FACULDADE DE MEDICINA
UNIVERSIDADE DO PORTO

MESTRADO INTEGRADO EM MEDICINA

2014/2015

José Pedro Portela Cidade Silva

Systematic review of the relation between intestinal microbiota and innate immunity in the Metabolic Syndrome: What do we know so far?

março, 2015

FACULDADE DE MEDICINA
UNIVERSIDADE DO PORTO

José Pedro Portela Cidade Silva

Systematic review of the relation between intestinal microbiota and innate immunity in the Metabolic Syndrome: What do we know so far?

Mestrado Integrado em Medicina

Área: Gastrenterologia

Tipologia:Monografia

Trabalho efetuado sob a Orientação de:

Professor Doutor Pedro Pimentel Nunes

E sob a Coorientação de:

Professor Doutor Adelino Leite Moreira

Trabalho organizado de acordo com as normas da revista:

Gut and Liver

março, 2015

U.PORTO
FMUP FACULDADE DE MEDICINA UNIVERSIDADE DO PORTO
UNIDADE CURRICULAR PROJETO DE OPÇÃO DISSERTAÇÃO / MONOGRAFIA

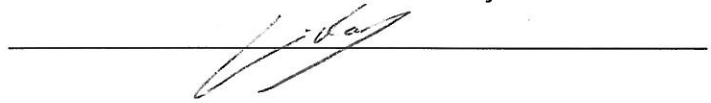
Projeto de Opção do 6º ano - DECLARAÇÃO DE INTEGRIDADE

Eu, _____José Pedro Portela Cidade Silva_____, abaixo assinado, nº mecanográfico ___200907044____, estudante do 6º ano do Ciclo de Estudos Integrado em Medicina, na Faculdade de Medicina da Universidade do Porto, declaro ter atuado com absoluta integridade na elaboração deste projeto de opção.

Neste sentido, confirmo que **NÃO** incorri em plágio (ato pelo qual um indivíduo, mesmo por omissão, assume a autoria de um determinado trabalho intelectual, ou partes dele). Mais declaro que todas as frases que retirei de trabalhos anteriores pertencentes a outros autores, foram referenciadas, ou redigidas com novas palavras, tendo colocado, neste caso, a citação da fonte bibliográfica.

Faculdade de Medicina da Universidade do Porto, _13_/_03_/__2015___

Assinatura conforme cartão de identificação:



U.PORTO
FMUP FACULDADE DE MEDICINA UNIVERSIDADE DO PORTO
UNIDADE CURRICULAR PROJETO DE OPÇÃO DISSERTAÇÃO / MONOGRAFIA

Projecto de Opção do 6º ano – DECLARAÇÃO DE REPRODUÇÃO

NOME

José Pedro Portela Cidade Silva

CARTÃO DE CIDADÃO OU PASSAPORTE (se estrangeiro)	E-MAIL	TELEFONE OU TELEMÓVEL
14005559	mimed09078@med.up.pt	917147616

NÚMERO DE ESTUDANTE	DATA DE CONCLUSÃO
200907044	Julho de 2015

DESIGNAÇÃO DA ÁREA DO PROJECTO

Gastroenterologia

TÍTULO ~~DISSERTAÇÃO~~/MONOGRAFIA (riscar o que não interessa)

SYSTEMATIC REVIEW OF THE RELATION BETWEEN INTESTINAL MICROBIOTA AND INNATE IMMUNITY IN THE METABOLIC SYNDROME:

WHAT DO WE KNOW SO FAR?

ORIENTADOR

Professor Doutor Pedro Pimentel Nunes

COORIENTADOR (se aplicável)

Professor Doutor Adelino Leite Moreira

É autorizada a reprodução integral desta ~~Dissertação~~/Monografia (riscar o que não interessa) para efeitos de investigação e de divulgação pedagógica, em programas e projectos coordenados pela FMUP.

Faculdade de Medicina da Universidade do Porto, _13_/_03_/_2015_

Assinatura conforme cartão de identificação: _____

Dedicatória

Quero dedicar a minha tese de Mestrado a todos os meus familiares, em especial à minha mãe, ao meu pai, e ao meu irmão, como fruto de uma longa jornada médica que percorremos juntos.

SYSTEMATIC REVIEW OF THE RELATION BETWEEN INTESTINAL MICROBIOTA AND INNATE IMMUNITY IN THE METABOLIC SYNDROME: WHAT DO WE KNOW SO FAR?

Running title: Intestinal Microbiota, Innate immunity and Metabolic Syndrome

José Pedro Portela-Cidade (1), Marta Borges-Canha (1), Adelino F. Leite-Moreira (1), Pedro Pimentel-Nunes (1,2,3).

1. Department of Physiology and Cardiothoracic Surgery, Cardiovascular Research & Development Unit, Faculty of Medicine, University of Porto, PORTUGAL.

2. Gastroenterology Department, Portuguese Oncology Institute, Porto, PORTUGAL.

3. CINTESIS / Department of Biostatistics and Medical Informatics, Porto Faculty of Medicine, Porto, PORTUGAL.

Correspondence address:

José Pedro-Cidade

Al. Prof. Hernâni Monteiro, 4200-319, Porto – PORTUGAL

Tel: + 351 918935390

Email: zencidade@gmail.com

Abstract

Backgound/Aims: Metabolic Syndrome is an emerging disease in developed countries and presents itself as a potential threat worldwide. The role of Diabetes, Dyslipidemia and Hepatic steatosis as pivotal participants in Metabolic Syndrome pathogenesis is well known. However, their common persistent chronic inflammation and its potential cause still elude. This systematic review aims to present evidence of the mechanisms that link the intestinal microbioma, innate immunity and Metabolic Syndrome.

Methods: A comprehensive research was made using pubmed database and 35 articles were selected.

Results: We found that metabolic syndrome is associated to increased levels of innate immunity receptors, namely, toll-like receptors, both at intestine and systemically and its polymorphisms may change the risk of metabolic syndrome development. Microbioma dysbiosis is also present in metabolic syndrome, with lower prevalence of *Bacteroidetes* and increased prevalence of *Firmicutes* populations. The data suggests that the link between Intestinal microbiota and Toll-like receptors can negatively endanger the metabolic homeostasis.

Conclusion: Current evidence suggests that innate immunity and intestinal microbiota may be the hidden link in the Metabolic Syndrome development mechanisms. In the near future, this can be the key in the development of new prophylactic and therapeutic strategies to treat Metabolic Syndrome patients.

Word count: 197

Key words: Innate immunity, intestinal microbiota and Metabolic Syndrome

Introduction

The Metabolic Syndrome presents itself as one as the principal chronic diseases of the developed countries and an important determinant of cardiovascular and metabolic mortality risk[1]. It is defined as a persistent pro-inflammatory state in which abnormal metabolic and physiological factors produce an increased risk of developing diabetes, obesity, dyslipidaemia and other cardiovascular risk factors[1-3]. Recent data reports a consistent activation of the innate immunity through the toll-like receptors (TLR) and its downstream signalling, suggesting not only a potential causative way, but also a possible perpetuator of its chronic immune stress to the organism[4,5]. On the other hand, new insights have revealed a pivotal role of the intestinal microbiota and its interaction with the host genetics, in the development of obesity and insulin resistance [6-8]. It has been also described the role of intestinal microbiota, its migration and its metabolic products systemic effects, in the activation of these TLR receptors in several organs, especially the Liver[9,10].

However, in spite of this new data, the relationship, causality and the mechanisms by which the intestinal microbiota can influence the expression of several immune receptors including TLR still eludes. Also, it is poorly understood the relationship between differential expression of TLR and the lesion of several organs presented in Metabolic Syndrome.

This systematic review aims to access the most recent data about the relevance of intestinal microbiota and TLR expression in the development of Hepatic lesion and Metabolic Syndrome.

Methods

A comprehensive search was performed in Pubmed and the following queries were used: [("metabolic syndrome"[All Fields] AND ("microbiome"[All Fields] OR "microbiota"[All Fields])) OR ("metabolic syndrome"[All Fields] AND ("toll-like receptors"[All Fields] OR "TLRs"[All Fields]) AND ("microbiome"[All Fields] OR "microbiota"[All Fields]))], and [(gut microbiota[Title] OR microbiota[Title]) AND (((TLR[Title]) OR Toll like receptor[Title]) OR Innate immunity[Title])]

From the search and after duplicates were removed, 230 studies were retrieved (Figure 1). Using inclusion and exclusion criteria, 35 articles were selected, analysed and included in this revision (Table 1).

The principal summary measures in an outcome level were different risk ratios of developing obesity, diabetes, dyslipidaemia or Metabolic Syndrome in every experimental conditions used, difference in means of RNA or biochemical expression levels of markers studied and differences on histological or phonotypical assessment of major organs studied in a variety of experimental conditions. Subgroup analyses within the selected studies were also considered and taken in account.

TLR expression and Metabolic Syndrome: the importance of the innate immunity

It is well known that the TLRs comprehend an extended family of pathogen-associated molecular patterns recognition (PAMP) receptors of the innate immunity. These receptors allow the prompt activation of downstream signalling of MyD88-dependent and MyD88-independent pathways in response to several antigens, mainly of Gram-positive and Gram-negative bacteria, and they are all linked to production of cytokines and enhancement of the inflammation.

Since it is established that subclinical inflammatory processes are major contributors to Metabolic Syndrome, obesity and diabetes, several lines of investigation were made to confirm the role of innate immunity receptors and prompt activation in these metabolic disturbances pathogenesis [11].

An initial study shows that there was a significant increase in both TLR2 and TLR4 cell surface expression and mRNA levels in monocytes of Metabolic Syndrome patients. This phenomenon persisted even when statistically adjusted its values to waist circumference and body mass index. This study also reveals that this TLR increased activity was significantly correlated with significant higher blood pressure and plasma glucose levels, and with Interleukin-1b (IL-1b), Monocyte Chemoattractant Protein-1 (MCP-1) and Necrosis Factor-kB (NF-kB) activity, concluding that the activation of the TLR can count as an independent factor not only to Metabolic Syndrome but also to the cardiovascular risk of these patients [12].

The impact of TLRs is also verified in other clinical situations that are strongly related to Metabolic Syndrome and that can explain its participation as a causative factor through indirect pathways.

On one hand, Cristina Cuda *et al* showed that different polymorphisms of TLR4, with different activation rates of this receptor, were statistically associated to different serum insulin levels and insulin sensitivities. The individuals with TLR4 with 299Gly allele were found to have higher levels of insulin and lower levels of insulin sensivity in comparison to the considered normal Asp homozygotes. They also found that a second polymorphism, an intronic SNP (rs5030728) can even modulate the relationship between dietary Saturated Fatty Acids and HDL cholesterol, showing that an increased intake of Saturated Fatty Acids was inversely related to significant lower levels of HDL cholesterol, in these individuals[13]. Steinhardt *et al*, on the other hand, strongly confirmed that another polymorphism of TLR4 is associated with syndromes of lipid accumulation[14], thereby sustaining that TLR could be involved in the regulation of several metabolically substrates and increase the risk of Dyslipidaemia, a known factor related to Metabolic Syndrome.

Another line of evidence using mice with +3725G/C polymorphism of TLR4, show that these mice have a functional decrease in their receptor activity to only 30% (P = 0.0001), and that they presented a statistically significant association with lower body mass indexes (25.5363.50 vs. 28.6064.62 kg/m2; age adjusted p = 0.023), waist circumferences ((89.27614.46 vs. 97.51612.59 cm; age adjusted p =0.025), and with higher adiponectine levels (14,5 ng/mL vs 10,6 ng/mL; p=0,021) [15]. These results may denote a positive relation between a prompt activation of the innate immunity and obesity, a known factor present in Metabolic Syndrome patients. These remarkable

results, however, were not so strongly verified in the smoking population, and the authors point this factor as a potential confounding variable that must be considered on future studies.

A strong association has been also described between the expression and activity of TLRs and the development of Type 1 and type 2 Diabetes, major factors in the Metabolic Syndrome pathogenesis[16].

A recent study described that Tlr2−/− genotype mice after twelve weeks of high fat diet, not only had more glucose tolerance and insulin sensivity than the control group, but also presented reduced levels of leptin, MCP-1 and Tumor Necrosis Factor-α (TNF-α) levels. Interestingly, insulin sensivity in TLR2 knockout mice were conserved in the liver causing a significant statistical reduction of hepatic glucose production and lower risk of developing hepatosteatosis under high-fat diet [17]. This data was consistent with L.-H. Kuo *et al* study that found that TLR2 knockout mice presented lower glucose and insulin levels than their wild counterpart, revealing an important role of this receptor in glucose homeostasis. This same study also stated that mice that knockout to TLR2 were associated with a phenotype of decreased body weight, fat mass, lower number (1.21×107 in Tlr2−/− vs 2.62×107 in WT) and smaller adipocyte cells (mean area821 μm2 in Tlr2−/− vs 1503 μm2 in WT) [18].

A meta-analysis using studies that linked Type 2 Diabetes, Metabolic Syndrome and D299G polymorphism of TLR4 also showed that this miss-sense variant was responsible for a lower response of this receptor to its ligand, Lipopolysaccharide, probably through a deficient recruitment of its down-signalling adapters MyD88 and TIR-domain-containing adapter-inducing interferon-β (TRIF) [19]. The study concluded

that the D299G-variant was, in the Caucasian subgroup, associated to a reduced risk of Diabetes type 2 and metabolic syndrome. However the results were not reproducible in other subgroups and the authors stated that it could be explained by the differences in the allele frequency among different populations [16].

On the other hand, Li Wen's *et al* study detected that in spite of the strong relevance of environmental stimuli, in specific-pathogen free NOD mice that lacked MyD88 protein, the rate of type 1 Diabetes was lower when compared to its wild counterpart suggesting that signalling through this receptor should be a strong participant in the immune response that causes type 1 Diabetes [20]. However, these data was not verified when individual KO-specific-TLR2 and 4 mice were used showing that these specific receptors were probably not necessary and that other innate immunity or TLR receptors using MyD88 intracellular signals might be responsible by the observed statistically significant lower responses to a diabetes-associated peptides recognized by specific CD8+ T cells, in type I Diabetes[20]. Interestingly though, this reduced risk observed in Myd88-negative mice was dependent on the presence of "beneficial" commensal microbes, since the use of antibiotics that produced germ-free MyD88-negative mice were linked to an increase in the risk of Diabetes. In reverse, the use of controlled intestinal colonization with specific "beneficial" bacterial populations, after the antibiotic treatment, could again attenuate this risk [20].

Microbiota and Metabolic Syndrome: a new link

Recently, the intestinal microbiota has been progressively studied and knowledge of its profound influence in maintaining the human physiology and nutrition

has astonishingly increased [21]. Using recent methods of pyrosequencing, the human gut microbial population has been described as the one with the highest in what concerns to density and variability of organisms, comprehending more than 5000 bacterial taxa and composed manly by *Bacteroidetes* and *Firmicutes* among other phyla less prevalent such as *Actinobacteria, Proteobacteria, Fusobacteria, Deferribacteres* and *Deinococcus* [22]. Although the preponderant presence of these microorganisms in our gut, their impact in the human homeostasis is still not fully known and its relationships with several chronic diseases, especially with the Metabolic Syndrome, has open new lines of pathological evidence and potential therapeutic targets.

In fact, it has become apparent that the microbiota can change metabolically the way we acquire nutrients or our ability to harvest energy through several mechanisms [23] . The preponderance of these bacteria in the catalytic processing of carbohydrates in the human digestion or even the precise location where these metabolic reactions occur can influence their availability to the human organism [23]. The bacteria are also associated to functions such as stabilizing tight junctions and promoting the secretion of antiinflamatory cytokines [23]. In the lipid metabolism, several types of conjugated and free fatty acids are generated by the intestinal microbiota and play important roles in stimulating paracrine and endocrine peptides such as Glucagon-like Peptide (GLP) and peptide YY, modulating the intestine function[24] . These facts point to influences to different energetic inputs and nutrients availability and therefore to an important contribution to metabolic disturbances similar to those described in obesity and diabetes.

Furthermore, it has become apparent that in spite of its location in the human intestine, the microbiota plays important systemic changes that are directly linked to the low-grade chronic inflammatory state, hugely present in the Metabolic Syndrome.

On one hand, the characterization of the intestinal bacteria in obese model mice (ob/ob) was consistent with 50% reduction of *Bacteroidetes* abundance and a reverse increase of *Firmicutes* when compared to lean mice [25]. This unbalanced microbiota was found to be modulated by the composition of the diet, manly rich in fatty acids, independently of previous obesity or obese stat, since resistant models to high-fat induced obesity mice did experiment the same microbiome changes that did its wild counterpart [25]. Moreover, it was found that a high-fat diet simulated the infusion of bacterial LPS defining a phenotype with development of obesity, diabetes and inflammatory cells infiltration in the adipose tissue. It was also found that the gut bacteria were able to induce the suppression of Fasting-induced adipocyte factor, thereby limiting the inhibition of Lipoprotein lipase in the adipocytes and promoting the body fat accumulation [26].

Analogous results were obtained by M. Remely *et al* study, since higher prevalence of *Firmicutes* and lactic bacteria were encountered in type 2 diabetic mice in opposition to *Bacteroidetes* and *F. prausnitzii*. The authors also tested the influence of these population changes in the methylation of exons of TLR2 and 4 in the intestinal epithelium and described significant statistical lower methylations rates for TLR2 in diabetic mice and lower methylation rates for TL4 in obese mice, suggesting that there are important epigenetic modulations by the intestinal bacteria and that those modulations could probably incur in exacerbated risks of Diabetes and obesity [27].

These results support the previous data from C. Zhang *et al* study that showed reduced levels of gut-protecting *Bifidobacteria*, a phylum associated to resistance to the effects of LPS from the gut microbiota, in mice with a high-calorie diet. The authors suggest that through the modification of intestinal microbiota to a "pathogenic-like" microbiome, a high-calorie diet can be responsible for a loss of integrity in the barrier function of the intestine, the systemic spread of bacterial endotoxins and pro-inflammatory state. In fact the study points that a high inflammatory state was present in all animals with impaired glucose tolerance and increased body fat, on high-fat diet. The authors also revealed a predominance of *Desulfovibrionaceae*, sulphate-reducing bacteria, in high-calorie diet mice, a fact that could be related to the development of Metabolic Syndrome in these mice, since the disruptive properties of the sulphate in the intestinal barrier were present. Finally, the authors described that this transformation to a "pathogen-like population" through diet, seem to play a relevant role in developing Metabolic Syndrome that is, apparently, also independent of the host genetics and genomic expression [28] .

Backhed F *et al* aimed to test the importance of microbiota during Infancy in the development of obesity later in life. This study showed that breastfeeding seems responsible for the colonization of the intestine mainly by *Bifidobacteria* and for a decrease in the *Enterobacteriaceae* family members. The variations to this intestinal microbiome ecosystem, with reduced levels of protective strains such as the *Bifidobacteria*, that are observed in 2 year old infants, had a profound impact on a regulator of the lipoprotein lipase called Angpt14/Fiaf, which seems to act as an inhibitor of the enzyme activity and responsible for an increase in the body fat

accumulation. This was observed in studies using mice models, which showed an increase in body fat tissue in germ-free Angpt14/Fiaf-deficient mice, and in non-germ free mice, with a functional Angpt14/Fiaf. Also, antibiotic use in infants can cause the elimination of *Bacteroids* and a reduction in *Bifidobacterium* population levels, thereby reducing two important antiobesogenic families in the intestinal microbiota [23].

TLR, Mycrobiota and Metabolic Syndrome: a global vision

The activation of innate immunity through the TLR cannot be dissociated from the intestinal microbiota and its mutual crosstalk playing a decisive factor in the development of Metabolic Syndrome.

In fact, the modulation and interaction between microbiota and TLR activation was investigated in a TLR2 Knockout mice study that revealed that, in spite of the genetic predetermination to insulin sensivity by these mice reported in studies before [17,18], this phenotype could be drastically reversed by gut microbiota. The study confirmed that these TLR2 knockout mice, in non-germ free facilities, had higher levels of Firmicutes and Bacteroidetes in comparison to wild-type mice, and increased lipopolysaccharides absorption, glucose intolerance, insulin resistance and obesity. The authors stated that these results can be explained by an increase activation of TLR4 receptors in the absence of TLR2 activation. They also verified increased endoplasmatic reticulum stress and JNK activation by the TLR4, which effects could be inhibited by a TLR4 antisense oligonucleotide causing an increase in glucose tolerance and sensivity when compared to the controls. Furthermore, in TLR2 Knockout mice, the use of a

mixture of antibiotics resulted in an improvement of insulin sensivity and metabolic status [6].

Another recent study by C. Ubeda *et al* further revealed that using knock-out mice to a specific TLR, the impact of the absence of that certain TLR was initially minimal in the bacteria communities in the intestine, after a treatment with antibiotics, when compared to their respective wild-type mice, gathering evidence that some caution is advised when valorising microbial communities' differences in these groups. Interestingly, the authors also stated that differences in mice's colonies with different TLR deficiencies was only verified after long term breeding in isolation from each other, and probably with great influence from maternal transmission [29]. However, contrary to the previous study of Vijay-Kumar M et al that described a major link between TLR-5 KO mice and the development of Metabolic Syndrome [30], this study didn't find any difference in the intestinal microbiota colonies or in the risk of Metabolic Syndrome. The authors attribute these results to differential exposures in husbandry conditions and mice types [29].

To add to this complex network of relations between host genetic expression and the environment, R. Kellermayer *et al* study showed an increased risk of epigenetic modification, DNA methylation and differential rates of transcripts in TLR2-knockout mice in the intestinal mucosa epithelium. The authors pointed out that these epigenetics and metagenomic effects, along with the modulation of the bacterial populations could explain the loss of the protective barrier function of the intestine and changes in the microbiome environment observed in the Metabolic Syndrome [31].

As the importance of the intestinal microbiome in the Metabolic Syndrome becomes more apparent, new lines of investigation have appeared, aiming the bacterial populations as a therapeutic hypothesis in the Metabolic Syndrome.

A study by J. Wang *et al* used high fat diet mice in which was induced Metabolic Syndrome and subjected them to administration of one of three probiotics (*Lactobacillus paracasei, L. rhamnosus and Bifidobacterium animalis*) in order to modulate the gut microbiota. The results showed that these strains were able to reduce the weight gain, increase insulin sensivity and limit hepatic steatosis, and also that they were able to modulate innate immunity and the pro inflammatory state, through the reduced infiltration of macrophages in de adipose tissue. Moreover, the probiotic treatment was capable of reducing key phylotypes keen to cause Metabolic Syndrome (as are Desulfovibrionaceae and clostridium examples), and capable of reduce inflammatory processes associated to sulfate sodium-induced lesion by these bacteria. On the other hand, certain strains and also certain combinations of probiotic strains can probably provide different benefits in reversing the metabolic syndrome by a different impact on certain mechanisms, such as the increase of acetate, a known molecule shown to be able to improve obesity and diabetes on these mice [32].

Another study, performed in humans, reported that infusion of intestinal microbiota from lean donors to Metabolic Syndrome subjects was able to increase insulin sensivity (median rate of glucose disappearance changed from 26.2 to 45.3 mol/kg/min; $P < .05$), after 6 weeks. These changes were associated to an increase of butyrate-producing bacteria in the gut's recipients, which is known to influence directly the glucose metabolism [33]. Another study using probiotics rich in Lactobacillus, verified that those who received the probiotics had lower body mass index, waist and hip

measurements, and presented a reduction of fat in the abdomen and subcutaneous areas ($p<0.01$) [26].

A randomized pilot study by B leber *et al* tried to unravel the effect of a *Lactobacillus* probiotic strain in the gut permeability and bacterial endotoxin presence, in human Metabolic Syndrome patients, covering two phenomena attributed to the pathogenesis of the syndrome. However, in spite of the significant increase of the gut permeability in the Metabolic Syndrome group of patients, the investigators didn't find any difference of the endotoxin, C-reactive protein, neutrophil function and TLR expression levels between the group who had received the probiotic and the control group [34].

Liver as a pivotal organ in Metabolic Syndrome

The liver is a major organ on the human organism which has a profound impact in its metabolic and immune homeostasis [9]. Indeed, its' high efficient metabolic regulation of glucose, fatty acids and immune regulators and its privileged location as an organ that first receives blood from the gastrointestinal tract, and protects the remain circulation from its potential threats, make the liver a crucial participant in several regulatory processes. Also, it has been progressively verified that these same functions can be unbalanced in the Metabolic Syndrome. Usually, a prompt activation of several immune receptors on the hepatic immune system occurs, in response to a breakthrough of the epithelial barrier in the intestine, and the rise of certain bacterial populations and translocation [9] and this inflammatory state, therefore, can explain the development of

non-alcoholic fatty liver disease, promoting an increased risk to Metabolic Syndrome and several comorbidities such as Diabetes, Dyslipidemia and obesity [1].

K. Sawada *et al* aimed to measure the expression of TLRs, TNF-α, Interleukin-1B and phospho-interleukin-1 receptor-associated kinase 1, in the liver and small intestine, in non-alcoholic fatty liver disease induced mice. The authors reported a significant statistical increase in the inflammatory cytokines and TLR2, 4, 5 and 9 expressions in the liver of 16 weeks mice, but no differences in the liver of 4 and 8 weeks mice. The study also revealed that, in the intestine, it could be observed exactly the opposite, since all parameters were significantly decreased indicating that these pathways are probably inhibited in mice with NAFLD. Moreover, the treatment of these mice with antibiotics could attenuate the higher expression of those inflammatory markers and TLRs in the liver but it had little impact on the expression of these molecules in the small intestine of NAFLD mice [35].

The authors further stated that, the increase on the liver TLRs expression appears to be selective since TLR2, 4, 5 and 9 were upregulated in primary kupffer cells while only TLR4 and 9 were increasingly expressed in the primary hepatocytes [35].

Another recent study tested the effect TLR4 in NASH in high fat, high cholesterol diet mice. The results stated that, compared with wild-type, the TLR4 mutated mice were resistant to the development of macrovesicular and microvesicular steatosis, hepatocellular ballooning and a fivefold increase in NASH scores. This phenotype was even associated with decreased TLR4 function manly in kupffer cells, and consequential lower levels of macrophages and cytokines. Interestingly, the study also included the measurement of a transcription factor named XBP-1, known to be

stimulated by reactive oxygen species. This protein was abrogated in the TLR4 mutant mice, with a simultaneously decrease of NF-kB activation and cytokine production [36].

Also, TLR4 has been described as a signalling way to NAFLD development, in high fat diet mice, and linked to hepatic steatosis, hepatic insulin resistance and hepatic weight gain in these experimental circumstances. The same results were found through other members of TLR family such as TLR9 and TLR5 pathways [37]. Finally TLR2 was also studied and is now apparent that its ligands are increased in obese mice and that its blockage can restrain its insulin resistance induction. In fact, TLR2 Knouckout mice exhibit lower expression of TNF-α and IL-1β inflammatory cytokines [18].

The same rational was presented in other studies, where fructose-induced hepatic steatosis in mice was associated with significant induction of TLR1-4 and TLR6-8 along with an increase of MyD88, TNF- and iNOS levels. The authors point that bacterial components translocation and the prompt activation of TLRs and TLR-depend pathways support a chronic inflammatory state that could, in the liver, end with the development of non-alcoholic liver disease [30,34,38].

Further studying the preponderance of the reactive oxygen species in the NAFLD pathology, J. Henao-Mejia *et al* tested two inflammasome molecules and its effector protein in bacterial modulation and Metabolic Syndrome outcome. The study revealed that the progress from NAFLD to NASH was positively influenced by these molecules since knockout mice to inflammasome proteins (Nlrp3 and Nlrp6) were associated to lower effector protein (IL-18) levels, different gut microbiota and increased TLR4 and TLR9 agonists. These mice also had more hepatic steatosis, obesity

and glucose intolerance and higher rate of *Porphyromonadaceae* in the intestinal gut, a known bacterial family that increases the risk of Metabolic Syndrome in both mice and humans [39].

Considering its importance, a modulation by probiotics can emerge as a therapeutic target to this "metabolic infection". A recent study by S. Liang *et al* using probiotics, showed an improvement in the obesity, glucose tolerance and insulin levels in high fat diet fed mice. It also revealed that hepatic steatosis was reduced with significantly lower hepatic triglyceride content and improved histology, with the use of this probiotics [40].

Another study by Y.R. Xie *et al* used normal and liver cirrhosis mice that were subjected to liver transplantation. The authors found that not only the liver cirrhosis groups had higher endotoxin levels and higher numbers of total bacteria, but also, that the referred group had an increased number of bacteria in liver and lymph nodes. The liver cirrhosis groups had higher rates of *Enterobacteriaceae* and lower *Lactobacilli* and *Bacteroides* and, simultaneously, had higher MUC2, MUC3 and TLR2 and 4 mRNA expression levels in the intestine and TLR2 and 4 increased mRNA expression levels in the liver. Astonishingly, these parameters had not improved until 1 month after the liver transplantation and, surprisingly, had not improved with the use of a lactobacillus-enriched probiotic, suggesting probably the need of longer times of therapy or search for different probiotic compositions specific for each metabolic level of disturbance, either hepatic or systemic [41]. This rational may account for important medical decisions in the future and possibly allow different therapeutical options become medically used in clinical practice.

Discussion

For all the data stated, it is well apparent that the interaction between intestinal microbiota and the innate immunity might have a pivotal role in the Metabolic Syndrome, which impact could go way beyond the limits of the intestine and have a direct and systemic impact through several organs.

Based on the results analysed, we propose that the composition of the intestinal microbiome, changing from a "symbiotic" population manly composed by *Bifidobacteria* and *Bacteroidetes*, to a more "unbalanced" one, where *Firmicutes, Porphyromonadaceae* and *Desulfovibrionaceae* emerge, can be modulated and provoked by diet and its relative composition in lipids and other factors. This dysbiotic environment can promptly activate several immune receptors present in the intestinal barrier though the recognition of PAMPs and bacterial antigens, culminating in an increase activation of TLR2, 4, 5 and 9, and others synergic inflammatory signalling pathways that were not described yet. The local pro-inflammatory profile can explain the altered phenotypic expression of these receptors in the intestine surface, the metabolic absorption disturbances observed, the increase of cytokines and inflammatory markers levels and the loss of barrier function of the intestine, which are all described in Metabolic Syndrome models [42,43].

With this break on intestine mechanic defences, this dysbiotic bacteria and its products will have an easy access to the vascular space, raising the level of exogenous provocative antigens to the immune innate system and through its translocation influence the homeostasis of different organs, specially the liver because of its metabolic preponderance and privileged location[44] (Figure 2).

These mechanisms synergistically account for the increase activation of TLR2, 4, 5 and 9 in the kuppfer cells and the TLR 2, 4 and 9 in the hepatocytes and the consequential increase in the inflammatory products of its pathways such as TNF-α, IL-1β and iNOS. This chronic inflammatory state can justify the appearance of macrovesicular and microvesicular steatosis, and hepatocellular ballooning that counts for the NASH transformation[45]. The role and overexpression of several other molecules as xBP1 and NFKb can further explain this modification probably through a shift in the genetic expression and/or epigenetic modulation of the liver cells.

On the other hand, throughout the organism, the antigenic activation of TLR2 and TLR4 in various cells can be a major way of explaining the glucose intolerance, insulin resistance and the increased risk of Diabetes, co-morbidity associated to this syndrome. Furthermore, the MyD88 pathway activated by these receptors seems to be preponderant to this outcome, since the absence of its signalling participants or TRIF could account for a reduced risk of developing Diabetes[5].

The innate immunity activation can even influence the milieu of the adipose tissue, being responsible for a pro-inflammatory state with increased activation of TLR4, the recruitment of different inflammatory cells and the production of inflammatory molecules. This can be the cause of the decreased adiponectine levels expression, the increased lipoprotein lipase functions and the explanation of the dyslipidaemia and obesity present in these patients [46] (Figure 3).

Furthermore, the chronic inflammatory state is known to disturb the balance of coagulation/anticoagulation factors and the metabolic production of oxidative stress throughout the organism. These factors can further influence the regulation of delivering

nutrients and energetic substrates to the cells and its internal management, causing a most profound impact in the homeostatic equilibrium of the human body[47].

These new data opens interesting lines of evidence that a modulation of intestinal microbiota can be a powerful therapeutic way of influence or even reverse the progression of Metabolic Syndrome. The use of probiotics has revealed that through changes the intestinal dysbiosis, a lower pro-inflammatory profile can be achieved with expressive benefits in glucose tolerance, insulin sensivity, lower weight and lower hepatic steatosis. Although several lines of investigation are still needed in this behalf, the data collected so far is consistent with a potential therapeutic target in the clinical control of Metabolic Syndrome development and treatment[48].

In conclusion, this review detects several lines of evidence of the profound preponderance of early activation of TLR receptors and its interactions with a dysbiotic intestinal microbiota in causing several disturbances known to be implicated in Metabolic Syndrome (Table 2). Thus, in the near future, the understanding if there is a specific set of TLR overexpression associated to the different chronic metabolic diseases that coexist in the Metabolic Syndrome, the characterization of the modulation of hepatic immune receptors and cytokine production and the clarification of which are the major bacterial molecules that work as a factor to propagate or ameliorate the Metabolic Syndrome state, shall be preponderant points in the breakthrough of the investigation in Metabolic Syndrome medical area.

Conflicts of Interest

The authors don’t have any conflicts of interest to declare

Acknowledgements

None

LEGENDS

FIGURE1- Fluxogram describing the methods of data collection and articles selection.

FIGURE2 – Consequences and inflammatory pathways activated during the development of Metabolic Syndrome in intestinal and liver tissues. The prompt activation in the intestinal epithelium by intestinal microbiota leads to an activation of Myd88 signaling pathways, with higher mRNA expression of TLR2, 4, 5 and 9, inflammatory cytokines, TNF-α, Interleucin 1β and MUC 2 and 3 receptors. This causes a loss barrier function of the intestine and bacterial translocation to other organs especially the liver. In the liver occurs Myd88 signaling pathways activation with increased mRNA expression of TLR2, 4 , 5, 6 and 9 in hepatocyte and kuppfer cells. It is also relevant the increase expression of inflammatory cytokines, NF-Kb, TNF-α and Interleucin-1β and decrease in anti-inflammatory pathways as XBP-1 culminating in transformation of hepatic tissue into non-alcoholic steatohepatitis and cirrhosis

FIGURE 3- Consequences and inflammatory pathways activated during the development of Metabolic Syndrome in adipose and other tissues. Present in the Metabolic Syndrome development are increased mRNA expression of TLR 4 and inflammatory cytokines and decreased adiponectine production and lipoprotein lipase, in the adipose tissue. It is also present a macrophage invasion and inflammatory pathways activation within this tissue. Systemically it also occurs a prompt activation of Myd88 signaling pathways with mRNA expression of TLR 2 and 4 and inflammatory cytokines that globally causes glucose intolerance and decreased insuline sensivity throughout the human organism

REFERENCES

1. Kaur J. A comprehensive review on metabolic syndrome. Cardiol Res Pract 2014;2014:943162.
2. Tilg H. Obesity, metabolic syndrome, and microbiota: multiple interactions. J Clin Gastroenterol 2010;44 Suppl 1:S16-18.
3. Mehal WZ. The Gordian Knot of dysbiosis, obesity and NAFLD. Nat Rev Gastroenterol Hepatol 2013;10:637-644.
4. Jialal I, Kaur H, Devaraj S. Toll-like receptor status in obesity and metabolic syndrome: a translational perspective. J Clin Endocrinol Metab 2014;99:39-48.
5. Jin C, Henao-Mejia J, Flavell RA. Innate immune receptors: key regulators of metabolic disease progression. Cell Metab 2013;17:873-882.
6. Caricilli AM, Picardi PK, de Abreu LL, et al. Gut microbiota is a key modulator of insulin resistance in TLR 2 knockout mice. PLoS Biol 2011;9:e1001212.
7. Wiest R, Lawson M, Geuking M. Pathological bacterial translocation in liver cirrhosis. J Hepatol 2014;60:197-209.
8. Frasinariu OE, Ceccarelli S, Alisi A, Moraru E, Nobili V. Gut-liver axis and fibrosis in nonalcoholic fatty liver disease: an input for novel therapies. Dig Liver Dis 2013;45:543-551.
9. Seki E, Schnabl B. Role of innate immunity and the microbiota in liver fibrosis: crosstalk between the liver and gut. J Physiol 2012;590:447-458.
10. Chassaing B, Etienne-Mesmin L, Gewirtz AT. Microbiota-liver axis in hepatic disease. Hepatology 2014;59:328-339.
11. Furukawa S, Fujita T, Shimabukuro M, et al. Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest 2004;114:1752-1761.
12. Jialal I, Huet BA, Kaur H, Chien A, Devaraj S. Increased toll-like receptor activity in patients with metabolic syndrome. Diabetes Care 2012;35:900-904.
13. Cuda C, Badawi A, Karmali M, El-Sohemy A. Polymorphisms in Toll-like receptor 4 are associated with factors of the metabolic syndrome and modify the association between dietary saturated fat and fasting high-density lipoprotein cholesterol. Metabolism 2011;60:1131-1135.
14. Steinhardt AP, Aranguren F, Tellechea ML, et al. A functional nonsynonymous toll-like receptor 4 gene polymorphism is associated with metabolic syndrome, surrogates of insulin resistance, and syndromes of lipid accumulation. Metabolism 2010;59:711-717.
15. Penas-Steinhardt A, Barcos LS, Belforte FS, et al. Functional characterization of TLR4 +3725 G/C polymorphism and association with protection against overweight. PLoS One 2012;7:e50992.
16. Belforte FS, Coluccio Leskow F, Poskus E, Penas Steinhardt A. Toll-like receptor 4 D299G polymorphism in metabolic disorders: a meta-analysis. Mol Biol Rep 2013;40:3015-3020.
17. Ehses JA, Meier DT, Wueest S, et al. Toll-like receptor 2-deficient mice are protected from insulin resistance and beta cell dysfunction induced by a high-fat diet. Diabetologia 2010;53:1795-1806.
18. Kuo LH, Tsai PJ, Jiang MJ, et al. Toll-like receptor 2 deficiency improves insulin sensitivity and hepatic insulin signalling in the mouse. Diabetologia 2011;54:168-179.
19. Figueroa L, Xiong Y, Song C, Piao W, Vogel SN, Medvedev AE. The Asp299Gly polymorphism alters TLR4 signaling by interfering with recruitment of MyD88 and TRIF. J Immunol 2012;188:4506-4515.

20. Wen L, Ley RE, Volchkov PY, et al. Innate immunity and intestinal microbiota in the development of Type 1 diabetes. Nature 2008;455:1109-1113.
21. Qin J, Li R, Raes J, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 2010;464:59-65.
22. Eckburg PB, Bik EM, Bernstein CN, et al. Diversity of the human intestinal microbial flora. Science 2005;308:1635-1638.
23. Backhed F, Ding H, Wang T, et al. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A 2004;101:15718-15723.
24. Backhed F, Manchester JK, Semenkovich CF, Gordon JI. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc Natl Acad Sci U S A 2007;104:979-984.
25. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nature 2006;444:1022-1023.
26. Parekh PJ, Arusi E, Vinik AI, Johnson DA. The role and influence of gut microbiota in pathogenesis and management of obesity and metabolic syndrome. Front Endocrinol (Lausanne) 2014;5:47.
27. Remely M, Aumueller E, Jahn D, Hippe B, Brath H, Haslberger AG. Microbiota and epigenetic regulation of inflammatory mediators in type 2 diabetes and obesity. Benef Microbes 2014;5:33-43.
28. Zhang C, Zhang M, Wang S, et al. Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. Isme j 2010;4:232-241.
29. Ubeda C, Lipuma L, Gobourne A, et al. Familial transmission rather than defective innate immunity shapes the distinct intestinal microbiota of TLR-deficient mice. J Exp Med 2012;209:1445-1456.
30. Vijay-Kumar M, Aitken JD, Carvalho FA, et al. Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. Science 2010;328:228-231.
31. Kellermayer R, Dowd SE, Harris RA, et al. Colonic mucosal DNA methylation, immune response, and microbiome patterns in Toll-like receptor 2-knockout mice. Faseb j 2011;25:1449-1460.
32. Wang J, Tang H, Zhang C, et al. Modulation of gut microbiota during probiotic-mediated attenuation of metabolic syndrome in high fat diet-fed mice. Isme j 2014.
33. Vrieze A, Van Nood E, Holleman F, et al. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. Gastroenterology 2012;143:913-916.e917.
34. Leber B, Tripolt NJ, Blattl D, et al. The influence of probiotic supplementation on gut permeability in patients with metabolic syndrome: an open label, randomized pilot study. Eur J Clin Nutr 2012;66:1110-1115.
35. Sawada K, Ohtake T, Hasebe T, et al. Augmented hepatic Toll-like receptors by fatty acids trigger the pro-inflammatory state of non-alcoholic fatty liver disease in mice. Hepatol Res 2014;44:920-934.
36. Ye D, Li FY, Lam KS, et al. Toll-like receptor-4 mediates obesity-induced non-alcoholic steatohepatitis through activation of X-box binding protein-1 in mice. Gut 2012;61:1058-1067.
37. Rivera CA, Adegboyega P, van Rooijen N, Tagalicud A, Allman M, Wallace M. Toll-like receptor-4 signaling and Kupffer cells play pivotal roles in the pathogenesis of non-alcoholic steatohepatitis. J Hepatol 2007;47:571-579.
38. Csak T, Velayudham A, Hritz I, et al. Deficiency in myeloid differentiation factor-2 and toll-like receptor 4 expression attenuates nonalcoholic steatohepatitis and fibrosis in mice. Am J Physiol Gastrointest Liver Physiol 2011;300:G433-441.

39. Henao-Mejia J, Elinav E, Jin C, et al. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature 2012;482:179-185.
40. Liang S, Webb T, Li Z. Probiotic antigens stimulate hepatic natural killer T cells. Immunology 2014;141:203-210.
41. Xie YR, Liu SL, Liu X, et al. Intestinal microbiota and innate immunity-related gene alteration in cirrhotic rats with liver transplantation. Transplant Proc 2011;43:3973-3979.
42. Henao-Mejia J, Elinav E, Thaiss CA, Flavell RA. The intestinal microbiota in chronic liver disease. Adv Immunol 2013;117:73-97.
43. Sommer P, Sweeney G. Functional and mechanistic integration of infection and the metabolic syndrome. Korean Diabetes J 2010;34:71-76.
44. Burcelin R, Garidou L, Pomie C. Immuno-microbiota cross and talk: the new paradigm of metabolic diseases. Semin Immunol 2012;24:67-74.
45. Moschen AR, Kaser S, Tilg H. Non-alcoholic steatohepatitis: a microbiota-driven disease. Trends Endocrinol Metab 2013;24:537-545.
46. Duseja A, Chawla YK. Obesity and NAFLD: the role of bacteria and microbiota. Clin Liver Dis 2014;18:59-71.
47. Manco M, Putignani L, Bottazzo GF. Gut microbiota, lipopolysaccharides, and innate immunity in the pathogenesis of obesity and cardiovascular risk. Endocr Rev 2010;31:817-844.
48. Miura K, Ohnishi H. Role of gut microbiota and Toll-like receptors in nonalcoholic fatty liver disease. World J Gastroenterol 2014;20:7381-7391.

TABLE 1 – Main Characteristics of animal and clinical studies included in the systematic revision

Title	Authors	Year	Type of study	Methods	Limitations	Main Conclusions
Polymorphisms in Toll-like receptor 4 are associated with factors of the metabolic syndrome and modify the association between dietary saturated fat and fasting high-density lipoprotein cholesterol	Cristina Cuda, et al.	2010	Prospective cohort study	- Dietary assessment; - Anthropometrics and energy expenditure assessment - Genotyping using PCR;	- Very homogeneous study population -Exclusion of Individuals from the study population with comorbidities associated to Metabolic Syndrome	-Carriers of the Asp299Gly polymorphism had significantly higher insulin levels, higher homeostasis model assessment of insulin resistance and family history of Diabetes; -Carriers of the intronic polymorphism (rs5030728) modified the relationship between dietary SFA and HDL cholesterol;
A functional nonsynonymous toll-like receptor 4 gene polymorphism is associated with metabolic syndrome, surrogates of insulin resistance, and syndromes of lipid accumulation	Alberto Penas Steinhardt et al.	2010	Cross sectional study	- Clinical measurements - Genotyping using PCR	-All individuals included in the study were men; - Individuals with comorbidities associated to Metabolic Syndrome were not included	- Carriers of Asp299Asp Tlr4 polymorphism had higher prevalence of enlarged waist elevated triglyceride syndrome, hypertriglyceridemic waist (HW), and overweight-lipid syndrome; - Carriers of Asp299Asp Tlr4 polymorphism had higher insulin levels, and were associated to Metabolic Syndrome in the group of individuals with C-Reactive Protein levels below the 95th percentile;
Functional Characterization of TLR4 +3725 G/C Polymorphism and Association with Protection against Overweight	Alberto Penas Steinhardt et al.	2012	Prospective cohort study	- Genotyping using PCR - Cell Culture and Transfections - Biochemical Analyses, Anthropometric and Clinical Measurements	- No exclusion criteria were used	- Carriers of 11350G/C TLR4 polymorphism was associated with a reduction of 30% of TLR4 gene activity; - Carriers of 11350G/C TLR4 polymorphism had also less weight, lower BMI, waist circumference and higher adiponectin levels;
The Asp299Gly Polymorphism Alters TLR4 Signaling by Interfering with Recruitment of MyD88 and TRIF	Leandra Figueiroa et al.	2012	Experimental study	- Cell culture and Transfections - RNA isolation, reverse transcription, and real-time quantitative PCR	- Antagonists of the TLR4-MyD88 were not studied	- Mice that carried D299G polymorphism of the TLR4 revealed comparable total TLR4 expression, TLR4–MD2 interactions, and LPS binding, in comparison to wild type mice. However, they were associated with macrophages that failed to elicit LPS-mediated induction of TNF-a and IFN-b mRNA levels and diminished LPS-driven interaction of MyD88 and TRIF with TLR4;
Toll-like receptor 4 D299G	F.S. Belforte,	2012	Meta-	-----	-Possible publication bias	- It was reported a significant association between

polymorphism in metabolic disorders: a meta-analysis	et al.		analysis		- Low number of studies included	TLR4 D299G polymorphism and metabolic disorders (T2DM and Met-S) risk (OR = 0.566, 95 % CI: 0.347–0.925, p =0.023) particularly in Caucasians;
Innate immunity and intestinal microbiota in the development of Type 1 diabetes	Li Wen, et al.	2008	Experimental study	- Histopathology evaluation - Sequencing and phylogenetic analysis	- Other parallel signalling pathways such as TRIF were not considered	- Specific-pathogen free (SPF) Non-Obese Diabetic mice lacking MyD88 protein did not develop Type 1 Diabetes - MyD88-deficiency changes the composition of the distal gut microbiota - The exposure to the microbiota of Specific Pathogen Free NOD.MyD88-negative donors attenuates Type 1Diabetes found in Germ Free NOD recipients;
Toll-like receptor 2-deficient mice are protected from insulin resistance and beta cell dysfunction induced by a high-fat diet	J.A. Ehses, et al.	2009	Experimental study	- Indirect calorimetry and physical activity assessment - Immunohistochemistry - RNA isolation, reverse transcription, and real-time quantitative PCR	- Inability to conclude whether the observed reductions in tissue inflammation in vivo are due to an immune cell, or parenchymal cell, origin.	- Mice with Tlr2−/− genotype were protected from the adverse effects of high fat diet compared with Tlr2+/+ littermate controls; - Female Tlr2−/− mice showed pronounced improvements in glucose tolerance, insulin sensitivity, and insulin secretion following 20 weeks of HFD feeding;
Toll-like receptor 2 deficiency improves insulin sensitivity and hepatic insulin signalling in the mouse	L.-H. Kuo, et al.	2010	Experimental study	- RNA isolation, reverse transcription, and real-time quantitative PCR - Protein analysis	- Further studies are needed to account for the mechanistic insight of the improvement of insulin sensivity with TLR2 deficiency	- Aged or high-fat-fed TLR2-deficient mice were protected from obesity and adipocyte hypertrophy compared with wild-type mice; - Mice lacking TLR2 exhibited improved glucose tolerance and insulin sensitivity regardless of feeding them regular chow or a high-fat diet; - The attenuated hepatic inflammatory cytokine expression and related signalling are correlated with increased insulin action specifically in the liver in TLR2-deficient mice;
Familial transmission rather than defective innate immunity shapes the distinct intestinal microbiota of TLR-deficient mice	Carles Ubeda, et al.	2012	Experimental study	- High-throughput sequencing of 16S rRNA genes - RNA isolation, reverse transcription, and real-time quantitative PCR	- Environmental intestinal colonization besides maternal origin was not studied	- The composition of intestinal microbiota was not significantly different between MyD88-, TLR2-, TLR4-, TLR5-, and TLR9-deficient mice and their respective wild-type (WT) littermates; - Differences between different knockout mice groups were due to long term divergence in isolation from each other; - The long term differences in intestinal microbiota

						composition resulted mainly from maternal transmission;
Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5.	Vijay-Kumar M., et al.	2010	Experimental study	- Bacterial trsnlocation to experimental purposes - RNA isolation, reverse transcription, and real-time quantitative PCR	Other potential correlations with other TLR were not considered limiting the rational for TLR5 role and preponderance	Toll-like receptor 5 knockout mice exhibited hyperphagia, hyperlipidemia, hypertension, insulin resistance, and increased adiposity; - Transfer of the gut microbiota from TLR5-deficient mice to wild-type germ-free mice conferred many features of metabolic syndrome to the recipients;
Colonic mucosal DNA methylation, immune response, and microbiome patterns in Toll-like receptor 2-knockout mice	Richard Kellermayer, et al.	2011	Experimental study	- Histological assessment - Pyrosequencing - RNA isolation, reverse transcription, and real-time quantitative PCR	- Intestinal microbiome changes were not as reproductive on human tissue colonic samples	- In the TLR2 knockout mice, the expression of genes involved in immune processes were found significantly different as well as epigenomic and transcriptomic modifications associated with altered microta composition; - Several bacterial species, including members of the Firmicutes were significantly different in abundance between WT and Tlr2_/_ animals;
A human gut microbial gene catalogue established by metagenomic sequencing	Junjie Qin, et al.	2010	Prospective cohort study	- Stool sampling; - real-time quantitative PCR - Library database construction	- Only usage of faecal samples.	- Over 99% of the genes are bacterial, indicating that the entire cohort harbours between 1,000 and 1,150 prevalent bacterial species and each individual at least 160 such species, which are also largely shared; - This Large intestinal microbiome is responsible of several functions known to be important to the host–bacterial interaction, such as degradation of complex polysaccharides, synthesis of short-chain fatty acids, indispensable amino acids and vitamins;
Microbiota and epigenetic regulation of inflammatory mediators in type 2 diabetes and obesity	M. Remely, et al.	2014	Experimental study	- Pyrosequencing - RNA isolation, reverse transcription, and real-time quantitative PCR	- The groups were not entirely comparable by possible confounders in the selection of mice characteristics	- higher ratio of Firmicutes/ Bacteroidetes in type 2 diabetics was observed when compared to lean controls and obese, as well as lactic acid bacteria; - Methylation analysis of four CpGs in the first exon of TLR4 showed significantly lower methylation in obese individuals and methylation of seven CpGs in the promoter region of TLR2 was significantly lower in type 2 diabetics mice;

						- The methylation levels of both TLRs were significantly correlated with body mass index
Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice	CHenhong Zhang, et al.	2010	Experimental study	- Pyrosequencing - RNA isolation, reverse transcription, and real-time quantitative PCR	The Bifidobacteria effects were not analysed since this group of bacteria was actually removed from guts fed with High Fat Diet possibly due to the much longer feeding time.	- Diet changes explained 57% of the total structural variation in gut microbiota, whereas genetic mutation accounted for no more than 12% - Desulfovibrionaceae, were dominant in all animals with impaired glucose tolerance, most significantly in the wild-type with high fat diet group, which had the highest calorie intake and the most serious MS phenotypes
Modulation of gut microbiota during probiotic-mediated attenuation of metabolic syndrome in high fat diet-fed mice	Jingjing Wang, et al.	2014	Experimental study	- Enzyme-linked immunosorbent assay - Histomorphology and immunohistochemistry - pyrosequencing - RNA isolation, reverse transcription, and real-time quantitative PCR	- Only usage of faecal samples.	-The use of the probiotics was responsible for a reduction of the bacteria positively related to metabolic Syndrome - A probiotic composed by Bifidobacterium animalis subsp. lactis I-2494 was even responsible by a decreased adipose and hepatic TNF-alfa gene expression.
Transfer of Intestinal Microbiota From Lean Donors Increases Insulin Sensitivity in Individuals With Metabolic Syndrome	ANNE VRIEZE, et al.	2012	Prospective cohort study	- Gut Microbiota Transfer Procedures - RNA isolation, reverse transcription, and real-time quantitative PCR - Biochemical analysis of Fecal samples	- Diet composition was not controlled in healthy control patients	- After infusion of microbiota from lean donors, all groups presented an increase in insulin sensivity of recipients and an increase of butyrate-producing intestinal microbiota
Increased Toll-like receptor Activity in Patients With Metabolic Syndrome	ISHWARLAL JIALAL, et al.	2012	Prospective cohort study	- Magnetic cell separation - RT-PCR - Fluorescent in-situ hybridization.	-The control group could have less than 2 features of Metabolic Syndrome -Potential differences in other cells expression	-Circulating levels of high-sensitivity C-reactive protein, interleukin (IL)-1b, IL-6, IL-8, and soluble tumor necrosis factor receptor 1 (sTNFR1) were significantly increased in MetS versus control subjects -Significant increase in both TLR2 and TLR4 surface expression and mRNA on monocytes after adjustment for waist circumference. - Plasma free fatty acids and endotoxin were increased in Metabolic Syndrome patients but only correlated significantly

						considering TLR4 expression.
The influence of probiotic supplementation on gut permeability in patients with metabolic syndrome: an open label, randomized pilot study	B. Leber, et al.	2012	Randomized pilot study	- Fluorescent in-situ hybridization.- - cytometric analysis	- Determination of endotoxin excluding important pathogens present in human gut	- Gut permeability was significantly increased in Metabolic Syndrome compared with controls - LBP and sCD14 levels were not significantly different from healthy controls. - High-sensitive C-reactive protein and LBP levels slightly but significantly increased after 3 months within the probiotics group. - Neutrophil function and TLR expression did not differ from healthy controls or within the patient groups.
Augmented hepatic Toll-like receptors by fatty acids trigger the pro-inflammatory state of non-alcoholic fatty liver disease in mice	Koji Sawada, et al.	2014	Experimental study	- Cell culture - Biochemical analyses - Histopathological evaluation - RNA isolation, reverse transcription, and real-time quantitative PCR - Immunohistochemistry/ immunocytochemistry	- The intestinal microbioma was not characterized nor its potential role in the results obtained	- The expression of inflammatory cytokines such as TNF, IL-1 β , and TLR-2, -4, -5, and -9 was increased in the liver, but decreased in the small intestine of High Fat Diet-fed mice in vivo. - The expression of TLRs in primary hepatocytes and Kupffer cells was increased by treatment with palmitic acid
Toll-like receptor-4 mediates obesity-induced non-alcoholic steatohepatitis through activation of X-box binding protein-1 in mice	Dewei Ye, et al.	2014	Experimental study	- Cell culture - RNA isolation, reverse transcription, and real-time quantitative PCR	- Use of a new understudied mice model for representation of human NASH	- The model used with a high fat and high calorie diet developed typical pathological features of NASH, which is associated with obesity and the metabolic syndrome - mice lacking functional TLR4 were resistant to HFHC diet-induced liver inflammation and injury and were less susceptible to the diet-induced production of reactive oxygen species (ROS) and proinflammatory cytokines - a transcription factor XBP-1 involved in the unfolded protein responses, was activated in the liver by an HFHC diet, whereas XBP-1 activation was abrogated in TLR4 knockout mice.
Toll-like receptors 1–9 are elevated in livers with fructose-induced hepatic steatosis	Sabine Wagnerberger, et al.	2012	Experimental study	- RNA isolation, reverse transcription, and real-time quantitative PCR - Immunostaining and histology	- Mechanisms of hepatic TLR activation were not studied	- The accumulation of Triacylglycerol found in the livers of fructose-fed mice was associated with a significant induction of TLR 1–4 and 6–8. - Plasma RBP4 concentration and hepatic mRNA

				evaluation - Western Blotting		expression levels of TNF-a, iNOS, MyD88 and number of F4/80 positive cells of fructose-fed animals were significantly higher than those of controls; - These effects of fructose were attenuated in antibiotic-treated mice
Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity	Jorge Henao-Mejia, et al.	2012	Experimental study	- RNA isolation, reverse transcription, and real-time quantitative PCR - Immunostaining and histology evaluation -Transmission electron microscopy -Bacterial 16S rRNA amplicon sequencing	- False negative and False positive results could be allocated to the measure of PAMPs method used	- Inflammasome deficiency-associated changes in the configuration of the gut microbiota are associated with exacerbated hepatic steatosis and inflammation through influx of TLR4 and TLR9 agonists and enhanced hepatic TNF-α - co-housing of inflammasome-deficient animals to wild type mice results in exacerbation of hepatic steatosis, glucose intolerance, andobesity - NLRP3 and NLRP6 inflammasomes govern the rate of progression of multiple metabolic syndrome-associated abnormalities,
Probiotics Antigens Stimulate Hepatic NKT cells	Shuwen Liang, et al.	2013	Experimental study	- Histology - Flow citometry - Immunofluorescence expression assessment	- NKT cells participation in the development of NASH was not clear	- High dose of selected probiotic was effective for the improvement of hepatic NKT cell depletion, obesity and steatosis. -Disruption of TLR4 signaling does not protect HF diet induced NKT cell depletion and metabolic dysfunction
Intestinal Microbiota and Innate Immunity-Related Gene Alteration in Cirrhotic Rats with Liver Transplantation	Y.R. Xie, et al.	2011	Experimental study	- RNA isolation, reverse transcription, and real-time quantitative PCR - Bacterial translocation for experimental groups protocol	- The experimental model acquisition method could influence intestinal bacteria directly and influence its modifications	- Liver cirrhosis and liver cirrhosis with transplant were associated with higher endotoxin levels and with higher incidence of bacterial translocation to liver and mesenteric lymph nodes, and with the number of total bacteria. -Mucins (MUC2, MUC3) and Toll-like receptors (TLR2, TLR4) messenger RNA (mRNA) expression were significantly higher in the cirrhosis groups than the control group
Diversity of the Human Intestinal Microbial Flora	Paul B. Eckburg, et al.	2005	Case control	- RNA isolation, reverse transcription, and real-time	- Pyrosequencing as the most accurate method of bacterial identification was	- A majority of the bacterial sequences corresponded to uncultivated species and

			study	quantitative PCR	not used	novel microorganisms - significant intersubject variability and differences between stool and mucosa community composition were observed
The gut microbiota as an environmental factor that regulates fat storage	Fredrik Ba¨ckhed, et al.	2004	Experimental study	- Measurement of Total Body Fat Content and Metabolic Rate - RNA isolation, reverse transcription, and real-time quantitative PCR	- Microbiota composition was not accessed	- adult germ-free mice with a normal microbiota harvested from the distal intestine (cecum) of conventionally raised animals produces a 60% increase in body fat content and insulin resistance despite reduced food intake. - microbiota promotes absorption of monosaccharides from the gut lumen, with resulting induction of de novo hepatic lipogenesis
Mechanisms underlying the resistance to diet-induced obesity in germ-free mice	Fredrik Ba¨ckhed, et al.	2007	Experimental study	- Immunoblotting - RNA isolation, reverse transcription, and real-time quantitative PCR	- The bacterial microbiota was not assessed or characterized and could explain for the differences observed in Fiaf-deficient mice - The preponderance of mechanisms in the explanation of the increased obesity was not ascertained statistically	- Germ-free mice were associated with a lean phenotype and increased levels of AMPK expression in liver and muscle samples - knockout mice lacking fasting-induced adipose factor are not protected from diet-induced obesity
Human gut microbes associated with obesity	Ruth E., et al.	2006	Prospective cohort study	- Pyrosequencing; - Real-Time quantitative PCR.	- Low number of participants in the study	- Bacterial lineages were remarkably constant within people over time - Before diet therapy, obese people had fewer Bacteroidetes ($P<0.001$) and more Firmicutes ($P=0.002$) than did lean controls - with time, the relative abundance of Bacteroidetes increased ($P<0.001$) and the abundance of Firmicutes decreased ($P=0.002$), irrespective of diet type
Deficiency in myeloid differentiation factor-2 and toll-like receptor 4 expression attenuates nonalcoholic steatohepatitis and fibrosis in mice	Timea Csak, et al.	2011	Experimental study	- histological evaluation - flow cytometry analysis - RNA isolation, reverse transcription, and real-time quantitative PCR	- Participation of other TLR that could account for the results obtained were not considered	- Features of NASH were significantly attenuated in MD-2 KO and TLR4 KO mice. -Serum alanine aminotransferase, was increased in controls but was attenuated in MD-2 KO and TLR4 KO mice. - Inflammatory activation, indicated by serum TNF-alfa and nictoinamide adenine dinucleotide phosphate oxidase complex mRNA expression and activation, was significantly

						lower in MCD diet-fed MD-2 KO and TLR4 KO compared with corresponding genotype control mice.
Increased oxidative stress in obesity and its impact on metabolic syndrome	Shigetada Furukawa, et al.	2004	Experimental study	- Biochemical measurements. - RNA isolation, reverse transcription, and real-time quantitative PCR	- The reactive oxygen species role was not determined - Diabetes and other comorbidities of the mice model were not taken in account in the statistical analysis	- Production of ROS increased selectively in adipose tissue of obese mice, accompanied by augmented expression of NADPH oxidase and decreased expression of antioxidative enzymes. - In cultured adipocytes, elevated levels of fatty acids increased oxidative stress via NADPH oxidase activation, and oxidative stress caused dysregulated production of adipocytokines - in obese mice, treatment with NADPH oxidase inhibitor reduced ROS production in adipose tissue, attenuated the dysregulation of adipocytokines, and improved diabetes, hyperlipidemia, and hepatic steatosis

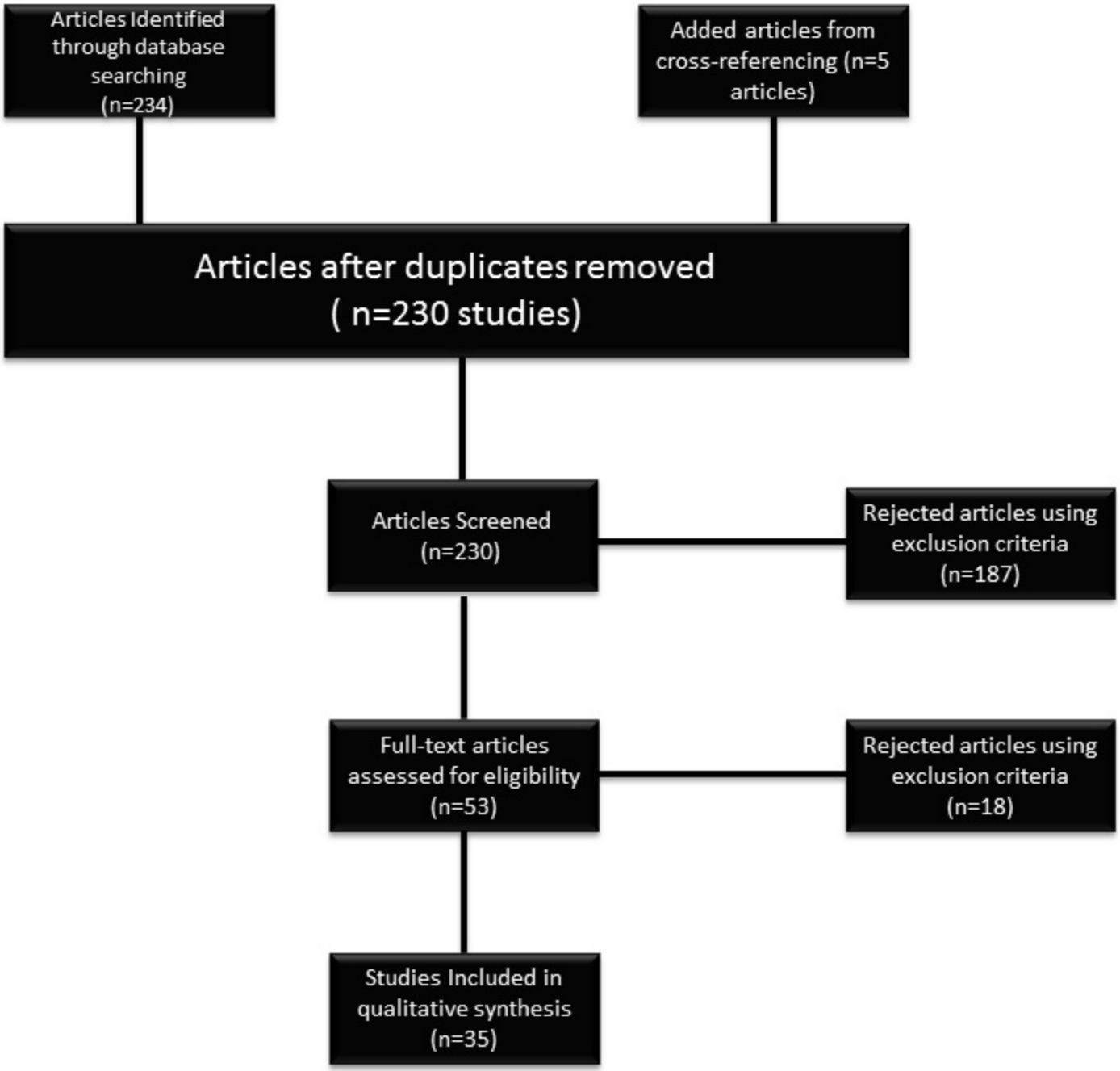
TABLE 1 – Main Characteristics of animal and clinical studies included in the systematic revision (includes “Authors”, “Year of publication”, “Type of Study”, “Methods”, “Limitations” and “Main Conclusions”

TABLE 2 – Principal proposed mechanisms in the development of Metabolic Syndrome

	Toll-like receptor activation	Biochemical molecules expression	Intestinal Microbiota modifications	Phenotypical/Hysthological Modifications
Diabetes	- ↑ TLR4 and TLR2 expression levels - ↑ MyD88 and TRIF signalling pathways	- ↑ Insulin levels - ↑ Leptin, MCP-1 and TNF-α expression levels - ↑ CD8+ T cells activation (↑ risk of Diabetes type 1)	- ↑ *Firmicutes* and ↓ *Bacteroidetes* and *F. prausnitzii*	- Glucose Intolerance, ↓ insulin sensivity
Dyslipidemia	- ↑TLR4 expression levels	- ↑ Saturated Fatty acids and ↓HDL cholesterol levels - ↓ Adiponectine levels -↑ Lipoprotein Lipase and ↓ Fasting-induced adipocyte factor (Angpt14/Fiaf) activities	- ↑ *Firmicutes* and ↓ *Bacteroidetes*	- Obesity - Increased macrophage infiltration in adipose tissue -Smaller Adipocytes
NASH	- ↑ TLR2 and TLR4, 5 and 9 expression levels	- ↑IL-1β, phospho-interleukin-1 receptor-associated kinase 1 and TNF-α expression levels - ↑ XBP-1, iNOS and NF-kB expression levels - ↑ Nlrp3, Nlrp6 and IL-18 expression levels - ↑MUC2 and 3 intestinal activity	- ↑ *Firmicutes*, *Pophyromonadaceae* and *Enterobacteriaceae* - ↓*Bacteroidetes*, *Lactobacilli* and *Bacteroides*	- Macrovesicular and microvesicular hepatic steatosis and hepatocellular ballooning - Obesity and glucose intolerance
Metabolic Syndrome	- ↑ TLR2 and TLR4 expression levels	- ↑IL-1β, MCP-1 and NF-kB expression levels; - ↑ Insulin levels and ↓Insulin sensivity	- ↑ *Firmicutes*, *Desulfovibrionaceae*, and *Pophyromonadaceae* - ↓*Bacteroidetes* and *Bifidobacteria*	- Higher waist circunferences and body weight - Diabetes (with all above) - Dyslipidemia (with all above) - NASH (with all above)

TABLE 2 – Principal proposed mechanisms in the development of Metabolic Syndrome [here, ↑ represents an augment and ↓ a decrease].

Figure 1


Articles Identified through database searching (n=234)
Added articles from cross-referencing (n=5 articles)
Articles after duplicates removed (n=230 studies)
Articles Screened (n=230)
Rejected articles using exclusion criteria (n=187)
Full-text articles assessed for eligibility (n=53)
Rejected articles using exclusion criteria (n=18)
Studies Included in qualitative synthesis (n=35)

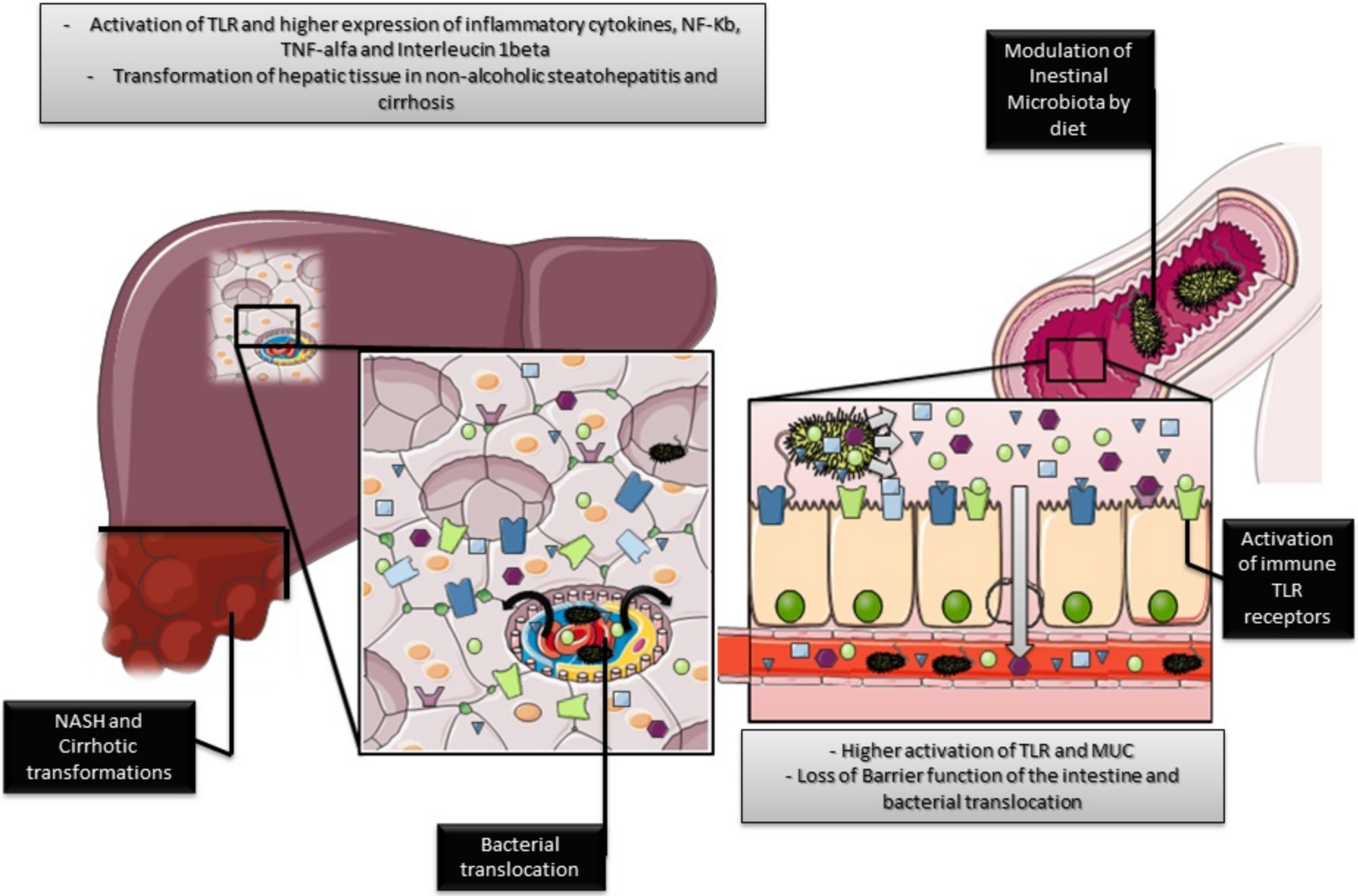
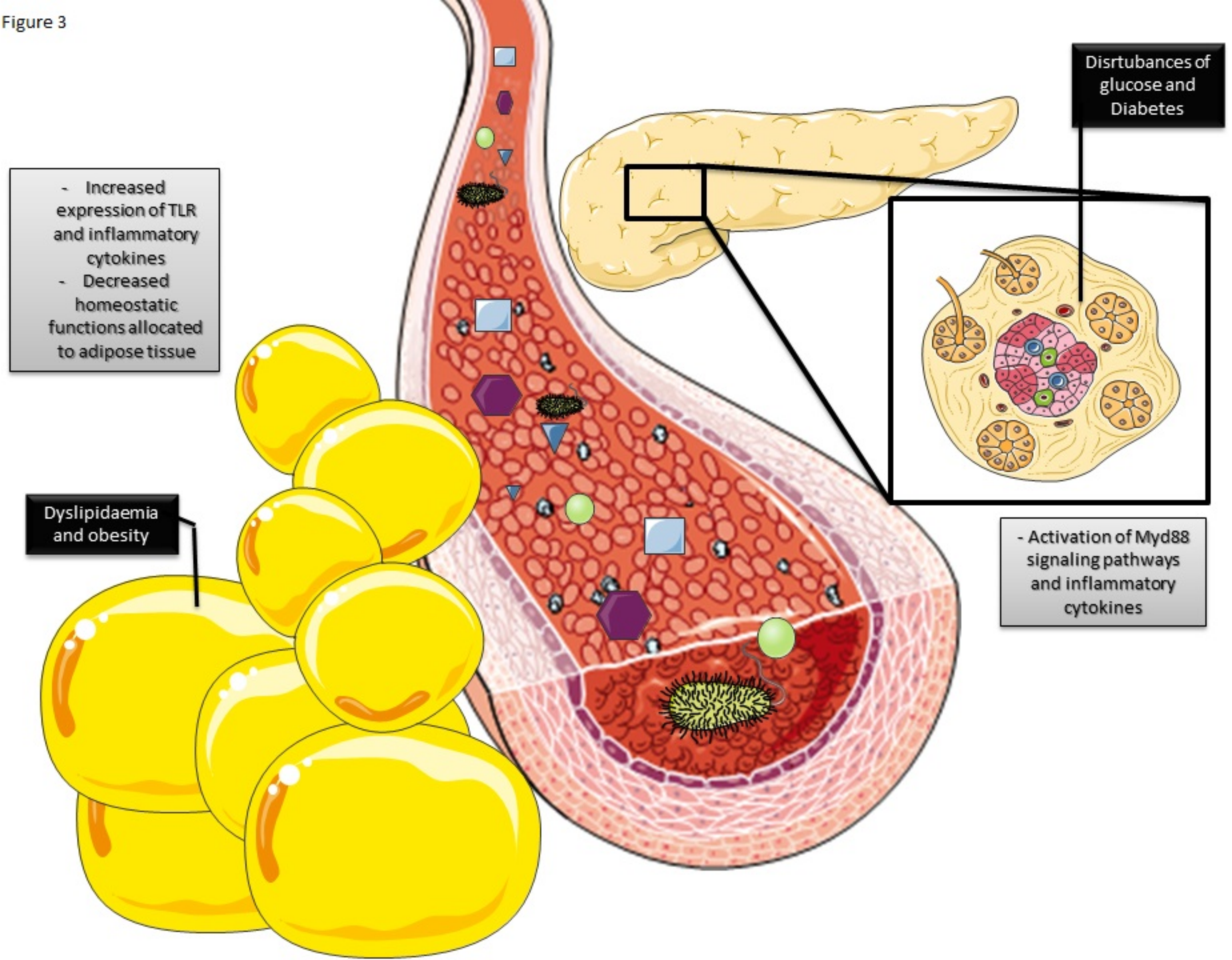

- Activation of TLR and higher expression of inflammatory cytokines, NF-Kb, TNF-alfa and Interleucin 1beta
- Transformation of hepatic tissue in non-alcoholic steatohepatitis and cirrhosis
Modulation of Inestinal Microbiota by diet
Activation of immune TLR receptors
- Higher activation of TLR and MUC
- Loss of Barrier function of the intestine and bacterial translocation
NASH and Cirrhotic transformations
Bacterial translocation

Figure 2

Figure 3


- Increased expression of TLR and inflammatory cytokines
- Decreased homeostatic functions allocated to adipose tissue
Dyslipidaemia and obesity
Disrtubances of glucose and Diabetes
- Activation of Myd88 signaling pathways and inflammatory cytokines

Anexos

Gut and Liver

Instructions to Authors

Gut and Liver is published and distributed jointly by the Korean Society of Gastroenterology, the Korean Society of Gastrointestinal Endoscopy, the Korean Society of Neurogastroenterology and Motility, Korean College of *Helicobacter* and Upper gastrointestinal Research, Korean Association for the Study of Intestinal Diseases, the Korean Association for the Study of the Liver, Korean Society of Pancreatobiliary Disease, and Korean Society of Gastrointestinal Cancer.

Gut and Liver is published bimonthly in a printed and an electronic version only in English. The Journal publishes original articles, case reports, imaging and issues, brief communications, letters to the editor, editorials, invited review articles, and special review section in the field of gastroenterology. Submissions are accepted only on the understanding that they have not been submitted elsewhere and have not been published elsewhere. Its editorial policies are the responsibility of the Editor-in-Chief, the associate editors, and the Editorial Board under the general authority of the Editorial Committee.

The Editorial Office Contact Information
Gut and Liver Editorial Office
305, Lotte Gold Rose II
890-59, Daechi 4-dong, Gangnam-gu
Seoul 135-839, Korea
Tel: +82-2-393-3344, Fax: +82-2-393-3344
E-mail: office@gutnliver.org

All submitted papers are peer-reviewed before it is decided whether they should be accepted, rejected, or returned for revision. The journal reserves the right to edit the language of papers accepted for publication for clarity and correctness, and to make formal changes to ensure compliance with this Journal. Proofs will be sent to the corresponding author for final approval. Accepted manuscripts become the permanent property of this journal and may not be reproduced by any means, in whole or in part without the written permission of both the author and the publisher.

EDITORIAL POLICY

1. Ethical guidelines

The journal adheres to the ethical guidelines for research and publication described in 'Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Writing and Editing for Biomedical Publication' (http://www.icmje.org; International Committee of Medical Journal Editors [ICMJE]), 'Good Publication Practice Guidelines for Medical Journals' (http://kamje.or.kr/publishing_ethics.html; Korean Association of Medical Journal Editors [KAMJE]), and 'Guidelines on Good Publication' (http://publicationethics.org/resources/guidelines; Committee on Publica¬tion Ethics [COPE]).

2. Authorship

All authors must have made a significant intellectual contribution to the manuscript according to the criteria formulated by the ICMJE. Each author should have participated sufficiently in the work to take public responsibility for the content. Authorship credits should be based only on substantial contributions to conception and design, analysis and interpretation of data, drafting the article, revising it critically for important intellectual content, or on final approval of the version to be published. General supervision of the research group is not sufficient for authorship. All authors must state they have approved the final draft submitted. Authors submitting to Gut and Liver will be required to indicate the contribution(s), each has made to the manuscript. Any changes to the author list after submission, such as a change in the order of the authors, or the deletion or addition of authors, needs to be approved by a letter requesting the change signed by all the authors participating.

3. Conflicts of interest

The authors must acknowledge any conflict of interest they may have in the submission. The conflicts of interest may fall under any of the following categories: guarantor of the article; specific author contributions; financial support; potential competing interests.

4. Copyright

In view of copyright laws, accepted papers must complete the assignment forms. These forms are available on the website http://www.gutnliver.org/thesis/File/Copyright_form.pdf. The papers will not be published until the copyright is completed.

5. Informed consent

Human study must conform to ethical standards, and be approved by the appropriate Institutional Review Board (IRB). A statement concerning IRB approval and consent procedures must appear at the beginning of the Methods section. Any systematic data gathering effort in patients or volunteers must be approved by an IRB or adhere to appropriate local/national regulations. Authors may be questioned about the details of consent forms or the consent process. On occasion, the Editor-in-Chief may request a copy of the approved IRB application from the author.

6. Statement of human and animal right

Clinical research studies must state that the work was done in accordance with the Ethical Principles for Medical Research Involving Human Subjects outlined in the Helsinki Declaration in 1975 (revised in 2000). Clinical studies that do not meet the Helsinki Declaration will not be considered for publication. Human subjects must not be identifiable. Patients' name, initial, hospital

number, date of birth, or other protected healthcare information must not be disclosed. Animal research studies must state that the work was performed according to National or Institutional Guide for the Care and Use of Laboratory Animals, and the ethical treatment of all experimental animals must be observed.

7. Originality and duplicate publication

All submitted manuscripts should be original and should not be considered by other scientific journals for publication at the same time. No part of the accepted manuscript should be duplicated in another scientific journal without permission by Editorial Board. If duplicate publications related to the papers of this journal are detected, the publication of the papers can be disapproved in *Gut and Liver* based on the decision of the Research Ethics Committee of *Gut and Liver*.

8. Obligation to register clinical trial

Clinical trial defined as "any research project that prospectively assigns human subjects to intervention and comparison groups to study the cause-and-effect relationship between a medical intervention and a health outcome" should be registered to the primary registry to be prior publication. *Gut and Liver* accepts the registration in any of the primary registries that participate in the WHO International Clinical Trials Portal (http://www.who.int/ictrp/about/details/en/index.html) as well as http://www.actr.org.au/, http://www.clinicaltrials.gov/, http://www.isrctn.org/, www.umin.ac.jp/ctr/index/htm, and http://www.trialregister.nl/. The clinical trial registration number shall be published at the end of the abstract.

Reporting of randomized controlled trials should follow the guidelines of The CONSORT Statement (http://www.consort-statement.org).

SUBMISSION

Manuscript may be sent either directly to the Editorial Office or submitted via online through the website (http://gutnliver.org). *Gut and Liver* uses an online submission and review system. Please register with our online submission system or contact the Editorial Office if you are unable to submit via online.

PEER REVIEW AND ACCEPTANCE

All papers submitted to *Gut and Liver* are first reviewed by the editorial team without being sent out for external peer review on the grounds of priority, insufficient originality, scientific flaws, or the absence of a message that is important to the readers of the journal. The decision on these papers is usually taken within 2-3 weeks.

The remaining articles are usually sent to two reviewers. It is very helpful if you suggest a selection of reviewers with their details. We may not always use the ones you recommend but if everyone suggests reviewers the reviewer database is much richer, and in the end everyone benefits. We reserve the right to return manuscripts in which no reviewers are suggested.

The final responsibility for the decision to accept or reject lies with the editors. In many case rejection may occur in spite of favorable reviews because of editorial policy or lack of space. The editor retains the right to determine priorities for publication and also to determine the style and if necessary request shortening of material accepted for publication.

COVER LETTER

Cover letter should inform the editor that neither the submitted material nor portions thereof have been published previously or are under consideration for publication elsewhere. When more than one related manuscript has been published or is under consideration for publication by this or other journals, authors are required to declare this in their letter and to enclose copies of those publications for an editorial perusal. Failure to do so may lead to automatic rejection of the submitted manuscript. The corresponding author should certify that all listed authors participated meaningfully in the study and that they have seen and approved the final manuscript. The cover letter must briefly explain each author's individual contributions.

PREPARATION OF MANUSCRIPT

The manuscript must be written with double-line spacing and 3 cm margins on A4 sized format. Number pages consecutively, beginning from the title page.

1. Title page

The title, each author's full name and institutional affiliation(s) should be typed on the title page and in the journal style. A running title should be added if a title exceeds 12 words. Corresponding author's name, full address, telephone and facsimile numbers, and E-mail address should be stated. A footnote may be placed.

2. Abstract

Authors of original scientific papers must supply a structured abstract of no more than 200 words under the following headings: Background/Aims, Methods, Results, and Conclusions. Nonstandard abbreviations, references or footnotes should not be used. A list of key words should be placed below the abstract using MeSH terms within 5 words.

3. Main text

1) Original articles

All clinical research papers submitted which involve human

or animal subjects must be accompanied by evidence of Institutional Review Board or Ethics Committee Review. Word count is up to 3500 words. References are limited to those critical and relevant to the manuscript, figure legends, tables, and illustrations. All original manuscripts must include the following:

• Introduction - The rationale for the study should be summarized and pertinent background material outlined. The introduction should not contain either results or conclusions.

• Materials and Methods - Methods should be described in sufficient detail so that another investigator could repeat the work. The location (manufacturer name, city, state, country) of a manufacturer listed in the text should be included. Statistical methods used should be outlined. Ethical guidelines for human or animal study should be described and approval of institutional human research review committee or animal welfare committee should be cited. Describe in detail hazardous procedures or chemicals involved, including precautions observed.

• Results - Results should be presented in logical sequence in the text. Tables and illustrations and repetitive presentation of the same data in different forms should be avoided. The results should not include material appropriate to the discussion.

• Discussion - Discussion should explain results in relation to any hypotheses advanced in the introduction. This may include an evaluation of the methodology and of the relationship of new information to the existing corpus of knowledge in that field. Data given in the results section should not be reiterated here.

2) Case reports

The format should include introduction, case report, and discussion which is similar to that of an original article. Word count is up to 1,500 words.

3) Brief communications

Brief communications are short articles describing clinical or experimental findings of importance or great advancement. A brief communication should be organized in the same way as case reports and should be limited to 1,500 words. The number of tables and figures in total should not exceed two.

4) Imaging and issues

This new section presents unusual or classic, challenging or informative images in the field of gastroenterology. Submissions will be reviewed by Imaging and issues Coordinating Editors. The format should include title page, case description, discussion, references and images. The case description should be in one page or less. It should include the followings: brief history, findings at physical exam, and laboratory results, clinical course and outcome. The images and all the labels within the images must be described in the case. Discussion and references should be in one page or less. Discussion should include important features of the images, differential diagnosis, and clinical significance. Number of references should be less than five. References must include definitive studies and pertinent reviews. Up to two figures of high quality are accepted.

5) Letters to the editor

The journal welcomes readers' comments on articles published recently in the journal or orthopedic topics of interest.

6) Reviews

Substantive reviews of systematic and clinical topics in gastroenterology and liver disease will be considered for publication. They will both be evaluated by peer review of the manuscript prior to consideration of publication. An abstract must be included.

7) Editorial

Editorials express opinions on current topics of interest or provide comments on papers published elsewhere in the same issue. Editorials are usually solicited by the Editor. Tables and/or figures may be included. Editorials should have fewer than five authors and should not exceed 1000 words. No subdivisions such as the Introduction, Materials and Methods, Results, and Discussion are required. The total number of references should be limited to a maximum of 10.

4. Conflicts of interest

Any potential conflict of interest relevant to this article is to be described.

5. Acknowledgements

A brief acknowledgement of persons who made a genuine contribution may be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustration. All funding related with the work should be specifically stated.

6. References

Number serially in the text in order of citation with numbers as superscripts. List all authors if the number of authors is less than 7. List first three authors followed by et al if the number of authors is 7 or more. Journal titles are abbreviated in accordance with the style of Index Medicus. For more on references, refer to the National Library of Medicine (NLM) Style Guide for Authors, Editors, and Publishers (http://www.nlm.nih.gov/citingmedicine).

• References to an article with 6 or fewer authors:

Meltzer SJ, Abnen DJ, Battifour A, Yokokota J, Cline MJ. Protooncogene abnormalities in colon cancer and adenomatous polyps. Gastroenterology 1987;92:1174-1180.

• References to an article with more than 6 authors:

Shim SG, Rhee JC, Rhee PL, et al. Mechanism of motilin action on smooth muscle of the human stomach. Korean J Gastro-

enterol 2002;39:4-12.

• Reference to a book:

Day RA. How to write and publish a scientific paper. 3rd ed. Phoenix: Oryx, 1988.

• Reference to a chapter in a book:

Costa M, Furness JB, Llewellyn-Smith IF. histo-chemistry of the enteric nervous system. In: Johnson LR, ed. Physiology of the gastrointestinal tract. Volume 1. 2nd ed. New York: Raven, 1987:1-40.

• Website:

World Health Organization (WHO). WHO statistical information system [Internet]. Geneva: WHO; c2010 [cited 2012 Jan 5]. Available from: http://www.who.int/whosis/en/menu.cfm.

7. Tables

Each table must be simple and typed on a separate page with its heading above it. Explanatory matter should be placed in footnotes below the tabular matter and should not be included in the heading. All non-standard abbreviations should be explained in the footnotes. Indicated by *, †, ‡, §, ||, ¶, #. Statistical measures such as SD or SEM should be identified. Vertical rules and horizontal rules between entries should be omitted. Each table should be referred in the text consecutively and numbered according in order of citation. Tables should also be typed double-spaced, including headings. Larger size sheets must not be utilized. Tables should be numbered consecutively in Arabic numerals beginning with 1.

8. Figures legends

Figure legends should be typed double-spaced on a separate sheet. Symbols, arrows, and letters should be used to indicate parts of illustrations. Each figure should be referred in the text consecutively and should be numbered according in order of citation.

9. Figures

We encourage authors to use color figures when they will enhance the presentation of the data. Only high-resolution figure files (Minimum 300 dpi) should be submitted, preferably in JPEG or EPS format. Adherence to these requirements from the outset will prevent the Production Editor from contacting the author at a later stage for better quality figures.

10. Terminology

Medical terminology should be followed by the recent terminology book published by the Korean Medical Association (http://term.kma.org/).

11. Abbreviations

Abbreviations should be used in case that they appear in the text at least 3 times and should be explained in parentheses in its first use in the text. Do not use abbreviation(s) in the title.

12. Units of measurement

Laboratory measurements should be used in SI units (International System of Units). The metric system is preferred for the expression of length, area, mass, and volume.

ARTICLE PROCESSING CHARGES

1. Page charges

Original articles, review articles - Page charges are $300 (300,000 KRW) per 6 printed pages or less, and $50 (50,000 KRW) per additional page.

Case reports, brief communications, letters - $250 (250,000 KRW) per 4 printed pages or less, and $50 (50,000 KRW) per additional page.

Invited review articles are exempt from page charges.

2. Color charges

US$100 (100,000 KRW) for each illustration containing color.

3. Author reprints

US$50 (50,000 KRW) for 100 copies. VAT will be added.