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Biliary atresia: From Australia to the zebrafish

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ABSTRACT

This review is based upon an invited lecture for the 52nd Annual Meeting of the British Association of Paediatric Surgeons, July 2015.

The aetiology of biliary atresia (BA) is at best obscure, but it is probable that a number of causes or pathophysiological mechanisms may be involved leading to the final common phenotype we recognise clinically.

By way of illustration, similar conditions to human BA are described, including biliary agenesis, which is the normal state and peculiar final pattern of bile duct development in the jawless fish, the lamprey. Furthermore, there have been remarkable outbreaks in the Australian outback of BA in newborn lambs whose mothers were exposed to and grazed upon a particular plant species (*Dysphania glomulifera*) during gestation. More recent work using a zebrafish model has isolated a toxic isoflavonoid, now named Biliatresone, thought to be responsible for these outbreaks.

Normal development of the bile ducts is reviewed and parallels drawn with two clinical variants thought to definitively have their origins in intrauterine life: Biliary Atresia Splenic Malformation syndrome (BASM) and Cystic Biliary Atresia (CBA). For both variants there is sufficient clinical evidence, including associated anomalies and antenatal detection, respectively, to warrant their aetiological attribution as developmental BA. CMV IgM + ve associated BA is a further variant that appears separate with distinct clinical, histological, and immunohistochemical features. In these it seems possible that this involves perinatal obliteration of a normally formed duct system. Although still circumstantial, this evidence appears convincing enough to perhaps warrant a different treatment strategy. This then still leaves the most common (more than 60% in Western series) variant, now termed Isolated BA, whereby origins can only be alluded to.

The aim of this article is to review the nature and aetiology of biliary atresia from the personal perspective of a surgeon whose career has been built largely on a quest to unravel or at least start to untangle some of the mystery surrounding this dread disease. Winston Churchill once described Russian foreign policy in the 1930s as "a riddle wrapped in a mystery inside an enigma" and for sure this equally well might describe the situation with the aetiology of BA. We Kasai surgeons are like the fossil hunters finding the jaw bone of some prehistoric man trying to determine what he last had for dinner!

So what do we know? We know that the condition is peculiar to the neonatal period. We know that it is irreversible — no one spontaneously got better from BA. We know it affects both intrahepatic and extrahepatic bile ducts. Certain truly genetic cholestatic conditions such as Alagille's syndrome are characterised by biliary hypoplasia, whereby the entire duct system from cholangiole to common bile duct is etiolated but patent and bile flow is constrained. Some infants are born with cystic choledochal malformations, detected antenatally, and therefore coming to relatively early surgery, who have a miniscule distal duodenal connection yet remain completely anicteric with perfectly pigmented stool. When drainage finally ceases, pressure rises and the intrahepatic ducts dilate.

So, what don't we know? Firstly, we don't even know when the pathology starts in most infants. The old dichotomy between "embryonic" and "perinatal" is simplistic and naïve [1]. The first labelled group is okay and I will explore this in a moment but the latter is simply a shield designed to deflect criticism for doctors who see these infants in the first weeks of life and don't diagnose what is actually going on. Something abnormal is going on in first days of life in most infants (i.e. it is congenital) and I will now review recent clinical observations later which seem to support that, possibly unfashionable, position.

It is possible to measure the levels of liver enzymes in amniotic fluid and they can only get there via the bile duct and gut. Francoise Muller from Paris assayed γ-glutamyl transpeptidase (amongst other enzymes) [2]. Normally levels in amniotic fluid rise from about the time of establishment of bile duct continuity and bile production at about 12 weeks' gestation to reach a plateau during the 2nd trimester and then fall off during the 3rd trimester as the anal sphincter closes. Retrospective evaluation of more than 8000 samples showed that lowest values during this plateau period were found in 3 infants who ultimately proved to have (isolated) BA (Fig. 1).

Here in the UK, Imran Mushtaq (latter a urologist) measured bile acids in the Guthrie blood spot (taken in first days of life) in an attempt to find a screening test for BA [3]. About 3/4 (77%) of 61 infants who later proved to have BA had elevated total bile acids (>97th percentile, 33 μmol/L). Most recently, Harpavat et al. from Texas [4] retrospectively identified

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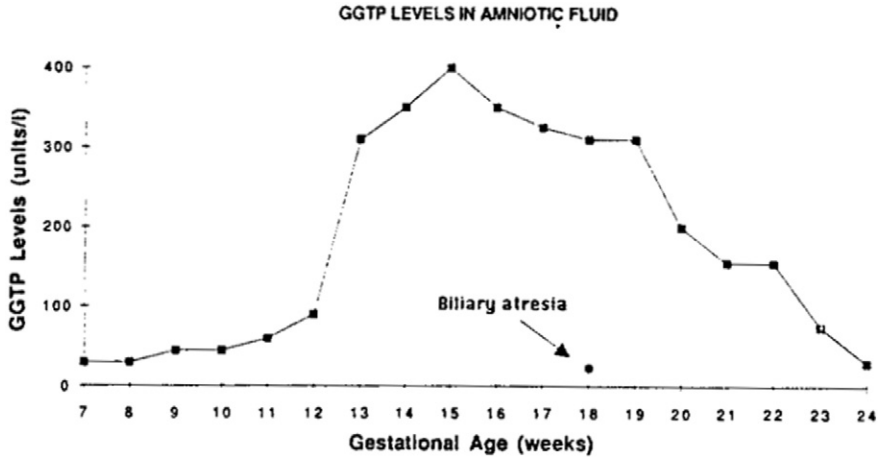

Fig. 1. ***In-Utero*** **Biliary Atresia.** Normal values for γ-glutamyl transpeptidase in amniotic fluid across 2nd trimester of fetal life. Indicated is level of infant later shown to have biliary atresia [reproduced with permission]. Red line indicates approximate beginning of bile secretion by hepatocyte.

split bilirubin values in 31 infants with BA obtained when they were <48 h old (about half their overall cohort with BA). All were abnormal and significantly higher than controls. These disparate studies strongly imply that the cause of the cholestasis is fully established at birth in more than half of infants with what we have been labelling as isolated BA — making even those "developmental" BA.

So, let us now turn to the complex process by which bile ducts develop in man.

1. Development of the extrahepatic biliary system and BASM

The biliary system is derived in two distinct ways with different time schedules — the extrahepatic bile duct being the first to form and is derived directly from the foregut endoderm whilst the intrahepatic ducts are derived later from hepatoblasts forming within the liver primordium.

The extrahepatic duct itself appears at about day 20 as an outpouching from distal foregut and develops into a funnel-shaped structure with a lumen and a gallbladder by day 45. It is lined by cholangiocytes derived from foregut endoderm, expressing transcription factors common to the pancreas and duodenum (e.g. *PDX-1*, *PROX-1*, *HNF-6*). The molecular mechanisms regulating this phase of biliary development are not well described in humans but mice deficient in Pdx-119 or Hes1 (a Notch-dependent transcription factor), Hnf-6, Hnf-1β, or Foxf1 (a transcription factor target for Sonic Hedgehog signalling) can cause altered development of the gallbladder with a common bile duct [5–8].

We do have a clinical variant of BA where the timing of origin can be put squarely within this period of extrahepatic bile duct development from 25 to 45 days. This we named **Biliary Atresia Splenic Malformation (BASM)** syndrome from the most obvious and consistent of the associated anomalies in 1993 [9]. More than 80% have polysplenia and the rest either double spleens or asplenia. These infants also show various combinations of situs inversus, a preduodenal portal vein, absence of the vena cava, malrotation and cardiac anomalies [10]. Typically their extrahepatic bile duct pathology shows an atrophic gallbladder and complete absence of the common bile duct, without much in the way of inflammation. Interestingly, we reported 3 infants with BASM (albeit unrecognised at the time) who had had laparotomy in the first week of life where a liver biopsy was performed [11]. These invariably showed no evidence of liver fibrosis. It appears that even in these infants the actual fibrotic process starts from the time of birth not from the time of biliary occlusion.

It has not been possible to define a genetic cause for infants with BASM. CFC-1 (on Ch 2q 21.1 and encoding for CRYPTIC protein) seemed the likeliest candidate gene given that knockout mice exhibit randomisation of abdominal situs, asplenia and cardiac anomalies such as transposition and double outlet right ventricle [12]. Five of 10 BASM infants in a small French series did show *CFC-1* mutations but so did about 10% of normal infants [13]. Recently a deletion of the gene *FOXA2*, a regulator of *NODAL* expression, was identified in a girl with BASM and her father who had other heterotaxy features (situs inversus and polysplenia) but not BA [14].

In our clinical series many of the infants seemed to have had abnormal 1st trimester events or complications including higher rates of *in vitro* fertilisation and maternal diabetes [9,10]. Both are associated with an increased frequency of birth defects and there is some overlap with some specific types of anomalies seen in the latter such as transposition of the great vessels. The actual cause of diabetic embryopathy is still elusive although a hyperglycaemic intrauterine environment may induce epigenetic effects by oxidative or nitrosative stress mechanisms in animal models [15].

2. Development of the intrahepatic biliary system

By 28 days, the embryonic liver is populated by hepatoblasts, derived directly from endoderm and haematopoietic cells initially derived from yolk sac. The former are arranged in plates, initially 3–4 cells thick, which line the vascular sinusoidal network. Hepatoblasts will give rise to both hepatocytes and ductal cells with Notch signalling promoting ductal cell development and inhibiting hepatocyte formation. The nascent intrahepatic bile ducts only appear as a distinct entity from about seven weeks' gestation [16,17]. At this point, each branch of the infiltrating portal venous network is surrounded by a layer of mesenchyme and then a cylindrical double cell layer of darkly staining cells termed the *ductal (or limiting) plate*. Remodelling of this layer occurs at about 12 weeks to form a single-cell-layer lined network of interconnected bile ducts enveloped into the mesenchyme. These remodelling and extension from the dual-layer depend upon a unique process of so-called *transient asymmetry* whereby ductal plate cells resembling cholangiocytes (expressing *SOX9* and CK19) on the side facing the portal tract are matched by ductal plate cells resembling hepatoblasts (expressing *HNF4*) on the side facing the parenchyma [18]. After the formation of a lumen, the nascent bile duct becomes symmetrical as "hepatoblasts" are replaced by "cholangiocytes" to form a double cell layer composed entirely of cholangiocytes and then intercalating to form a single cell layer. Ductal progression and elongation proceed from the hilum to the periphery and appear to be controlled by the non-canonical Wnt pathway.

Bile is first observed in primitive cholangioles and then transported into the fetal gut from about 12–14 weeks' gestation implying completion of biliary continuity. At some point, of course, extrahepatic and intrahepatic systems coalesce at the interface of the porta hepatis though again the process is imperfectly understood. The so-called