Review Article:
Extrahepatic Biliary Atresia is an Aflatoxin Induced Cholangiopathy in Infants with Null GSTM1 Genotype with Disrupted P53 and GSTPi to Mothers Heterozygous for GSTM1 Polymorphism: Damage Control is Mediated through Neutrophil Elastase and CD14+ Activated Monocytes: Kotb Disease

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Abstract
Extrahepatic biliary atresia (EHBA) has long been defined as a progressive cholangiopathy of infancy of obscure aetiology. It is a grave disease with serious morbidity, mortality and economic burden. EHBA is currently treated surgically by Kasai portoenterostomy. In 10 years post-portoenterostomy the survival does not exceed 30%, and 60-80% of children with EHBA eventually develop liver cirrhosis. EHBA is the leading indication for liver transplantation in children. Evidence supports that immune mediated destruction of extrahepatic portal tracts that extends to intrahepatic bile ductules is an integral part of pathogenesis of EHBA. Yet initiator of immune mediated pathogenesis was still unknown. Evidence supports that the aetiology of EHBA is an aflatoxin induced cholangiopathy in glutathione S transferase (GST) M1 deficient infants having disrupted p53 and GSTPi, whose mothers are heterozygous for GSTM1. Neutrophil elastase and CD14+ mediated damage control and integral in EHBA. These factors combined result in hepatotoxicity and bile duct damage followed by defective regeneration, and disruption of ontology respected development. EHBA is a multiple hit disease. Aflatoxins B1 and B2 are transmitted through foods, they are highly substituted coumarins with short half life that need timely cytochrome P450 family and GST detoxification. Bacterial lipopolysaccharide increase aflatoxins induced hepatocyte and bile duct damage up to 20 folds. Evidence supports that placental intrauterine transfer of aflatoxins is recognized, and that maternal detoxification of aflatoxins protects foetus during pregnancy, which will be lost post-partum. Postpartum infantile incompetent detoxification of the aflatoxin hepatic stores would result in hepatic toxicity, in proliferated bile ducts and fibrosis hence EHBA. Maternal detoxification products of aflatoxins delivered in breast-milk punctuate the course of infants with EHBA by attacks of cholangitis. EHBA is potentially preventable. We recommend prompt diagnosis by assessment of aflatoxins and GSTM1 phenotype in any neonatal hepatitis. Stringent monitoring of upper limits of mycotoxins in our food, and in food of poultry and cattle is a must.


Introduction
EXTRAHEPATIC Biliary Atresia (EHBA) has long been of unknown aetiology, and was defined as a chronic progressive obstructive cholangiopathy of infancy starting in the extrahepatic bile ducts with scarring, fibrosis, lumen obstruction followed by spread to intrahepatic bile ducts [1]. Management of EHBA is palliative relief of obstruction by Kasai portoenterostomy [2,3]. Despite timely intervention most of infants will run a progressive course of fibrosis, cirrhosis and portal hypertension that is punctuated by attacks of cholangitis [24]. Evidence supports that results are best when performed within first 60 days of life [1]. Scientists proposed aetiologies such as vascular accident, reovirus type 3 infection, hepatitis B virus infection, autoimmune disease, an early first trimester arrest of biliary development, ductal plate malformation, genetic metabolic bile abnormality and a mere genetic susceptibility [1].

Involvement of immune system is by far most consistent and integral in EHBA [1,5-8], but pattern
of involvement does not support that EHBA is an “auto” immune disease [9].

To date no proposed theory of aetiology could explain the multifaceted pathogenesis of what is known about EHBA.

**What do we know about EHBA?**

1- It is a hepatic disease that develops and progresses early in life ending in obstruction of extrahepatic bile ducts, commonly preceded with a disease free interval with possible previous intrauterine accident [10,11].

2- The progression results in fibrosis and adhesions that would end in multi level obliterations of bile ducts [12].

3- Small vessel disease, ischemia, and portal vein disease [13-25].

4- Lack of rule in progression of disease, with seeming seasonal variation that was not definitely proven as infectious [26-28].

5- Prompt chaotic attempts at regeneration defying ontogeny [2,10,14].

6- EHBA progression is halted by debulking of portahepatis i.e. portoenterostomy [2,3].

7- EHBA progression is punctuated by attacks of cholangitis that does not comply with a predicted model [2,10,14].

8- Post debulking, steroids and antibiotics have inconsistent effects [29,30].

9- Selective burden of disease on cholangiocytes, and on portahepatis [1].

10- Neutrophil elastase involvement in clearing aftermath is a must [6,8].

Any proposed aetiology has to include all aforementioned characteristics of EHBA, otherwise it will fall short of being the underlying cause. Based upon the evidence published in our previous works EHBA is an aflatoxin induced cholangiopathy in infants with null glutathione S transferase (GST) M1 genotype with disrupted p53 and GSTPi [1,6,8,31-33]. Neutrophil elastase and CD14+ mediated damage control is integral in EHBA [8].

**Why aflatoxins?**

Aflatoxins B 1 and B2 are a group of highly substituted coumarins that are acute coagulants produced by Aspergillus flavus [34,35]. They are grouped with other microfungi products as mycotoxins to man and other animals. They cause acute and chronic aflatoxicosis, they are mutagenic, teratogenic, carcinogenic, immunosuppressive [36], and cause hepatocellular damage [37,38]. Aflatoxin B1 ranks as the most potent carcinogen [39]. Aflatoxins B 1 and B2 are natural contaminants of figs, oilseed, cereals, nuts, peanuts, tobacco, corn, wheat, pistachio and many other foods. Aflatoxins contamination can occur before harvest and during storage, and might exhibit seasonal variations [40]. Upon ingestion, aflatoxins B 1 and B2 are transported to the liver via the portal vein where they get detoxified by cytochrome p450 (CYP) 1A2 into the water soluble aflatoxins M1 and M2 to be excreted in urine [41]. Aflatoxins M1 and M2 can also be excreted in milk when lactating mothers or cattle ingest aflatoxins B1 and B2 contaminated foods [40]. Another mode of exposure to aflatoxins B1 and B2 is through placental transfer to the developing embryo and foetus [42,43].

Aflatoxins can also be detoxified by CYP 3A4 to produce the toxic aflatoxin B1-8, 9 epoxide which can be further detoxified by the GST family [44]. Aflatoxins B1 and B2 hepatic intoxication characteristic features include centrilobular scarring, hepatic venous occlusion, ductular proliferation, cholestasis, focal syncytial giant cell transformation of hepatocytes, and pericellular fibrosis [37,38]. Prenatal repeated exposure to 100 gg aflatoxin B1 in pregnant rats result in extensive cystic lesions in progeny along with bile ductular proliferation, neoplastic nodules and hepatocellular carcinoma. These findings were accentuated in the progeny of rats who were prenatally exposed to aflatoxins B1 and after delivery their mothers continued aflatoxins ingestion, and nursed their progeny milk containing aflatoxins M1. Hepatotoxicity developed 2-3 months post partum. The least encountered hepatic lesions were in progeny exposed only to aflatoxins M1 in nursing milk [45]. It is important to notice that silencing of GSTPi gene in aflatoxicosis is associated with hepatocellular carcinoma [46]. Bacterial lipopolysaccharide augments hepatoxicity of aflatoxins, and channels brunt upon hepatocytes, through damaged sinusoidal endothelial cells and activation of coagulation system [47-48]. Bacterial lipopolysaccharide also up-regulates CD14+ monocytes. CD 14+ when activated by binding to bacterial lipopolysaccharide result in a cascade of cytokines and nitric oxide which is a cytotoxic effector in cell killing [49].

**Why did we suspect Aflatoxins in Infants with EHBA?**

Because:

1- Aflatoxins produce a diverse spectrum of disease depending upon amount of ingested afla-
toxins, number of ingestions, their type, type of food and host detoxification system integrity and harmony of phase I, phase II and phase III, i.e. cytochrome P450 superfamily, glutathione S-transferase, epoxide hydrolase, and others [51-53]. According to type of enzyme ontogeny, deficiency or disruption, aflatoxins can result in malignancy [29,30], immune diseases, failure to thrive, hepatocellular damage and cirrhosis [37, 54-57].

2- Aflatoxins cross placenta, and induce neonatal bile duct disease in rats [45], and EHBA is a neonatal bile duct disease. In fact, we found that all infants with EHBA had aflatoxins B 1 and only a small number had B2 [32]. Since infants feed on milk, which will carry aflatoxins M 1 and M2 but not B 1 or B2 [40,58-60], then aflatoxins B 1 and B2 must have been transmitted through the placenta from their mothers during intrauterine life [42,43].

3- Aflatoxins should undergo detoxification [41,44]. Detoxification during pregnancy is the responsibility of maternal liver, fetal liver and placenta [61-66]. Detoxification is decreed by ontogeny [61,62], interaction of the 3 detoxifying bodies and the amount and timing of exposure to the contaminated foods. It is crucial to stress that aflatoxins have a relatively short life in terms of hours in individuals with intact detoxification system [57,67]. Evidence supports that placental intrauterine transfer of aflatoxins is recognized, and that maternal detoxification of aflatoxins protects the foetus during pregnancy [42,43], which will be lost immediately post-partum. Aflatoxin induced hepatotoxicity in progeny of rats with intact detoxification manifests in 2-3 months [45], which is the case in EHBA [1].

4- Aflatoxin amount and timing of exposure dictate different clinical disease expression. Spectrum of disease begins by embryonic exposure to aflatoxins that increases DNA adducts 20 folds and carcinogenesis [68] up to failure to thrive [69]. Thus, aflatoxin ingestion during early pregnancy and embryogenesis might result in the ductal plate malformation, which is named Meyenberg complexes in rats exposed during embryogenesis to aflatoxins B1 and B2 [45]. Aflatoxins could be responsible for the early embryonic or foetal ductal malformation that was reported to contribute to pathogenesis [10] in EHBA. In humans, foetal biliary system develops around the 5th-8th week, from the endodermal hepatic diverticulum of foregut, which proliferates to form septum transversum and extrahepatic bile ducts. It is to be noted that hepatoblasts develop from epithelial cells arising from gut endoderm. From 7th week the ductal plate begins reduplication forming a double layer of cells around the portal tract. Bile ducts are always patent during embryogenesis until delivery [70]. Thus aflatoxins could result in inflammation of developing ductal plate or bile ducts resulting in non-obliterative adhesions, which would progress early in life to complete obliteration of bile ducts. CYP 1A2 ontogenesis is delayed in foetal life, up to being undetected [61], i.e., in the setting of maternal ingestion of aflatoxins in food in mid and late first trimester we assume that CYP 1A2 will not detoxify ingested aflatoxins. Pregnancy is associated with decreased maternal activity of CYP 1A2 during early, middle and late pregnancy [63]. Human placenta has decreased expression of CYP 1A2 as well [64]. Yet, GST activity in human placenta is increased in first trimester, and decreases gradually to one third of this activity by full term [68], and is expressed in foetal liver throughout pregnancy [66]. Endogenous antioxidant defenses in plasma and erythrocytes of the pregnant woman differ across the three trimesters of pregnancy. A window of susceptibility to aflatoxins is recognized during embryogenesis to aflatoxins [68]. It is to be noted that first trimester is associated with nadir of catalase, superoxide dismutase and aminolevulinic dehydratase as well [71]. First trimester is governed by other factors as well that allow a window of susceptibility during embryogenesis to aflatoxins [68]. It is to be noted that CYP 3A4 responsible for detoxification of aflatoxins yielding the deleterious aflatoxin B1-8, 9 epoxide is not detectable in first trimester in human placenta, yet it is inducible [64]. During the later stages of pregnancy ontogeny developed maternal detoxification will protect against further damage and will remove aflatoxin loads in foetus. It seems that transfer of aflatoxins immediately before, during delivery and milking of cord will transmit aflatoxins that will be transferred by portal vein to portahepatis. Actually newborns with EHBA have loads of aflatoxins in their portahepatis [1,32] and eventually exhibit hepatic toxicity that is accentuated in presence of bacterial lipopolysaccharide [8]. Hence the development and progression of EHBA early in life, with possible previous intrauterine accident [10,11]. It is interesting that CYP3A4 activity in humans is depressed at delivery and increases insidiously by end of first month of life to reach 30% [72], thus in a newborn with lack of GSTM1 aflatoxins B1 will not be promptly detoxified. The un-detoxified aflatoxins B1 and B2 will remain as such in newborn damaging its liver [32]. Rats with intact phase I and II detoxification system when prenatally exposed to aflatoxins B1, express hepatotoxicity clinically by 2-3 month of age [45].
5- Aflatoxin-induced hepatitis in rats exposed to repeated intrauterine doses of aflatoxins, is associated with cystic biliary dilatations besides centrilobulular scarring, hepatic venous occlusion, ductular proliferation, cholestasis, focal syncytioc giant cell transformation of hepatocytes, and pericellular fibrosis [37,38]. These cystic dilatations are not constantly present in EHBA, but reported in some cases with EHBA and in EHBA associated with choledocal cysts [73], and/or polycystic disease of liver [74]. These cystic dilatations are not constantly present in EHBA, and are not encountered as a whole mark in aflatoxicosis acquired post-partum [37,38]. It is not clear if endotoxemia and CD14+ are responsible for this augmented effect of aflatoxicosis on the bile ducts with development of secondary inflammatory adhesions annulling the “bile duct dilatation” effect on extra-and intrahepatic bile ducts.

6- Aflatoxins become detoxified in humans and animals. Since the host detoxification system fidelity is a crucial factor for the expression of hepatotoxicity, we can get postpartum infant hepatic damage with no maternal expression of liver disease in the infant with null GSTM1, and heterozygote mother for GSTM1 [32,33]. She will protect her baby during intrauterine life by active detoxification, but she will not be able to maintain detoxification post-partum, on the contrary she overloads newborn liver by aflatoxins M 1 and M2 transmitted to him in her milk if she re-ingests aflatoxins [32] or in the artificial infant formula [75,76].

7- Aflatoxins induced epidemic of toxic hepatitis in India in 1978 was associated with characteristic features of centrilobular scarring, hepatic venous occlusion, ductular proliferation and cholestasis, focal syncytioc giant cell giant cell transformation of hepatocytes, and pericellular fibrosis [37,38], which are same findings are present in livers of infants with EHBA [1]. EHBA is characterized by destructive inflammatory process that affects intrahepatic and extrahepatic bile ducts leading to fibrosis, adhesions and obliteration of the biliary tract with the eventual development of biliary cirrhosis [1,14,15].

8- Aflatoxins being highly substituted compounds are associated with coagulation defects [77,78], that might mount to hepatic ischemia [47], which is also a whole mark of EHBA [1]. Evidence of vasculitis in EHBA is abundant, including small vessel disease, up to large vessel disease. Portal vein “atresia” and decreased hepatic portal flow in EHBA is recognized [17-21].

9- Aflatoxins damage p53, resulting in regeneration that defies ontogeny, thus hepatic regeneration nodules do not respect structure, and subject develops cirrhosis and malignancy [79-81]. p53 is responsible for DNA fidelity. In EHBA p53 is disrupted [31] and relentless attempts at healing end in prompt early life cirrhosis.

10- Aflatoxins ingestion in presence of bacterial lipopolysaccharide result in serious damage to sinusoidal endothelial cells nearly 20 folds [47]. activates coagulation system [48], through a neutrophil elastase dependent or independent damage ending in adhesions [47]. Aflatoxins are blood borne, thus portal hepatitis is loaded with aflatoxins, and immune process involvement is accentuated at porta hepatis [1]. Kasai has long been recognized as a palliative procedure, we suppose that Kasai would debulk this load of portalhepatis aflatoxins, as demonstrated in our previous work, where portalhepatis liver tissues post portoenterostomy were loaded with aflatoxins [1].

11- Aflatoxin hepatotoxicity is augmented by bacterial lipopolysaccharide [47,48], and all biopsies of EHBA have unaminous CD 14+ monocytes [5,7,8]. CD14+ monocytes when activated by binding to bacterial lipopolysaccharide result in a cascade of cytokines and nitric oxide which is a cytotoxic effector in cell killing [49]. This explains the why antibiotics have a role in EHBA, yet inconsistent.

12- Liver damage is reported with aflatoxins B1 and B2 [32,33]. It is peculiar that aflatoxins M1 and M2 ingestion in milk is not particularly associated with hepatocellular carcinoma [82,83] but with accentuation of hepatitis in hepatitis C and B infected subjects [84]. Aflatoxin M 1 and M2 are associated with failure to thrive and neonatal cholestasis [69].

13- Aflatoxins induced DNA adducts, i.e. aflatoxins bonding to DNA result in long term effects of malignant transformation [85], and these DNA adducts increase 20 folds upon embryonic exposure to aflatoxins [68], which is encountered in children with EHBA, as they are known to develop hepatocellular carcinoma [86-89].

_Why is EHBA a Kotb Disease? What is a Kotb Disease?_

Pathogenesis of diseases involving neutrophil activation is generally regarded as “auto” immune [90], or “malignant” [91] while our work clearly defies this interpretation. Our work provides evidence that neutrophil involvement is for damage control, and initiation of regeneration [8].
Exposure of susceptible hosts to chemicals/toxins/substances that should be detoxified but “cannot/do not” get detoxified through phase I, II and III detoxification, will accumulate thus resulting in cellular damage and involvement of neutrophil elastase and other enzymes. This will be followed by a cascade of events labeled “autoimmune” or “immune” or “malignant”. This subset of diseases will be expressed during exposure of subject, and course will always be associated with exacerbations related to exposure of offending chemical/toxin/substance. Load and type of offending chemical/toxin/substance, duration of exposure and specific defect of detoxification will dictate the phenotype of the previously labeled “auto” immune disease, or “malignancies”. During the “attack” and its “exacerbations” there will always be a back-stage player, nor seen or looked for, i.e. offending chemical/toxin/substance. Research in the forthcoming era will definitely change a lot of our appreciation and understanding of diseases currently considered “auto” immune or “malignant” hence the spectrum of Kotb Disease.

Where do we go from here?

Prompt diagnosis of EHBA by assessment of aflatoxins B 1 and B2 in is a venue for future research. Alternative modes of medical therapy should be sought to replace the surgical Kasai portoenterostomy that removes the bulk of liver tissue studded by aflatoxins. Until then prevention seems a very important tool of disease control. Implementation of screening programs for aflatoxins in infants and lactating mothers will afford characterization of the infant at risk of development of EHBA.

Aflatoxins and mycotoxin levels in food and in infants' milk formulae should be stringently controlled.

Conclusion:

EHBA is an aflatoxin induced cholangiopathy in infants with null GSTM1 genotype with disrupted p53 and GSTPi to mothers heterozygous for GSTM1 polymorphism [31-33], and liver damage control is mediated through neutrophil elastase and CD14+ bacterial lipopolysaccharide activated monocytes [8]. Infants with EHBA have disrupted p53 and GSTPi [44] resulting in regeneration that does not respect ontogeny with subsequent development of cirrhosis. Course of EHBA is punctuated by aflatoxin M1 induced cholangitis.

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