not known whether their use improves fetal prognosis. Future experiments using this model will allow us to evaluate whether these agents may be of benefit to the fetus. We anticipate that subsequent studies in animals and humans may elucidate further the precise role of fetal cardiomyocyte damage in the unexplained late fetal death in OC.

ACKNOWLEDGMENTS

Ventricular myocytes were kindly provided by Peter H. Sugden, National Heart and Lung Institute Division, Imperial College. This work was supported by grants from the Wellcome Trust and the British Heart Foundation.

REFERENCES

1 Reid, R., Ivey, K. J., Rencoret, R. H. and Storey, B. (1976) Fetal complications of obstetric cholestasis. Br. Med. J. **i**, 870–872
2 Reyes, H. (1982) The enigma of intrahepatic cholestasis of pregnancy: lessons from Chile. Hepatology **2**, 87–96
3 Fisk, N. M. and Storey, G. N. B. (1988) Fetal outcome in obstetric cholestasis. Br. J. Obstet. Gynaecol. **95**, 1137–1143
4 Rioseco, A. J., Ivankovic, M. B., Manzur, A. et al. (1994) Intrahepatic cholestasis of pregnancy: a retrospective case-control study of perinatal outcome. Am. J. Obstet. Gynecol. **170**, 890–895
5 Sjovall, K. and Sjovall, J. (1966) Serum bile acid levels in pregnancy with pruritus. Clin. Chem. Acta **13**, 207–211
6 Bacq, Y., Myara, A., Brechot, M. C. et al. (1995) Serum conjugated bile acid profile during intrahepatic cholestasis of pregnancy. J. Hepatol. **22**, 66–70
7 Laatikainen, T. J. and Ikonen, E. (1977) Serum bile acids in cholestasis of pregnancy. Obstet. Gynecol. **50**, 313–318
8 Laatikainen, T. J. and Tulenheimo, A. (1984) Maternal serum bile acid levels and fetal distress in cholestasis of pregnancy. Int. J. Gynaecol. Obstet. **22**, 91–94
9 Heikkinen, J., Maentausta, O., Tuimala, R., Ylostalo, P. and Janne, O. (1980) Amniotic fluid bile acids in normal and pathologic pregnancy. Obstet. Gynecol. **56**, 60–64
10 Reference deleted
11 Reference deleted
12 Laatikainen, T. (1975) Fetal bile acid levels in pregnancies complicated by maternal intrahepatic cholestasis. Am. J. Obstet. Gynecol. **122**, 852–856
13 Kimura, H., Oyamada, Y., Ohshika, H., Mori, M. and Oyamada, M. (1995) Reversible inhibition of gap junctional intercellular communication, synchronous contraction, and synchronism of intracellular Ca^{2+} fluctuation in cultured neonatal rat cardiac myocytes by heptanol. Exp. Cell Res. **220**, 348–356
14 Soen, Y., Cohen, N., Lipson, D. and Braun, E. (1999) Emergence of spontaneous rhythm disorders in self-assembled networks of heart cells. Phys. Rev. Lett. **82**, 3556–3559
15 Husse, B. and Wussling, M. (1996) Developmental changes of calcium transients and contractility during the cultivation of rat neonatal cardiomyocytes. Mol. Cell. Biochem. **163/164**, 13–21
16 Barrigon, S., Wang, S. Y., Ji, X. and Langer, G. A. (1996) Characterization of the calcium overload in cultured neonatal rat cardiomyocytes under metabolic inhibition. J. Mol. Cell. Cardiol. **28**, 1329–1337
17 Hori, M., Sato, H., Iwai, K. et al. (1992) Disruption of microtubules in cultured neonatal rat cardiomyocytes during rapid contractions; protective effects of beta-adrenoceptor antagonist. Jpn. Circ. J. **56**, 62–68

18 Jongsma, H. J., Masson-Pevet, M. and Tsijernina, L. (1987) The development of beat-rate synchronization of rat myocyte pairs in cell culture. Basic Res. Cardiol. **82**, 454–464
19 Iwaki, K., Sukhatme, V. P., Shubeita, H. E. and Chein, K. R. (1990) α and β-adrenergic stimulation induces distinct patterns of immediate early gene expression in neonatal rat myocardial cells. J. Biol. Chem. **265**, 13809–13817
20 Miyamoto, Y., Umebayashi, Y. and Nishisaka, T. (1999) Comparison of phototoxicity mechanism between pulsed and continuous wave irradiation in photodynamic therapy. J. Photochem. Photobiol. B **53**, 53–59
21 Lopez, J. R., Jovanovic, A. and Terzic, A. (1995) Spontaneous calcium waves without contraction in cardiac myocytes. Biochem. Biophys. Res. Commun. **214**, 781–787
22 Williams, D. A., Delbridge, L. M., Cody, S. H., Harris, P. J. and Morgan, T. O. (1992) Spontaneous and propagated calcium release in isolated cardiac myocytes viewed by confocal microscopy. Am. J. Physiol. **262**, C731–C742
23 Korchev, Y. E., Gorelik, J. V., Lab, M. J. et al. (2000) Cell volume measurement using scanning ion conductance Microsc. Biophys. J. **78**, 451–457
24 Zimniak, P., Little, J. M., Radominska, A., Oelberg, D. G., Anwer, M. S. and Lester, R. (1991) Taurine-conjugated bile acids act as Ca2+ ionophores. Biochemistry **30**, 8598–8604
25 Thibault, N. and Ballet, F. (1993) Effect of bile acids on intracellular calcium in isolated rat hepatocyte couplets. Biochem. Pharmacol. **45**, 289–293
26 Coquil, J. F., Berthon, B., Chomiki, N. et al. (1991) Effects of taurolithocholate, a Ca^{2+}-mobilizing agent, on cell Ca^{2+} in rat hepatocytes, human platelets and neuroblastoma NG108–15 cell line. Biochem. J. **273**, 153–160
27 Bountra, C., Kaila, K. and Vaughan Jones, R. D. (1988) Effect of repetitive activity upon intracellular pH, sodium and contraction in sheep cardiac Purkinje fibres. J. Physiol. (London) **398**, 341–360
28 White, R. L., Spray, D. C., Schwartz, G. J., Wittenberg, B. A. and Bennet, M. V. L. (1985) Some electrical and pharmacological properties of gap junctions between adult ventricular myocytes. Am. J. Physiol. **249**, C447–C455
29 Page, E. and Shibata, Y. (1981) Permeable junctions between cardiac cells. Annu. Rev. Physiol. **43**, 431–441
30 Rook, M. B., van Ginneken, A. C., de Jonge, B., el Aoumari, A., Gros, D. and Jongsma, H. J. (1992) Differences in gap junction channels between cardiac myocytes, fibroblasts, and heterologous pairs. Am. J. Physiol. **263**, C959–C977
31 Levy, M. N. and Wiseman, M. N. (1991) Electrophysiologic mechanisms for ventricular arrhythmias in left ventricular dysfunction: electrolytes, catecholamines and drugs. J. Clin. Pharmacol. **31**, 1053–1060
32 Di Diego, J. M. and Antzelevich, C. (1994) High [Ca2+]o-induced electrical heterogeneity and extrasystolic activity in isolated canine ventricular epicardium. Phase 2 reentry. Circulation **89**, 1839–1850
33 Abu-Ekteish, F., Daoud, A., Rimawi, H., Kakish, K. and Abu-Heija, A. (2000) Neonatal exchange transfusion: a Jordanian experience. Ann. Trop. Paediatr. **20**, 57–60
34 Tajuddin, M., Tariq, M., Bilgrami, N. L. and Kumar, S. (1980) Biochemical and pathological changes in the heart following bile duct ligation. Adv. Myocardiol. **2**, 209–212
35 Hirvioja, M. L., Tuimala, R. and Vuori, J. (1992) The treatment of intrahepatic cholestasis of pregnancy by dexamethasone. Br. J. Obstet. Gynaecol. **99**, 109–111
36 Nicastri, P. L., Diaferia, A., Tartagni, M., Loizzi, P. and Fanelli, M. (1998) A randomised placebo-controlled trial of ursodeoxycholic acid and S-adenosylmethionine in the treatment of intrahepatic cholestasis of pregnancy. Br. J. Obstet. Gynaecol. **105**, 1205–1207
37 Palma, J., Reyes, H., Ribalta, J. et al. (1992) Effects of ursodeoxycholic acid in patients with intrahepatic cholestasis of pregnancy. Hepatology **15**, 1043–1047
38 Palma, J., Reyes, H., Ribalta, J. et al. (1997) Ursodeoxycholic acid in the treatment of cholestasis of pregnancy: a randomized, double-blind study controlled with placebo. J. Hepatol. **27**, 1022–1028

Received 14 June 2000/15 September 2000; accepted 11 December 2000

BJOG: an International Journal of Obstetrics and Gynaecology
May 2003, Vol. 110, pp. 467–474

Dexamethasone and ursodeoxycholic acid protect against the arrhythmogenic effect of taurocholate in an *in vitro* study of rat cardiomyocytes

J. Gorelik[a], A.I. Shevchuk[a], I. Diakonov[a], M. de Swiet[b], M. Lab[c], Y. Korchev[a], C. Williamson[a,b,*]

Objective To establish whether the therapeutic agents ursodeoxycholic acid and dexamethasone protect cardiomyocytes from taurocholate-induced arrhythmias in an *in vitro* model.

Design Laboratory study.

Setting Imperial College London, Hammersmith Campus.

Sample Neonatal rat cardiomyocytes.

Methods Using scanning ion conductance microscopy, we measured the rate, rhythm, amplitude of contraction and calcium dynamics of ventricular myocytes from one to two day old rats. Cells were pre-incubated for 16 hours in dexamethasone (80 or 800 nM) or 0.1 mM ursodeoxycholic acid before adding taurocholate at different concentrations (0.3–4.5 mM).

Main outcome measures Changes in rate and amplitude of contraction, calcium dynamics and rhythm.

Results Taurocholate at concentrations of up to 3 mM induces abnormal changes including reductions in rate, amplitude of contraction, abnormal calcium dynamics and dysrhythmias. Although dexamethasone had no immediate protective effect on these changes, pre-incubation with dexamethasone was protective. Ursodeoxycholic acid pre-incubation was protective at taurocholate concentrations up to 1 mM.

Conclusion The therapeutic agents dexamethasone and ursodeoxycholic acid appear protective against the arrhythmogenic effect of taurocholate on cardiomyocytes.

INTRODUCTION

Obstetric cholestasis, also called intrahepatic cholestasis of pregnancy, is a liver disease of pregnancy associated with third trimester intrauterine death, fetal distress and spontaneous preterm labour[1–4]. It affects approximately 0.6% of pregnancies in UK white Caucasians, and double this number of pregnancies in Indian and Pakistani Asians[5,6]. The perinatal mortality rate has fallen from 9–11% in older studies[2,3] to 2–3.5% in more recent studies where the fetus was delivered by 38 weeks of gestation[1,3,4].

The aetiology of the fetal complications of obstetric cholestasis pregnancies is poorly understood. Intrauterine death is thought to occur suddenly, as there is no evidence of preceding intrauterine growth restriction or uteroplacental insufficiency and fetal autopsy is normal[1]. Placental histology shows non-specific changes consistent with hypoxia[7]. An abnormal fetal heart rate ($\leq$100 or $\geq$180 beats/minute) has been observed in previous studies[2,8,9].

Obstetric cholestasis causes maternal pruritus and abnormal liver function tests, including raised serum primary bile acids[9–12]. The increase in cholic acid is much more marked than that of chenodeoxycholic acid or deoxycholic acid[12–14].

It has not been established whether the absolute levels of maternal total serum bile acids correlate with adverse pregnancy outcomes in obstetric cholestasis. In one study of 86 pregnant women with obstetric cholestasis in which the severity of the disease was classified according to the level of the serum bile acids, the incidence of meconium-stained amniotic fluid was significantly increased in the subgroup of women with the most severe cholestasis[11]. In another series of 117 cases that were classified in the same way, reduced fetal heart rate variability and/ or late decelerations on cardiotocography were seen more commonly in the subgroup with most severe cholestasis compared with the group with mild cholestasis[9]. There has also been one case report of primary sclerosing cholangitis in pregnancy in which there was marked fetal bile acidaemia associated with a variety of fetal complications including fetal bradycardia[15].

[a]*Division of Medicine, Imperial College London, MRC Clinical Sciences Centre, UK*
[b]*Division of Paediatrics, Obstetrics and Gynaecology, Imperial College London, UK*
[c]*National Heart and Lung Institute, Imperial College London, UK*

* **Correspondence**: Dr C. Williamson, Maternal and Fetal Disease Group, Institute of Reproductive and Developmental Biology, Hammersmith Campus, Du Cane Road, London W12 0NN, UK.

doi:10.1016/S1470-0328(03)02973-2

We hypothesised that raised fetal serum bile acids in obstetric cholestasis may result in the development of a fetal dysrhythmia and sudden intrauterine death. Previously, using an *in vitro* model of rat one to two day neonatal cardiomyocytes, we have demonstrated that the primary bile acid taurocholate can alter the rate and rhythm of cardiomyocyte contraction and cause abnormal Ca^{2+} dynamics[16]. We have also shown that superfusion of a culture with taurocholate has different arrhythmogenic effects in individual cells, and that taurocholate causes reduced amplitude of contraction as well as dysrhythmias[17]. These data support our hypothesis that raised fetal serum bile acids in obstetric cholestasis may result in the development of a fatal dysrhythmia in the fetus and sudden intrauterine death.

Taurocholate was used because cholic acid is the main bile acid that is raised in obstetric cholestasis. In humans, the majority of bile acids are conjugated with either glycine or taurine, and glycoconjugates predominate. However, in obstetric cholestasis, the normal glycine/taurine ratio of 1.4 is reversed to 0.8[18].

Two drugs, ursodeoxycholic acid and dexamethasone, are clinically useful in obstetric cholestasis. There is more experience of the use of ursodeoxycholic acid than dexamethasone in the treatment of obstetric cholestasis. When the results of 10 studies with a total of 85 affected women who were treated with ursodeoxycholic acid are combined, 74 (87%) showed clinical or biochemical improvement, or both[18–26]. Ursodeoxycholic acid treatment has been shown to improve the serum bile acid levels measured in cord blood and amniotic fluid at the time of delivery[23]. In addition, there is very little accumulation of ursodeoxycholic acid in the amniotic fluid or in cord blood[23]. However, ursodeoxycholic acid treatment does not decrease total bile acid concentrations in the meconium[27]. There have been no reports of an adverse fetal outcome following maternal treatment with ursodeoxycholic acid. There has been one report of a series of 10 women treated with dexamethasone. In this series, all had a clinical and biochemical response[28].

In vitro experiments using placentas from women with obstetric cholestasis have demonstrated an impaired rate and affinity of bile acid transport in the basal membrane and reduced efficiency of transport at the apical membrane. All of the observed changes were reversed in placentas from women treated with ursodeoxycholic acid[29]. However, there have been no studies of the effect of either ursodeoxycholic acid or dexamethasone treatment on features of fetal dysrhythmia. As it has not been possible to perform controlled clinical trials of the effects of ursodeoxycholic acid and dexamethasone on fetal outcome in obstetric cholestasis, *in vitro* animal models such as the one presented in this paper are an important source of knowledge about the effects of these therapeutic agents. Therefore, in order to further investigate the association between elevated bile acid and fetal heart abnormalities in this study, we aimed to investigate whether ursodeoxycholic acid and dexamethasone have a protective effect on the arrhythmogenic effect of the bile acid taurocholate on rat neonatal cardiomyocytes.

METHODS

Ventricular myocytes were isolated from the hearts of one to two day old rats[30], cultured as previously described[16,17], and used following three to four days culture on glass coverslips (small network of cardiomyocytes). Taurocholate (Sigma-Aldrich, Dorset, UK) was added to give a final concentration of 0.3, 1.0 and 3.0 mM to a network of synchronously beating cells. The rate of contraction was calculated as described previously[16]. In some experiments cells were pre-incubated for 16 hours (overnight) with either 0.1 mM ursodeoxycholic acid (Sigma-Aldrich) or with either 80 or 800 nM dexamethasone (David Bull Laboratories, Warwick, UK) prior to the addition of taurocholate. In experiments where the therapeutic agents protected the cardiomyocytes from the effect of 0.3, 1.0 and 3.0 mM taurocholate, a higher dose of 4.5 mM was used. Taurocholate, ursodeoxycholic acid and dexamethasone were diluted in phosphate buffered saline to make stock solutions prior to addition to culture medium; the concentrations of the stock solutions were 150 mM, 10 mM and 80 μM, respectively. To investigate cardiomyocyte recovery, cells were transferred to taurocholate-free medium for 1 hour.

To investigate the changes of the rhythmicity of cardiomyocyte contraction caused by the addition of taurocholate, we recorded the vertical cell displacement of individual cells using a scanning ion conductance microscope as described previously[31].

The cardiomyocytes were loaded with the visible wavelength fluo-4 Ca^{2+} indicator by cell incubation with the esterified derivative of fluo-4 (fluo-4 acetoxymethyl) (Molecular Probes, Leiden, Netherlands) in a medium containing equal volumes of Leibovitz's L-15 (Gibco, Paisley, UK) and Hank's balanced salt solution buffer (Gibco) at room temperature for 15 minutes. Cells were then rewashed five times with the medium, followed by a post-incubation period of 20 minutes to allow for complete intracellular dye cleavage[32]. Scanning laser confocal microscopy was used to study the Ca^{2+} dynamics in the cardiac myocyte network as described previously[16,31].

Cells were fixed with 4% formalin solution in phosphate buffered saline, permeabilised with 0.1% Triton X-100 in phosphate buffered saline for 20 minutes. Blocking solution of 10% normal goat serum was then applied, followed by monoclonal antibodies to sarcomeric α-actinin, clone EA-53 (Sigma-Aldrich, A7811) in 1:100 dilution. The antigen was detected using fluorescein-conjugated secondary antibody. Slides were observed under a Nikon-Eclipse TE300 microscope. To localise fibrillar actin, cells were counterstained with TRITC-phalloidin (Sigma-Aldrich) in 1:500 dilution.

To establish the proportion of cardiomyoctes in the geneticin-treated cultures, cells were stained with antibodies to detect sarcomeric α-actinin and phalloidin to reveal the distribution of filamentous actin. Immunodetection showed that almost 90% of the cells possessed the myocyte marker, α-actinin (data not shown). Cells incubated normally display synchronous contractile behaviour, and taurocholate induces arrhythmia[16].

RESULTS

We present here the results of several independent experiments for each experimental condition analysed. Figures 1–3 illustrate recordings that we typically obtained, and which were consistent between experiments. In the first set of experiments, cells were pre-incubated for 16 hours with either ursodeoxycholic acid or dexamethasone. Taurocholate was then added to the culture medium. With dexamethasone or ursodeoxycholic acid alone under standard bright-field microscopy cells displayed synchronous beating. After cell loading with the calcium indicator fluo-4 synchronous calcium waves were observed that spanned the entire network of cells (Figs 1A and 1E), that is, the cells were coupled. When taurocholate was added to the culture medium of cells that had been pre-incubated with dexamethasone to give a final taurocholate concentration of up to 3 mM, the concentration that has been previously reported to cause severe disruption of cardiomyocyte contraction[17], there was no change in the rate or synchronisation of contraction (Fig. 1B) (four experiments). Only at the higher dose of 4.5 mM taurocholate did the cells show contractile irregularities (Fig. 1C). Neighbouring cells within the network had different patterns of calcium wave propagation, consistent with abnormal cell–cell communication. As soon as the medium was changed to taurocholate-free medium, the cells displayed signs of recovery. Although the rate of contraction did not return to that seen prior to taurocholate addition, it became regular and calcium waves showed intercellular coupling (Fig. 1D).

Pre-incubation with ursodeoxycholic acid protected cells from the effects of 0.3 mM taurocholate (Fig. 1F) but 3.0 mM taurocholate produced fibrillation/tachycardia and loss of synchronous contraction (Fig. 1G) and subsequent irreversible cellular overload with calcium (Fig. 1H) (four experiments).

The protective effect of dexamethasone was dependent on both the dose of taurocholate added and the concentration of dexamethasone in the pre-incubation medium (Table 1). Without pre-incubation with dexamethasone, the integrity of the network of cardiomyocytes was lost in the presence of both 3.0 and 4.5 mM taurocholate, and the effects of the latter concentration were irreversible. Pre-incubation with 80 nM dexamethasone protected cells from the effect of 0.3 and 3.0 mM taurocholate, but not from the higher dose of 4.5 mM. After transfer to taurocholate-free medium, the cells recovered but had a slower rate of contraction than before taurocholate treatment. Addition of 800 nM dexamethasone to the pre-incubation medium protected cells from the effect of 0.3 and 3.0 mM taurocholate concentrations, but cells exposed to 4.5 mM taurocholate

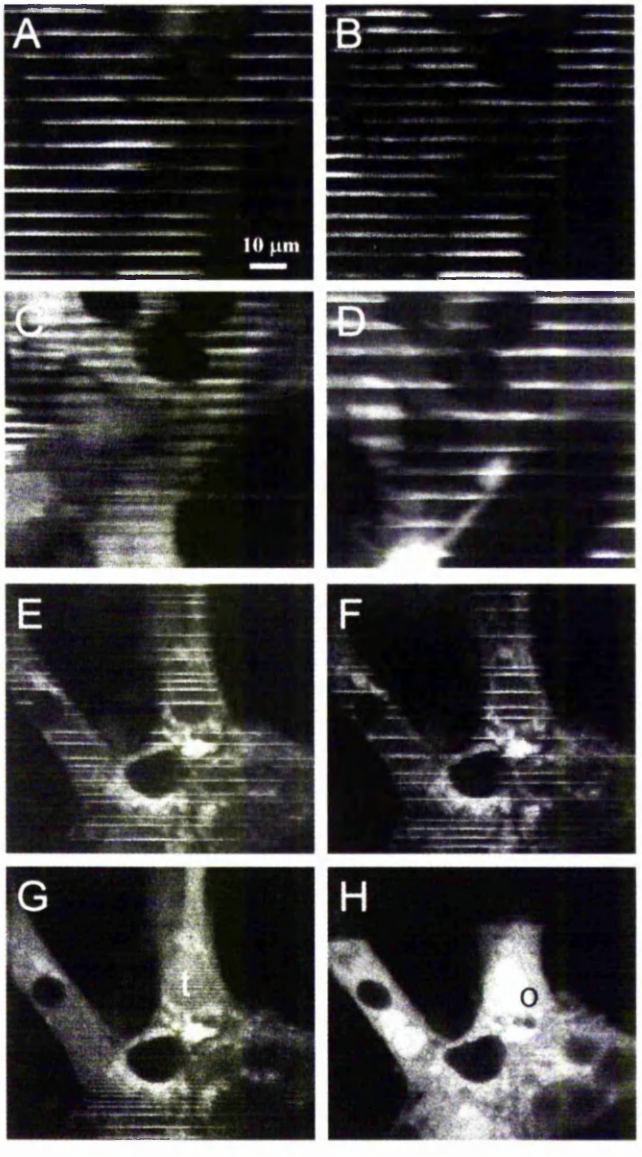


Fig. 1. Effect of taurocholate on calcium dynamics in cardiomyocytes pretreated with dexamethasone and ursodeoxycholic acid. (A) The cardiomyocyte network was incubated for 16 hours with 800 nM dexamethasone (A–D). Horizontal lines represent calcium waves which extend across adjacent cells, consistent with synchronous beating. The spacing between the lines provides an indication of frequency. (B) The addition of taurocholate up to a concentration of 3 mM does not alter calcium dynamics. (C) Addition of taurocholate to a concentration of 4.5 mM alters calcium dynamics. (D) Restoration of rhythmic contraction after transfer to taurocholate-free medium. (E) Network of cardiomyocytes incubated for 16 hours with 0.1 mM ursodeoxycholic acid. (F) The addition of 0.3 mM taurocholate does not alter calcium waves. (G) The addition of 3 mM taurocholate alters calcium wave dynamics, consistent with tachycardia—closely spaced lines (t), (H) this did not return to normal following transfer to taurocholate-free medium, and calcium overload (o) was seen with cessation of beating.

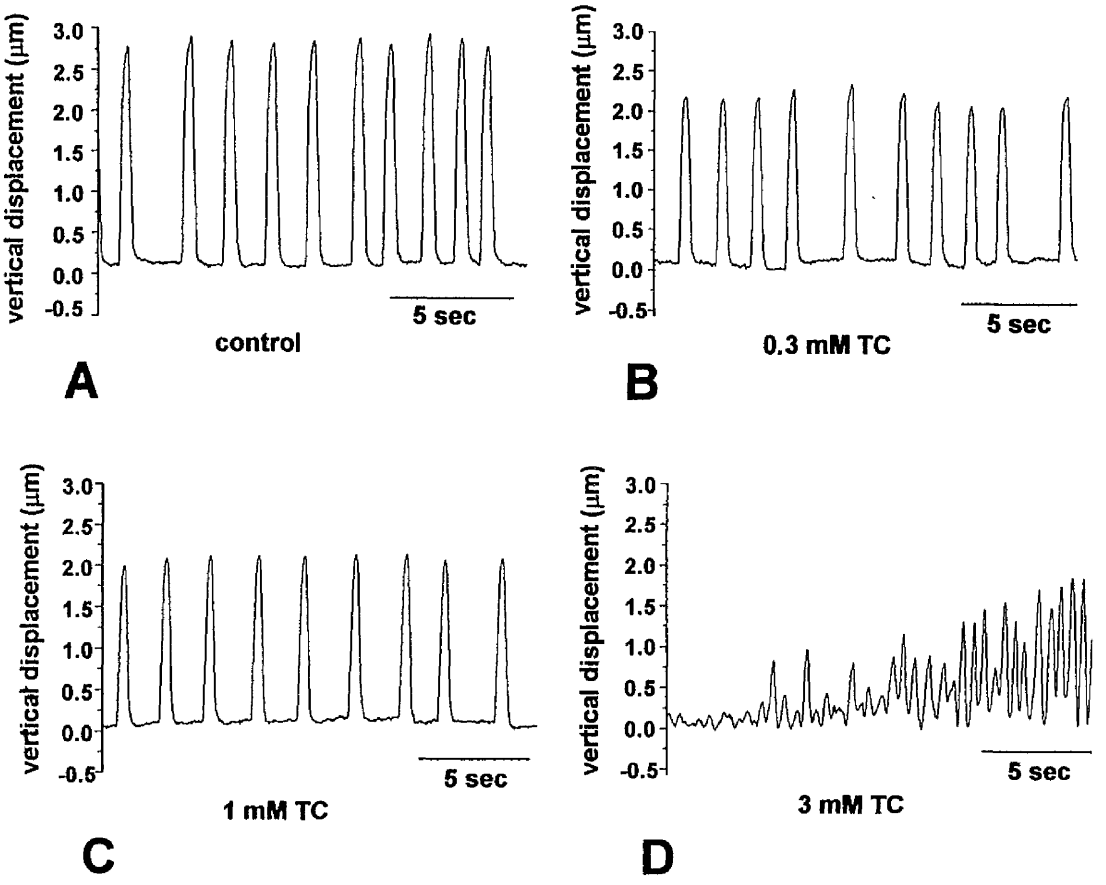


Fig. 2. Effect of various concentrations of taurocholate on the contraction of a newborn rat cardiomyocyte pre-incubated with ursodeoxycholic acid (0.1 mM, 16 hours). (A) Control; The addition of 0.3 mM taurocholate (B) and 1.0 mM taurocholate (C) does not decrease the rate of contraction; (D) Addition of 3 mM taurocholate causes an appearance consistent with fibrillation of the cardiomyocyte.

had a slower rate of contraction than before treatment. However, they made a full recovery after taurocholate was removed.

Table 2 shows that ursodeoxycholic acid could only protect cells from 1 mM taurocholate. When the cells were exposed to 3 mM taurocholate, there was loss of integrity and synchronous beating within the network of cells. When the cardiomyocytes were subsequently transferred to taurocholate-free medium, they recovered but had a slower rate of contraction than before treatment.

In our previous paper, we demonstrated a reduced rate of contraction and proportion of beating cells when cardiomyocytes were exposed to increasing concentrations of taurocholate (0.1–3.0 mM), more marked at higher concentrations ($P < 0.001$). Using scanning ion conductance microscopy, we also demonstrated reduced amplitude of contraction and calcium transients with taurocholate[17].

Figure 2 shows a representative single-point scanning ion conductance microscope measurement of contraction of a small aggregate. The aggregate was pre-incubated with ursodeoxycholic acid for 16 hours then increasing doses of taurocholate were added (eight experiments). The rate of contraction did not change after addition of 0.3 or 1 mM taurocholate, but a small reduction of amplitude from 3 to 2.2 μM was seen (Figs 2B and 2C). Addition of 3 mM taurocholate caused movement oscillations which look like fibrillation (Fig. 2D).

In Fig. 3A, a typical example of a single-point scanning ion conductance microscope measurement is shown where a contracting cardiomyocyte pre-incubated with 800 nM dexamethasone for 16 hours was exposed to increasing concentrations of taurocholate (four experiments). There was no change in contraction when up to 1 mM taurocholate was added (data not shown). After 3 minutes, incubation with 3 mM taurocholate caused a slight decrease in amplitude of contraction from 3 to 2.2 μM, but the rate remained the same.

Pre-incubation with dexamethasone curtails the effects of taurocholate (Figs 1A–1D), and the question arises whether pre-incubation is a requirement for dexamethasone's action. When the cells were not pre-incubated with dexamethasone, and 800 nM dexamethasone and 1 mM taurocholate were added to the culture medium together, this lower dose of taurocholate caused a similar effect to that seen in cells that were not incubated with dexamethasone (four experiments) (Fig. 3B).

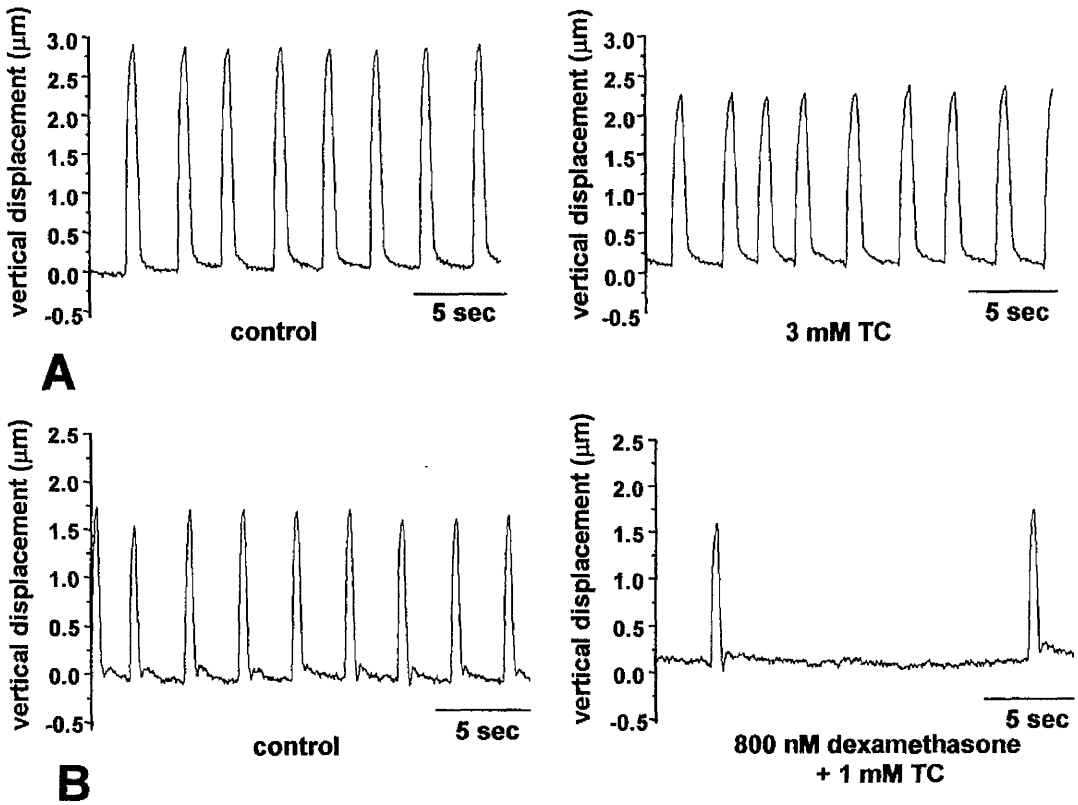


Fig. 3. (A) Effect of 3 mM taurocholate on the contraction of cardiomyocytes pre-incubated with dexamethasone (800 nM, 16 hours). The addition of 3 mM taurocholate slightly decreases the amplitude of contraction but not the rate (3 minutes after addition of taurocholate to the culture). (B) Effect of simultaneous addition of 1.0 mM taurocholate and 800 nM dexamethasone to the cardiomyocyte culture for 2 hours. The addition of taurocholate decreases the rate of contraction.

DISCUSSION

This study shows that the therapeutic agents dexamethasone and ursodeoxycholic acid protect neonatal rat cardiomyocytes from the arrhythmogenic effects of the bile acid taurocholate. We have previously demonstrated that when single cells or networks of cardiomyocytes are cultured in medium containing taurocholate, this results in abnormalities in the rate and amplitude of contraction, altered calcium dynamics and in the loss of synchronous contraction.

Table 1. Effect of 16 hours pre-incubation with dexamethasone (80 or 800 nM) prior to addition of 3.0 or 4.5 mM taurocholate on the rate of contraction of cardiomyocytes (beats per minute). Values are mean (SD).

	No dexamethasone	80 nM dexamethasone	800 nM dexamethasone
10 minute treatment			
Control	105.7 (7.9)	99.6 (9.2)	104.1 (13.0)
3.0 mM taurocholate	LI	88.2 (15)	96.5 (11.3)
4.5 mM taurocholate	LI	LI	62.1 (8.7)*
1 hour treatment			
Control	105.5 (7.6)	96.6 (8.4)	100.2 (8.0)
3.0 mM taurocholate	LI	92.2 (12.8)	96.8 (9.7)
4.5 mM taurocholate	LI	LI	61.0 (10.0)*
1 hour recovery			
Control	105.9 (8.7)	101.9 (7.2)	99.0 (11.0)
3.0 mM taurocholate	46.1 (8.0)*	93.7 (7.3)	97.7 (9.1)
4.5 mM taurocholate	LI	67.7 (13.0)*	87.7 (10.0)

LI = loss of integrity of the network.
* $P < 0.001$ when compared with control data.

Table 2. Effect of 16 hours pre-incubation with ursodeoxycholic acid (0.1 mM) prior to addition of 1.0 or 3.0 mM taurocholate on the rate of contraction of cardiomyocytes (beats per minute). Values are mean (SD).

	No ursodeoxycholic acid	0.1 mM ursodeoxycholic acid
10 minute treatment		
Control	88.0 (6.4)	88.0 (7.4)
1.0 mM taurocholate	47.2 (4.5)*	84.0 (6.7)
3.0 mM taurocholate	LI	LI
1 hour treatment		
Control	89.2 (4.6)	85.5 (6.7)
1.0 mM taurocholate	43.2 (3.2)*	85.5 (5.2)
3.0 mM taurocholate	LI	LI
1 hour recovery		
Control	90.5 (5.2)	88 (7.4)
1.0 mM taurocholate	57.6 (6.0)*	86.5 (9.5)
3.0 mM taurocholate	53.3 (4.9)*	52.8 (3.9)*

LI = loss of integrity of the network.
* $P < 0.001$ when compared with control data.

We have proposed that the results in this *in vitro* model may offer an explanation for the aetiology of intrauterine death in obstetric cholestasis[16]. If this hypothesis is correct, the protective effect of ursodeoxycholic acid and dexamethasone in the current study is a clinically important finding. Dexamethasone was more protective than ursodeoxycholic acid. This may reflect different mechanisms of action. We have shown that dexamethasone protects against taurocholate-induced loss of physiological integrity of the cardiomyocyte network. Dexamethasone has previously been shown to maintain the structural integrity of myocytes for periods of at least 45 days in the absence of any damaging agents[33].

Dexamethasone also protects against the taurocholate-induced reduction in rate of contraction. These data are in agreement with a report that demonstrated that dexamethasone protected against a decreased contraction rate and multiform arrhythmias that were induced in cultured rat myocardial cells following infection with Coxsackie B-2 virus[34–36]. In addition, dexamethasone inhibits endotoxin-induced changes in calcium and contractility in rat isolated papillary muscle. Pretreatment of rats with dexamethasone prevented the endotoxin-induced decrease in peak tension and inhibited the elevation in resting $[Ca^{2+}]$, with a resultant maintenance of Ca^{2+} transient magnitude[37]. The effect of dexamethasone is often attributed to its ability to induce gene expression or stabilise mRNA in target cells[38].

The cardioprotective effect of ursodeoxycholic acid may be a consequence of protection from the apoptosis that is induced by more hydrophobic bile acids or may be due to membrane stabilisation. Most of the studies of the protective effect of ursodeoxycholic acid have compared it with more hydrophobic bile acids (e.g. glycochenodeoxycholic acid). In rat and human hepatocytes, the mechanism of cytotoxicity of glycochenodeoxycholic acid varies at different concentrations. At the higher concentrations that are seen in severe cholestasis (i.e. 0.5 mM), cytolytic cell destruction has been demonstrated[39]. At lower pathological concentrations of 0.05–0.1 mM, apoptosis is the predominant mechanism of bile acid toxicity[40,41]. Studies of human and rat hepatocytes have shown that ursodeoxycholic acid protects against these cytotoxic effects of hydrophobic bile acids[39,40] and ursodeoxycholic acid at millimolar concentrations protects against membrane damage caused by more hydrophobic bile acids in *in vitro* studies of rat and human hepatocytes, and in artificial membranes[42–44].

Ursodeoxycholic acid may also exert its cardioprotective effect by increasing ATP levels. Studies of the mechanism by which ursodeoxycholic acid promotes bile flow have demonstrated that it stimulates secretion of ATP in isolated rat hepatocytes[45]. In cardiac myocytes, ATP is the endogenous ligand for the P2X receptor, and separate studies of rat and mouse hearts have shown that both ATP and another P2X receptor agonist (2-meSATP) stimulated large increases in the myocyte contractile amplitude[46]. Thus, it is possible that ursodeoxycholic acid-induced rise of ATP in cardiomyocytes has a cardioprotective effect.

Ursodeoxycholic acid increases the density of the hepatic apical conjugate export pump, Mrp2, fourfold in rat canalicular membranes[47]. The authors are not aware of other studies of the effect of ursodeoxycholic acid on the insertion of other potential bile acid transporters into the canalicular membrane.

Both ursodeoxycholic acid and dexamethasone could have immediate effects on cell membrane transporters, or compete with bile acid. But it seems unlikely that this plays a part in the effect of both agents on cardiomyocyte contraction, as both agents act only after being applied to cells well before the addition of taurocholate.

It is possible that the cardioprotective effects of ursodeoxycholic acid and dexamethasone are the result of altered expression of genes that influence bile acid transport or metabolism in cardiomyocytes. We are currently not aware of studies of the expression of these bile acid transporter genes in cardiomyocytes. If the transporters are expressed in cardiomyocytes, it is also possible that ursodeoxycholic acid and dexamethasone could act by a direct effect on the function of the transporters.

This current study suggests that dexamethasone may have a more potent cardioprotective effect on the fetus, although this study is relatively small. However, there is evidence from animal and human studies that fetal exposure to glucocorticoids may have an adverse effect on subsequent blood pressure[48] and brain maturation[49]. Our data do not suggest routine use of dexamethasone as a first line treatment for obstetric cholestasis, but we have provided evidence that it may have a beneficial effect in the prevention of intrauterine death in women who have not responded to ursodeoxycholic acid.

The results of the experiments reported in this paper imply that the clinical indications for the use ursodeoxycholic acid

and dexamethasone in obstetric cholestasis include protection from the fetal complications of obstetric cholestasis in addition to treatment of the maternal symptoms and biochemical abnormalities. Some patients will be treated with a combination of ursodeoxycholic acid and dexamethasone and therefore it will be of interest to perform subsequent studies of pretreatment of cardiomyocytes with these agents in combination. In addition, it will be of interest to vary the concentration of ursodeoxycholic acid used in subsequent experiments. These experiments report the effect of 0.1 mM ursodeoxycholic acid, a similar but slightly higher concentration than has been reported in maternal serum in ursodeoxycholic acid-treated women (i.e. approximately 0.03 mM)[18,49]. The concentration of dexamethasone used was similar to the serum levels of dexamethasone in patients following intravenous or intramuscular boluses of the drug[50].

The authors are not aware of any studies of the effect of high serum bile acids on the human heart. It would be of interest to study the effect of cholestasis in adults on cardiac function, and to investigate the effect of taurocholate on an *in vitro* model of the adult human heart.

CONCLUSION

We have demonstrated that dexamethasone, and to a lesser extent, ursodeoxycholic acid, protects against the arrhythmogenic effect of taurocholate in an *in vitro* model of the cardiac effects of bile acids.

Acknowledgements

Ventricular neonatal rat myocytes were provided by Peter H. Sugden, National Heart and Lung Institute Division, Imperial College. This work was supported by grants from the Wellcome Trust, the Graham Dixon Cardiovascular Fund, the Biotechnology and Biological Science Research Council and the British Heart Foundation.

References

1. Fisk NM, Storey GN. Fetal outcome in obstetric cholestasis. *Br J Obstet Gynaecol* 1988;**95**:1137–1143.
2. Reid R, Ivey KJ, Rencoret RH, Storey B. Fetal complications of obstetric cholestasis. *BMJ* 1976;**1**:870–872.
3. Reyes H. The enigma of intrahepatic cholestasis of pregnancy: lessons from Chile. *Hepatology* 1982;**2**:87–96.
4. Rioseco AJ, Ivankovic MB, Manzur A, et al. Intrahepatic cholestasis of pregnancy: a retrospective case–control study of perinatal outcome. *Am J Obstet Gynecol* 1994;**170**:890–895.
5. Abedin P, Weaver JB, Egginton E. Intrahepatic cholestasis of pregnancy: prevalence and ethnic distribution. *Ethn Health* 1999;**4**:35–37.
6. Kenyon AP, Piercy CN, Girling J, Williamson C, Tribe RM, Shennan AH. Obstetric cholestasis, outcome with active management: a series of 70 cases. *Br J Obstet Gynaecol* 2002;**109**:282–288.
7. Costoya AL, Leontic EA, Rosenberg HG, Delgado MA. Morphological study of placental terminal villi in intrahepatic cholestasis of pregnancy: histochemistry, light and electron microscopy. *Placenta* 1980;**1**:361–368.
8. Laatikainen TJ. Fetal bile acid levels in pregnancies complicated by maternal intrahepatic cholestasis. *Am J Obstet Gynecol* 1975;**122**: 852–856.
9. Laatikainen T, Tulenheimo A. Maternal serum bile acid levels and fetal distress in cholestasis of pregnancy. *Int J Gynaecol Obstet* 1984;**22**:91–94.
10. Milkiewicz P, Elias E, Williamson C, Weaver J. Obstetric cholestasis. *BMJ* 2002;**324**:123–124.
11. Laatikainen T, Ikonen E. Serum bile acids in cholestasis of pregnancy. *Obstet Gynecol* 1977;**50**:313–318.
12. Sjovall K, Sjovall J. Serum bile acid levels in pregnancy with pruritus (bile acids and steroids 158). *Clin Chim Acta* 1966;**13**:207–211.
13. Bacq Y, Myara A, Brechot MC, et al. Serum conjugated bile acid profile during intrahepatic cholestasis of pregnancy. *J Hepatol* 1995;**22**:66–70.
14. Heikkinen J, Maentausta O, Tuimala R, Ylostalo P, Janne O. Amniotic fluid bile acids in normal and pathologic pregnancy. *Obstet Gynecol* 1980;**56**:60–64.
15. Nolan DG, Martin LS, Natarajan S, Hume Jr RF. Fetal compromise associated with extreme fetal bile acidemia and maternal primary sclerosing cholangitis. *Obstet Gynecol* 1994;**84**:695–696.
16. Williamson C, Gorelik J, Eaton BM, Lab M, de Swiet M, Korchev Y. The bile acid taurocholate impairs rat cardiomyocyte function: a proposed mechanism for intra-uterine fetal death in obstetric cholestasis. *Clin Sci (Colch)* 2001;**100**:363–369.
17. Gorelik J, Harding SE, Shevchuk AI, et al. Taurocholate induces changes in cardiomyocyte contraction and calcium dynamics. *Clin Sci (Colch)* 2002;**103**:191–200.
18. Berkane N, Cocheton JJ, Brehier D, et al. Ursodeoxycholic acid in intrahepatic cholestasis of pregnancy. A retrospective study of 19 cases. *Acta Obstet Gynecol Scand* 2000;**79**:941–946.
19. Brites D, Rodrigues CM, Oliveira N, Cardoso M, Graca LM. Correction of maternal serum bile acid profile during ursodeoxycholic acid therapy in cholestasis of pregnancy. *J Hepatol* 1998;**28**:91–98.
20. Davies MH, da Silva RC, Jones SR, Weaver JB, Elias E. Fetal mortality associated with cholestasis of pregnancy and the potential benefit of therapy with ursodeoxycholic acid. *Gut* 1995;**37**: 580–584.
21. Floreani A, Paternoster D, Melis A, Grella PV. *S*-Adenosylmethionine versus ursodeoxycholic acid in the treatment of intrahepatic cholestasis of pregnancy: preliminary results of a controlled trial. *Eur J Obstet Gynecol Reprod Biol* 1996;**67**:109–113.
22. Mazzella G, Rizzo N, Salzetta A, Iampieri R, Bovicelli L, Roda E. Management of intrahepatic cholestasis in pregnancy. *Lancet* 1991; **338**:1594–1595.
23. Mazzella G, Nicola R, Francesco A, et al. Ursodeoxycholic acid administration in patients with cholestasis of pregnancy: effects on primary bile acids in babies and mothers. *Hepatology* 2001;**33**:504–508.
24. Nicastri PL, Diaferia A, Tartagni M, Loizzi P, Fanelli M. A randomised placebo-controlled trial of ursodeoxycholic acid and *S*-adenosylmethionine in the treatment of intrahepatic cholestasis of pregnancy. *Br J Obstet Gynaecol* 1998;**105**:1205–1207.
25. Palma J, Reyes H, Ribalta J, et al. Effects of ursodeoxycholic acid in patients with intrahepatic cholestasis of pregnancy. *Hepatology* 1992;**15**:1043–1047.
26. Palma J, Reyes H, Ribalta J, et al. Ursodeoxycholic acid in the treatment of cholestasis of pregnancy: a randomized, double-blind study controlled with placebo. *J Hepatol* 1997;**27**:1022–1028.
27. Rodrigues CM, Marin JJ, Brites D. Bile acid patterns in meconium are influenced by cholestasis of pregnancy and not altered by ursodeoxycholic acid treatment. *Gut* 1999;**45**:446–452.
28. Hirvioja ML, Tuimala R, Vuori J. The treatment of intrahepatic cholestasis of pregnancy by dexamethasone. *Br J Obstet Gynaecol* 1992;**99**:109–111.

29. Serrano MA, Brites D, Larena MG, et al. Beneficial effect of ursodeoxycholic acid on alterations induced by cholestasis of pregnancy in bile acid transport across the human placenta. *J Hepatol* 1998; **28**:829–839.
30. Iwaki K, Sukhatme VP, Shubeita HE, Chien KR. Alpha- and beta-adrenergic stimulation induces distinct patterns of immediate early gene expression in neonatal rat myocardial cells. fos/jun expression is associated with sarcomere assembly; Egr-1 induction is primarily an alpha 1-mediated response. *J Biol Chem* 1990;**265**:13,809–13,817.
31. Shevchuk AI, Gorelik J, Harding SE, Lab MJ, Klenerman D, Korchev YE. Simultaneous measurement of Ca2+ and cellular dynamics: combined scanning ion conductance and optical microscopy to study contracting cardiac myocytes. *Biophys J* 2001;**81**:1759–1764.
32. Lopez JR, Jovanovic A, Terzic A. Spontaneous calcium waves without contraction in cardiac myocytes. *Biochem Biophys Res Commun* 1995;**214**:781–787.
33. Muir TM, Hair J, Inglis GC, Dow JW, Lindop GB, Leckie BJ. Dexamethasone-induced differentiation of atrial myocytes in culture. *Am J Physiol* 1992;**263**:H722–H729.
34. Yang YZ, Guo Q, Jin PY, et al. Effect of dexamethasone on Coxsackie B-2 virus-infected rat beating heart cells in culture. *Zhongguo Yao Li Xue Bao* 1989;**10**:346–349.
35. Yang YZ, Yuan WL, Guo Q, et al. Effect of dexamethasone on Coxsackievirus B2-infected rat beating heart cells in culture. *Eur Heart J* 1991;**12**:39–43.
36. Yuan WL, Chen HZ, Yang YZ, et al. Influence of dexamethasone on electrical activities in cultured rat beating myocardial cells infected with Coxsackie B-2 virus. *Zhongguo Yao Li Xue Bao* 1990;**11**:328–331.
37. Reilly AM, Sun X, Williams DA, Dusting GJ. Dexamethasone inhibits endotoxin-induced changes in calcium and contractility in rat isolated papillary muscle. *Cell Calcium* 1999;**26**:1–8.
38. Reid L, Morrow B, Jubinsky P, Schwartz E, Gatmaitan Z. Regulation of growth and differentiation of epithelial cells by hormones, growth factors, and substrates of extracellular matrix. *Ann N Y Acad Sci* 1981;**372**:354–370.
39. Galle PR, Theilmann L, Raedsch R, Otto G, Stiehl A. Ursodeoxycholate reduces hepatotoxicity of bile salts in primary human hepatocytes. *Hepatology* 1990;**12**:486–491.
40. Benz C, Angermuller S, Tox U, et al. Effect of tauroursodeoxycholic acid on bile-acid-induced apoptosis and cytolysis in rat hepatocytes. *J Hepatol* 1998;**28**:99–106.
41. Benz C, Angermuller S, Otto G, Sauer P, Stremmel W, Stiehl A. Effect of tauroursodeoxycholic acid on bile acid-induced apoptosis in primary human hepatocytes. *Eur J Clin Invest* 2000;**30**:203–209.
42. Heuman DM, Pandak WM, Hylemon PB, Vlahcevic ZR. Conjugates of ursodeoxycholate protect against cytotoxicity of more hydrophobic bile salts: in vitro studies in rat hepatocytes and human erythrocytes. *Hepatology* 1991;**14**:920–926.
43. Heuman DM, Mills AS, McCall J, Hylemon PB, Pandak WM, Vlahcevic ZR. Conjugates of ursodeoxycholate protect against cholestasis and hepatocellular necrosis caused by more hydrophobic bile salts. In vivo studies in the rat. *Gastroenterology* 1991;**100**: 203–211.
44. Heuman DM, Bajaj R. Ursodeoxycholate conjugates protect against disruption of cholesterol-rich membranes by bile salts. *Gastroenterology* 1994;**106**:1333–1341.
45. Nathanson MH, Burgstahler AD, Masyuk A, LaRusso NF. Stimulation of ATP secretion in the liver by therapeutic bile acids. *Biochem J* 2001;**358**:1–5.
46. Mei Q, Liang BT. P2 purinergic receptor activation enhances cardiac contractility in isolated rat and mouse hearts. *Am J Physiol Heart Circ Physiol* 2001;**281**:H334–H341.
47. Beuers U, Bilzer M, Chittattu A, et al. Tauroursodeoxycholic acid inserts the apical conjugate export pump, Mrp2, into canalicular membranes and stimulates organic anion secretion by protein kinase C-dependent mechanisms in cholestatic rat liver. *Hepatology* 2001; **33**:1206–1216.
48. Doyle LW, Ford GW, Davis NM, Callanan C. Antenatal corticosteroid therapy and blood pressure at 14 years of age in preterm children. *Clin Sci (Colch)* 2000;**98**:137–142.
49. Modi N, Lewis H, Al Naqeeb N, Ajayi-Obe M, Dore CJ, Rutherford M. The effects of repeated antenatal glucocorticoid therapy on the developing brain. *Pediatr Res* 2001;**50**:581–585.
50. Tsuei SE, Moore RG, Ashley JJ, McBride WG. Disposition of synthetic glucocorticoids. I: Pharmacokinetics of dexamethasone in healthy adults. *J Pharmacokinet Biopharm* 1979;**7**:249–264.

Accepted 8 January 2003

To establish the proportion of cardiomyoctes in the geneticin-treated cultures, cells were stained with antibodies to detect sarcomeric α-actinin and phalloidin to reveal the distribution of filamentous actin. Immunodetection showed that almost 90% of the cells possessed the myocyte marker, α-actinin (data not shown). Cells incubated normally display synchronous contractile behaviour, and taurocholate induces arrhythmia[16].

RESULTS

We present here the results of several independent experiments for each experimental condition analysed. Figures 1–3 illustrate recordings that we typically obtained, and which were consistent between experiments. In the first set of experiments, cells were pre-incubated for 16 hours with either ursodeoxycholic acid or dexamethasone. Taurocholate was then added to the culture medium. With dexamethasone or ursodeoxycholic acid alone under standard bright-field microscopy cells displayed synchronous beating. After cell loading with the calcium indicator fluo-4 synchronous calcium waves were observed that spanned the entire network of cells (Figs 1A and 1E), that is, the cells were coupled. When taurocholate was added to the culture medium of cells that had been pre-incubated with dexamethasone to give a final taurocholate concentration of up to 3 mM, the concentration that has been previously reported to cause severe disruption of cardiomyocyte contraction[17], there was no change in the rate or synchronisation of contraction (Fig. 1B) (four experiments). Only at the higher dose of 4.5 mM taurocholate did the cells show contractile irregularities (Fig. 1C). Neighbouring cells within the network had different patterns of calcium wave propagation, consistent with abnormal cell–cell communication. As soon as the medium was changed to taurocholate-free medium, the cells displayed signs of recovery. Although the rate of contraction did not return to that seen prior to taurocholate addition, it became regular and calcium waves showed intercellular coupling (Fig. 1D).

Pre-incubation with ursodeoxycholic acid protected cells from the effects of 0.3 mM taurocholate (Fig. 1F) but 3.0 mM taurocholate produced fibrillation/tachycardia and loss of synchronous contraction (Fig. 1G) and subsequent irreversible cellular overload with calcium (Fig. 1H) (four experiments).

The protective effect of dexamethasone was dependent on both the dose of taurocholate added and the concentration of dexamethasone in the pre-incubation medium (Table 1). Without pre-incubation with dexamethasone, the integrity of the network of cardiomyocytes was lost in the presence of both 3.0 and 4.5 mM taurocholate, and the effects of the latter concentration were irreversible. Pre-incubation with 80 nM dexamethasone protected cells from the effect of 0.3 and 3.0 mM taurocholate, but not from the higher dose of 4.5 mM. After transfer to taurocholate-free medium, the cells recovered but had a slower rate of contraction than before taurocholate treatment. Addition of 800 nM dexamethasone to the pre-incubation medium protected cells from the effect of 0.3 and 3.0 mM taurocholate concentrations, but cells exposed to 4.5 mM taurocholate

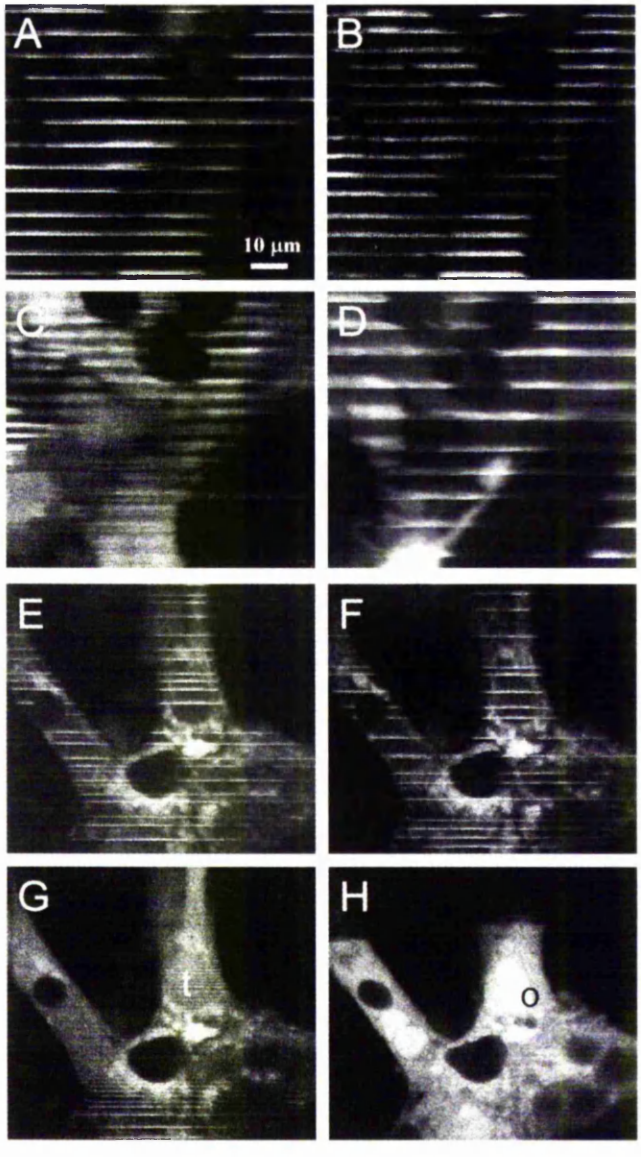


Fig. 1. Effect of taurocholate on calcium dynamics in cardiomyocytes pretreated with dexamethasone and ursodeoxycholic acid. (A) The cardiomyocyte network was incubated for 16 hours with 800 nM dexamethasone (A–D). Horizontal lines represent calcium waves which extend across adjacent cells, consistent with synchronous beating. The spacing between the lines provides an indication of frequency. (B) The addition of taurocholate up to a concentration of 3 mM does not alter calcium dynamics. (C) Addition of taurocholate to a concentration of 4.5 mM alters calcium dynamics. (D) Restoration of rhythmic contraction after transfer to taurocholate-free medium. (E) Network of cardiomyocytes incubated for 16 hours with 0.1 mM ursodeoxycholic acid. (F) The addition of 0.3 mM taurocholate does not alter calcium waves. (G) The addition of 3 mM taurocholate alters calcium wave dynamics, consistent with tachycardia—closely spaced lines (t), (H) this did not return to normal following transfer to taurocholate-free medium, and calcium overload (o) was seen with cessation of beating.