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Non-alcoholic fatty liver disease: Is it microbe related?

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Abstract: *Non-alcoholic fatty liver disease (NAFLD) is defined as spectrum of liver disease ranging from simple steatosis to steato-hepatitis (NASH). The microbiome-host interactions shape a number of phenomenon that occur in human body. The mechanism of interaction between host and the gut microbiota is complex in nature. The gut microbiota has many other important functions beyond the role in modulating immune response. These include maintenance of gut barrier integrity and other metabolic functions especially generation of short chain fatty acids from complex indigestible carbohydrates. Disruption in gut barrier leads to leaky gut which may result in metabolic endotoxemia, increased lipogenesis leading to NASH. The gut microbiota also contributes to the human health by virtue of the metabolic functions performed by it. This involves bacterial bile acid biotransformation, breakdown of oxalate, prodrug activation, vitamin production (Vitamin K, folate, B_{12} and biotin) and polysaccharide degradation by colonic bacteria resulting in production of short-chain fatty acids (SCFA).The increase in obesity appears to have fuelled the increase in occurrence of metabolic syndrome and its hepatic component-NASH. Deficiency of choline has been implicated in causation of hepatic steatosis. It has been reported that gut microbes are involved in metabolism of choline into methylamine leading to choline deficiency and thereby may have a role in genesis of NAFLD. Small bacterial overgrowth may also play a role in causation of NAFLD. Higher prevalence of SIBO causes increased intestinal permeability in patients with NAFLD when compared with healthy controls. The possible role of changes in microbes present in gut in NAFLD may provide opportunities for therapeutic intervention by modulation of gut microbiota.*
Keywords: *Microbiota, NAFLD, NASH, Steatosis, SIBO.*

I. Introduction

Non-alcoholic fatty liver disease (NAFLD) is defined as spectrum of liver disease ranging from simple steatosis to steatohepatitis(NASH). There is close interaction between gastrointestinal tract and the liver.The nutrients, micro-nutrients, chemical compounds and toxins absorbed by gut first reaches the liver for its metabolism.Changes in the gut microbiota have been reported in NAFLD, hepatic encephalopathy, alcohol related liver disease and in hepatocellular carcinoma. The gut microbiota is an area of immense interest amongst both clinicians and researchers presently[1].[2]A sea of microbes practically exists inside and on the surface of a single human being and is termed microbiota. Residing at many sites on or inside the human body, the microbes outnumber the human cell number by at least 10-folds. They probably count to around 10^{14} cells in all. The numbers of genes they harbour is even greater and estimated to be more than 100-fold of the human genes [1], [3]. The entire set of genome that exists in the microbiota is labelled as microbiome. The microbiome-host interactions shape a number of phenomenon that occur in human body. The mechanism of interaction between host and the gut microbiota is complex. It involves recognition of gut microbiota as non-pathogenic and a diminished immune response to the large number of microbes existing in the human gastrointestinal tract[4]. This complex interaction is mediated through recognition of bacterial antigens (labelled as Commensal associated molecular patterns or CAMPs) by the host. These molecular patterns are conserved across species. The host side of interaction is done by the pattern recognition receptors (PRR) which primarily exist as the toll like receptors (TLRs) and nucteotide-oligomerisation domains (NOD)[4]. These interactions and their possible role in pathogenesis of many acute and chronic diseases has fuelled an interest in human microbiota [2]. The present review focuses on the current understanding of gut microbiota and their role in causation of non-alcoholic steato-hepatitis (NASH).

The gut epithelium is unique as a single layer of cells separates a multitude of microbes from the human body. The constant interaction of the epithelium with the microbiota means that the epithelium must distinguish commensals from pathogens and have mechanisms to deal with the huge antigenic load it encounters. The armamentarium available to tackle this massive antigenic load is diverse. The human gut epithelium has a remarkable ability to produce a controlled inflammatory response to the antigenic loads [4]. The exposure to the commensals has an important role in modulation and maturation of the gut immune system. The bacterial sampling and recognition is mediated by interaction of pathogen associated molecular patterns (PAMPs) present on the commensal bacteria and the pattern recognition receptors (PRRs) present on the epithelium of the gastrointestinal tract. The PAMPs are conserved molecular patterns which are found on the microbes (pathogens and commensals alike). The PRRs are the corresponding proteins present in the host

epithelium (either the cell membrane or the cytoplasm) which recognise the PAMPs. The role of PRRs is played by the toll-like receptors (TLRs) and the nucleotide oligomerisation domains (NOD)[4, 7, 8]. The PAMP-PRR interaction modulates the further inflammatory cascade resulting in production of various pro-inflammatory signals. If this process is unregulated the result will be a massive immune response which will be detrimental to the host. A number of mechanisms contribute in preventing an over-activation of inflammatory response. The end result is a contained physiologic inflammation in response to the commensal microbiota[3, 4, 9]. For instance the gram negative bacterial endotoxin, the lipopolysaccharide (LPS), is recognised by the TLR4 present in the epithelium. However for this interaction to proceed other co-receptor molecules like the MD-2 (v-myb regulated gene 2) and CD-14 are required. The availability TLRs and their co-receptors, the localisation of TLRs (cell membrane or cytoplasm), expression of TLR signal suppressors like the Tollip protein and neutralisation of the antigens by the secretory IgA play an important role in limiting the immune response to the gut microbiota[3, 4].

The gut microbiota has many other important functions beyond therole in modulating immune response. These include maintenance of gut barrier integrity as also metabolic functions especially generation of short chain fatty acids from complex indigestible carbohydrates[2]. The mechanisms contributing to the barrier function include physical, chemical and immune components. The antimicrobial peptides (like the defensins, mucins and angiogenin 4) and the secretory IgA are important for the luminal chemical and immune mechanisms to maintain the gut barrier function [10]. Transcellular transport of antigens and toxins is prevented by an efficient endosomal system which excludes these antigens[11]. Similarly the paracellular transport of antigens is restricted by the presence of tight junctions and zonula occludens[12]. However, this barrier is disrupted in stressful situations during pathogen-enterocyte interaction, inflammation and certain drugs etc[13-17]. The disruption of this barrier provides an opportunity for the hitherto excluded antigens and LPS to enter the enterocytes into the systemic circulation[10]. This condition is the 'leaky gut' and may result in 'metabolic endotoxemia'.

The gut microbiota also contributes to the human health by virtue of the metabolic functions performed by it. This involves bacterial bile acid biotransformation, breakdown of oxalate, prodrug activation, vitamin production (including Vitamin K, folate, B_{12} and biotin) and polysaccharide degradation by colonic bacteria resulting in production of short-chain fatty acids (SCFA)[18]. The short chain fatty acids are produced by fermentation of non-digestible carbohydrates mediated by colonic bacteria. This generates various SCFA including acetate, proprionate, and butyrate. While acetate is the dominant SCFA, butyrate is the primary source of energy for the colonocytes[19]. Butyrate seemingly also plays a role in regulating colonic cell proliferation as also maintaining barrier function[18, 20]. The SCFA are an important source of energy for the coloncytes and assume immense importance in certain situations like the short bowel syndrome [21]. All in all the SCFA produced may play a role in reducing the risk of colon cancer, inflammatory bowel disease and reducing infection by pathogenic bacteria[22]. They may also have a role in reducing ammonia absorption from the gut by virtue of ensuring an acidic pH in the colon [22]. The gut microbiota in causation of NASH has a role.

II. Microbiota

(a) Microbiota in Obesity and Non-alcoholic Steatohepatitis (NASH)

Recent years have seen a tremendous increase in the prevalence of obesity. The causation of obesity is multifactorial. The global pandemic of obesity is primarily driven by increases in food supply [23]. This increase in obesity appears to have fuelled the increase in occurrence of metabolic syndrome and its hepatic component-NASH [24]. Gut microbes have been implicated in genesis of obesity. Germfree animals have a lower body fat than those who have normal microflora in their gut. Even more convincing evidence came from the gain in weight which was noted once germfree animals received microbiota from the genetically obese mice [25]. The microbial patterns of lean humans are different from obese individuals- the latter have lesser Bacteroides and a higher Firmicutes counts[26]. Others have failed to confirm this [27] .Weight losing diets have been shown to cause favourable changes in gut microbiome [26].

Colonic fermentation of polysaccharides resulting in SCFA production is believed to supply upto 10% of human body energy needs. This is inspite of the fact that the energy available per gram of glucose from SCFA (1.5 kcal) is less than that from small intestinal hydrolysis of digestible polysaccharides (3.9 kcal)[28, 29]. This colonic microbial fermentation and SCFA production may be more efficient in obese vis-à-vis the lean individuals. The difference in the microbial makeup may influence the energy extraction efficiency [30]. The methanogens have also been shown to possibly increase the efficiency of energy extraction [31] . This may be due to the variation in the type of microbial organisms in the colon of the obese individuals and hence a variation in the type of SCFA which is produced[27]. This has often been termed as 'energy harvesting'[29] .Energy harvesting can increase glycaemia and insulinemia thereby causing an enhanced tendency to lipogenesis[32]. Other possible mechanism is by creation of a leaky gut. As previously mentioned the gut

epithelium has an important barrier function which excludes many bacterial antigens including LPS from entering the systemic circulation. Changes in the gut microbial community can alter the gut physiology, disturbing the barrier function and result in 'metabolic endotoxemia' causing increased lipogenesis[29]. LPS has been implicated as a key molecule responsible for inflammation, obesity and diabetes mellitus linked to high fat diet [32]. It has been shown in a mice model that mice fed on high fat diet had elevation in serum LPS levels [33]. Further, these perturbations including endotoxemia and metabolic dysfunction were abolished by treatment with antibiotics suggesting the contribution of gut microbiota for development of NASH [34]. Use of prebiotic preparation to increase the representation of Bifidobacterium spp in gut microbiota also abolished the metabolic endotoxemia related to high fat diet [35]. Also the fact that diet influences the gut microbiota is established and the genesis of obesity may be routed through diet induced changes in the gut microbiota. Others have implicated the microbiota and changes in its composition in modulating the satiety. It is hypothesised that the physical and psychological stressors change the microbial composition in the gut and also modulate secretion of various satietogenic molecules [30]. It is now believed that the gut-brain axis is a two-directional talk. The central nervous system influences the gut microbiome by the release of various signalling molecules which modulate gastrointestinal motility [36]. Use of oligofructose in Wistar rats resulted in an increase in levels of anorexogenic peptides (GLP-I, PYY) in portal blood and reduced levels of orexigenic ghrelin [37, 38].Stress can increase intestinal permeability thereby allowing antigen-epithelium interaction, activation of immune response and changing the microbial environment in the gut [39]. All these mechanisms interact to result in genesis of obesity. However the complete role of each of these factors and their relative contribution in causation of obesity is under evaluation and the final word is not yet out.

(b) Choline deficient diets and microbiota

Choline is the source of methyl groups to the major methyl donor in human body, S-adenosyl-methionine (SAM). Endogenous production cannot meet the entire human needs for choline. Deficiency of choline has been implicated to result in causation of hepatic steatosis. It is also implicated in pathogenesis of total parenteral nutrition related steatosis [44]. Interestingly in a study involving 15 female subjects humans fed choline-deficient diet showed a change in the gut microbiota with change in dietary choline levels [45]. The changes were especially noted for the Gammaproteobacteria. Whether fatty liver occurs in patients with choline deficiency secondary to microbial changes in the gut is a matter of speculation. Interestingly when mice were fed high fat diet, the microbial changes in the gut microbiota were similar: an increase in levels of Firmicutes and Proteobacteria with a fall in Bacteroides level was noted [46]. It has been reported that gut microbes are involved in metabolism of choline into methylamine leading to choline deficiency and thereby may have a role in genesis of NAFLD. This mimics the state of a choline-deficient dietary state[47]. All in all it is possible that the disruptions in choline metabolism which may play a role in NAFLD-genesis may be routed through the gut microbiota.

(c) Role of diet induced microbiota changes

Both animal and human studies have shown evidence of the role of microbiota in extraction of energy and thereby a role in causing various manifestations of metabolic syndrome [48]. This is mediated by various factors including higher glycemia and insulinemia (resulting in increased hepatic lipogenesis), increased TLR4/LPS interaction and effect on hepatic fibrogenesis and increase in lipoprotein lipase activity (LPL) mediated by suppression of FIAF (Fasting induced adipocity factor). Elevated LPL levels mediate an increase in hepatic fat content by free fatty acids from triglyceride rich lipoproteins in plasma [49]. While we have already discussed how high fat diet might affect microbial organisation and changes in body fat as also the role of microbiota of gut in genesis of obesity, it is now apparent that the gut microbiota may contribute to low grade chronic inflammation related to obesity. This link between the microbiota and to the chronic low grade inflammation in NAFLD and other manifestations of metabolic syndrome is mediated via the bacterial cell wall lipopolysaccharidase[32]. When mice with CD14 knockout phenotype were challenged with high fat diet they failed to hepatic steatosis proving beyond reasonable doubt the role of LPS in mediation of hepatic steatosis which results in mice fed with high-fat diet [33]. Of interest is the possible role of the LPS/TLR4 interaction in determining the histologic severity of NAFLD. In a comparison of human NAFLD patients with controls it was noted that the plasma endotoxin levels and hepatic TLR4 mRNA levels were significantly higher in patients with NAFLD [43]. Furthermore a study which assessed the gut leakiness in patients with NAFLD using aspirin indicated an increased susceptibility to increased gut permeability in NAFLD patients [50]. A study which evaluated for levels of endotoxemia using the LPS binding protein (LBP) in obese patients concluded that the levels of endotoxemia had a correlation with severity of NAFLD: the patients with steatohepatitis had higher endotoxemia vis-à-vis the patients with simple steatosis. The increases in endotoxemia correlated with the rise in the expression of TNF-α in the liver [51].Recent evidence has indicated that the progression of NAFLD is mediated via inflammasomes. Inflammasomes are protein complexes present in cell cytoplasm composed of

one of several NLR proteins and sense endogenous or exogenous pathogen-associated molecular patterns (PAMPs). A study indicates that the mediation of metabolic effects of microbiota on liver involves interactes of the TLRs and NLRs (Nod-like receptor protein). Deficiency of inflammasomes results in changes in gut microbiota which might mediate progression of NAFLD. This occurs via increased influx of TLR agonists in situations of inflammasome deficiency[52]. Therefore evidence suggests that 'metabolic endotoxemia' not only mediates the pathogenesis of NAFLD but may also determine its severity. A study in mice provided evidence that TLR4 activation can mediate hepatic fibrogenesis through activation of hepatic stellate cells. TLR4 activation down regulates the transforming growth factor (TGF)-beta pseudo-receptor Bambi and eventually results in sensitisation of stellate cells to TGF-beta [53].

III. NAFLD

The causation of NASH, like obesity, is believed to be multifactorial [40]. The reasons include, other than an increased recognition, a change in lifestyle resulting in a global epidemic of obesity[41].Gut microbiota may cause NAFLD by luminal ethanol production resulting a leaky gut causing metabolic endotoxemia or by excess metabolism of choline leading to choline deficiency in liver [42].Overall NASH is recognised as the hepatic component of the metabolic syndrome resulting from an interaction of multiple factors including genes and their interaction with environmental factors[24].An increase in fat consumption has been known to cause increased hepatic fat. An increase in fructose consumption is also being implicated in causation of non-alcoholic steatosis [43]. Furthermore there is growing evidence which points to the gut microbiota in causation of fatty liver [41].

(a) SIBO in NAFLD

The role of small intestinal bacterial overgrowth (SIBO) in possible NAFLD-genesis has also been evaluated. Small bacterial overgrowth may also play a role in causation of NAFLD [48]. The histology of NAFLD is similar to the alcohol related liver disease and has led to speculation that the ethanol produced by the gut microbes may have a role in genesis of liver injury[54]. Another study noted a higher prevalence of SIBO as also increased intestinal permeability in patients with NAFLD when compared with healthy controls [55]. SIBO is more common in obese individuals and correlates with severe hepatic steatosis [56]. The evidence therefore points to an association between NAFLD and SIBO. The increased bacterial load in the gut of obese might, through an increased intestinal permeability, result in elevated endotoxin levels and result in hepatic steatosis[49]. Increased intake of fructose has been implicated in NAFLD causation. Fructose intake has been associated with leaky gut and increased endotoxin levels. Fructose-fed mice were noted to have an increase in portal endotoxin levels and a decrease in tight junction occluding in duodenum [57]. Also fructose seems to induce several TLR dependent pathways resulting in an increase in translocation of microbial components across the intestine and thereby mediating hepatic steatosis [58]. Hence the hepatic steatogenesis resulting from increased fructose intake might also be routed through the gut microbiota.

(b) Probiotics in NAFLD

In view of the strong evidence implicating microbiota in causation of NAFLD the obvious implication is whether modulation of microbial environment may be used in halting the progression of NAFLD or to treat it. Probiotic use has been attempted to improve NAFLD. Probiotics may have a role by modulation of the composition of gut microbiota, reducing intestinal permeability, suppressing inflammation and reducing endotoxemia [59]. VSL#3, a probiotic strain with seven different strains of bacteria, has been used in NAFLD bit the results are unconvincing. A study which evaluated the role of VSL#3 in improving the pro-inflammatory cytokine profile in NAFLD found that the levels of malondialdehyde and 4-hydroxynonenal improved. However, the clinical implication of this finding remains uncertain [60]. In a choline-deficient mouse model of NASH the use of VSL#3 did not decrease steatosis or inflammation but seemed to reduce fibrosis probably by increasing the expression of liver peroxisome proliferator-activated receptors and decreased expression of procollagen and matrix metalloproteinases[61]. Another mice study indicated some benefical role by way of modulation of nuclear factor κ B pathway [62]. However in a human study which evaluated the role of VSL#3 administration on patients with NAFLD the results were disappointing as all four patients had evidence of increased hepatic fat on magnetic resonance spectroscopy [63]. Also a Cochrane review done in 2007 found insufficient availability of randomised trials to recommend for or against the use of probiotic therapy in NAFLD [64]. Remarkably a recent study which evaluated the effect of administration of Lactobacillus bulgaricus and Streptococcus thermophiles in NAFLD concluded that this probiotic improved transaminases in the treatment arm vis-à-vis the placebo[65]. This might suggest that the type of probiotic strain which is utilised might affect the possible outcome. Similar results came from a paediatric study wherein the role of Lactobacillus rhamnosus GG was evaluated in obese patients with liver disease. The transaminase levels reduced while there was no changes in TNF-α levels and ultrasound determined hepatic brightness [66]. Indeed the effects of probiotics in

NAFLD might depend on the strain used and it will be inappropriate to club all probiotics as a single pharmaceutical agent. While probiotic are live organisms usually utilised in form of spores, prebiotic are indigestible polysaccharides which are metabolised by colonic bacteria and may stimulate activity of beneficial bacteria[67]. In a study evaluating role of prebiotic oligofructose supplementation (OFS) in high fat diet fed mice found a reduction in endotoxemia and pro-inflammatory cytokines. This also correlated with an OFS related increase in Bifidobacterium levels in the gut microbiota [35]. However direct evaluation of prebiotics in amelioration of NAFLD are not available. An animal study demonstrated lowering of serum cholesterol and inhibition of triglyceride levels in liver after prebiotic supplementation [68]. A study using Bifidobacterium longum and OFS combined showed reduction in liver enzyme AST and ALT in both NAFLD study group as well in control group[69]. A systemic review on randomised clinical trials (RCTs) testing probiotics, prebiotics or both in treatment of NAFLD concluded that available evidence precludes recommendations on use of pre and probiotics in NAFLD cases[70].

To summarise the increased/altered microbial load in the gut so alters the metabolic functions vis-à-vis the polysaccharide metabolism as also the intestinal permeability as to create a situation akin to metabolic endotoxemia and thereby enforces a state of chronic low grade inflammation eventually causing metabolic syndrome and its hepatic manifestation in form of non-alcoholic steatosis. The possible role of microbial changes in gut in NAFLD may provide opportunities for therapeutic intervention by modulation of gut microbiota.

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