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Gene expression regulation microRNA-dependent in two different pathological conditions: celiac disease and hypoxia

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Stay hungry, Stay foolish.

SUMMARY

ABSTRACT

miRNAs are oligonucleotides acting as negative regulators of gene expression; the aim of our study was to verify their role in the regulation of genes involved in two specific pathological conditions: celiac disease and hypoxia. Celiac disease (CD) is an autoimmune disease of the intestine that culminates in villous atrophy. We performed a microarray analysis comparing the duodenum of adult CD patients to that of healthy subjects. We detected the down-regulation of 7 miRNAs; 4 of them were confirmed by qRT-PCR, and their level correlated with the gravity of the mucosal damage. In silico analyses identified the target genes of these miRNAs, proteins involved in the immune response. In particular miR-192-5p acts on NOD2 and CXCL2, miR-31-5p on FOXP3, whereas miR-338 and miR-197 target RUNX1 and IL18, respectively. mRNA and protein levels of all targets resulted increased in CD patients compared to controls, and a significant inverse correlation with the respective miRNA was observed. Clinical manifestations of CD are very heterogeneous and age-dependent, thus we hypothesized that the underlying molecular mechanisms could be influenced by age. We analyzed the same miRNAs and target genes in pediatric CD patients compared to controls and the results showed the same trend of expression observed in adult ones, except for NOD2 and CXCL2 that did not show any expression variation. On the contrary, another miR-192-5p target, MAD2L1, involved in the cell cycle and intestinal mucosa remodeling, resulted up-regulated only in CD pediatric patients whereas it did not show any variations in adults. These data support our hypothesis on the presence of age-dependent molecular mechanisms. Finally, we hypothesized that a panel of plasmatic miRNAs could improve the current diagnostic tools; we analyzed the same miRNAs in the plasma of CD subjects detecting a trend of expression similar to that observed in the biopsies, but in patients on a gluten free diet (GFD) we did not observe a return to normal. The other analyzed pathological condition is hypoxia, characterized by a reduction of oxygen levels, and observed in various situations including anemia. We focused our attention on the mechanisms of iron homeostasis in these conditions, hypothesizing a role played by miRNAs. We analyzed the liver of mice exposed to hypoxia (12, 24 and 48 hours) and compared to mice on normoxia.

We detected a down-regulation of the entire Bmp/Smad pathway, in particular we analyzed gene and protein expression of Bmp6, HJV, BmpR1a, BmpR2, Smad1/5/8, Id1 and Hamp, the main regulator of iron homeostasis. Hypothesizing a role of miRNAs, we performed a microarray analysis on the liver of mice exposed to hypoxia, identifying several differentially expressed miRNAs. We verified by qRT-PCR the expression of miR-22-3p, miR-101a-3p e miR-351 that resulted up-regulated according to the time of exposure and inversely correlated with their targets Bmp6, BmpR1a and Atoh8 (Hamp transcriptional regulator), respectively. In conclusion, we suggested the pivotal role hypoxia-dependent of miRNAs in the regulation of genes involve in iron metabolism and in particular of Bmp/Smad pathway.

I.

Introduction

INTRODUCTION

1. microRNA

1.1 BIOGENESIS AND ROLE OF miRNAs AS REGULATORS OF GENE EXPRESSION

MicroRNA (miRNA) are a class of non-coding RNA, discovered just over a decade ago, now recognized as one of the major regulator of gene expression. Thousands of different miRNAs have been identified in animals and plants, and about 2500 in humans; a large number of miRNAs are conserved in closely related species and many have homologs in distant species, suggesting that their functions could also be conserved throughout the evolution *(Kim VN et al. 2006)*. The main characteristic of animal miRNAs is that their genes are often closely clustered on the genome, and in many cases are processed from the same polycistronic precursor transcript. When clustered miRNAs have similar sequences and they usually contribute additively to the regulation of a set of mRNA targets; on the other hand, when clusters contain miRNAs of distinct sequences, they are coordinately deployed towards their various targets *(reviewed in Ambros V, 2004).*

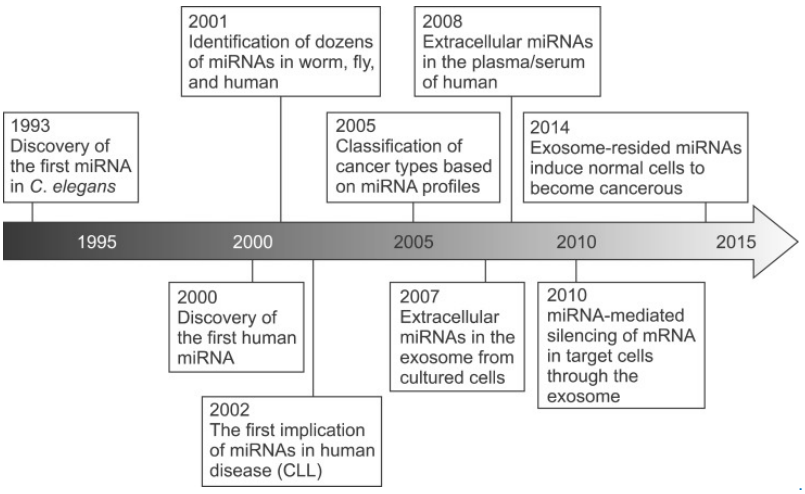


***Fig 1. Timeline of miRNA discovery**. Kym YK, 2015.*

The genes encoding for miRNAs are present in our genome and contain the TATA box necessary for the binding of the RNA polymerase II (Pol II) responsible of their transcription; however, the possibility that a few miRNA genes might be transcribed by other types of RNA polymerases

cannot be completely excluded. Transcription of miRNA genes generates long primary transcripts (pri-miRNAs) that contain a stem-loop structure which is cleaved to release the precursor of miRNA (pre-miRNA, about 70 nucleotides). This cleavage is performed by the nuclear RNase III Drosha, a large protein that requires a cofactor, DGCR8 (or Pasha), a protein which contains two double-stranded RNA-binding domains (dsRBDs) necessary to assist Drosha in substrate recognition. After that, pri-miRNA is exported to the cytoplasm by exportin-5 where is processed into 22-nt miRNA duplexes by the cytoplasmic RNase III Dicer. Mature miRNAs are incorporated into the effector complexes, which are known as 'miRISC' (miRNA-containing RNA induced silencing complex) *(reviewed in Engels BM et al., 2006).* During RISC assembly, miRNA duplexes are rapidly converted into single strand, which is the mature miRNA; one strand is called -5p, whereas the second strand of this short-lived duplex disappears or it is later incorporated in RISC and called miRNA-3p. The mature miRNA that binds to complementary sequences in the 3' UTR of target mRNA causes the silencing of the gene. The effect of this binding depends on the presence of perfect pairing or of mismatches: if the pairing it's complete, it will cause the degradation of the target mRNA, whereas a mismatch will result in translational inhibition without the reduction of the mRNA level. Because a single miRNA can target numerous mRNAs, often in combination with other miRNAs, their involvement in complex regulatory networks needs to be better understood *(Cannell IG et al., 2008).*

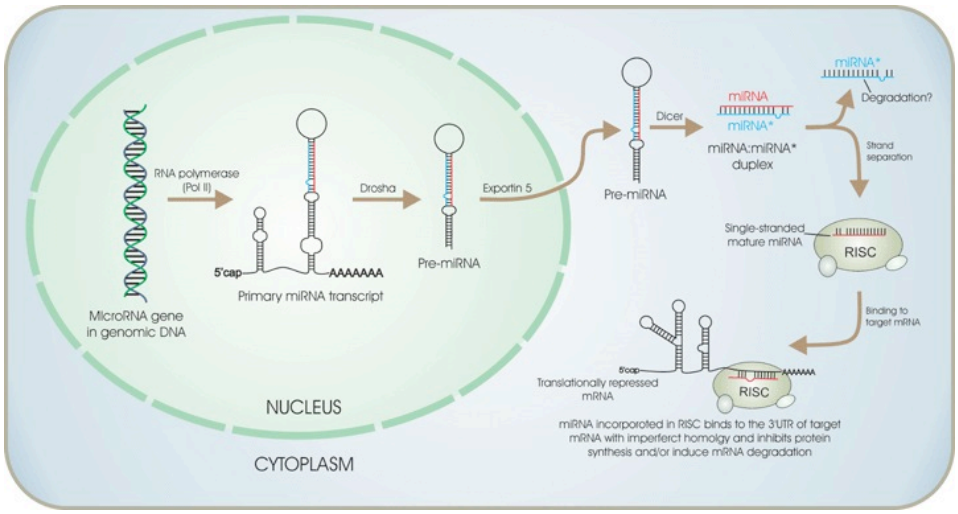


Fig 2. miRNA biogenesis. *Sonkoly E., 2009.*

1.2. miRNAs AS DIAGNOSTIC AND THERAPEUTICS TOOLS

Analyses of miRNA levels in human plasma and serum showed that extracellular miRNAs are unexpectedly stable in body fluids; moreover, many studies showed that the miRNAs profile is altered in the blood of patients affected by various diseases compared with that of healthy subjects *(Li H et al., 2015; Le large TY et al., 2015);* these discoveries have attracted much interest in utilizing the miRNAs as novel biomarkers. Compared to currently used biomarkers, which are generally based on the levels of specific proteins in the blood, biomarkers based on miRNA levels have, in theory, some advantages: they can be measured rapidly and accurately using high-throughput sequencing technology but, even more importantly from a diagnostic standpoint, since miRNAs are expressed differentially in different tissues and cellular states, the combination of a panel of miRNAs could provide a wealth of information.

For example in Wu study *(Wu F et al., 2011)* the results showed that peripheral blood miRNAs can be used to distinguish active Crohn Disease and Ulcerative Colitis from healthy controls; these data support the evidence that in these diseases, different circulating miRNAs may form the basis of future diagnostic tests. Finally, the high stability and their availability in the body fluids makes them good candidates as biomarkers for different diseases lacking specific biomarkers, such as gastric cancer *(Chen S et al., 2015)* or lung diseases *(Ebrahimi A et al., 2015).*

On the contrary, one of the most critical problems in utilizing circulating miRNAs is the difficulty in univocally identifyi miRNAs as up- or down-regulated, as demonstrated by the lack of consistency between studies: in fact different and non-overlapping sets of miRNAs have been reported as biomarkers for the same disease *(Kym YK, 2015).* Thus, despite many new discoveries about extracellular miRNAs, more researches are needed to increase their reliability as promising disease markers. Moreover, because the experimental method for measuring the amount of miRNAs in the body fluids is still not optimized and more technical advances are needed. With the complementation of experimental techniques and the increase in our knowledge about extracellular miRNAs, their use as noninvasive biomarkers will become an invaluable tool. The development of drug delivery methods may increase also the potential of miRNAs as therapeutic agents. Two main strategies are employed for pharmacological modulation of miRNA activity in vivo: restoring the

function of a miRNA using either synthetic miRNA mimics or viral expression constructs, or inhibiting miRNA functions by chemically modified anti-miR oligonucleotides, which sequester endogenous mature miRNAs, rescuing target gene expression *(Van Rooij E et al., 2014).*

These approaches can be used in different fields and some trials are currently underway, for example preclinical trials in non-human primates shows encouraging pharmacokinetic properties of anti-miRNA, considering miRNAs as therapeutic targets.

In 2008, Elmen et al. *(Elmen J et al., 2008)* published findings using intravenous injection of anti-miR-122 in monkeys showing the depletion of mature miR-122 and a long-lasting and reversible decrease in total plasma cholesterol without any evidence of toxicities or histopathological changes in the animals.

Moreover, one of the major field of applications of these approaches is cancer-related; for example in pancreatic adenocarcinoma problems related to resistance to multi-chemotherapeutic drugs could be improved using miRNAs as therapeutics agents, in particular miR-206 could act as tumor suppressor, targeting directly the oncogenes KRAS and blocking cell cycle progression, cell proliferation, migration and invasion *(Keklikogluo I et al., 2015).* With regard to the involvement of miRNAs in the pathogenesis of intestinal diseases, miR-21 has been shown to have potential clinical application: according to published data, its expression is regulated by *NF-κB* which is a master gene involved in various immune diseases and this miRNA could be targeted for example, in Inflammatory Bowel Diseases (IBD) to implement current therapies (*Zhang L et al., 2014).*

2. CELIAC DISEASE

2.1. PATHOGENESIS OF CELIAC DISEASE

Celiac Disease (CD) is a common inflammatory disease of the small intestine, triggered and maintained by the ingestion of gluten from wheat, barley, and rye in genetically predisposed individuals. The required genetic background is the presence of the HLA class II heterodimer HLA-DQ2 or HLA-DQ8 expressed on antigen-presenting cells (APCs). The HLA complex is a highly polymorphic region that contains the loci DQA and DQB, which encode, respectively, the alpha and beta chain of the class II heterodimer. The combination of HLA-DQA1*0501 and DQB1*0201 generate the HLA-DQ2 heterodimer. Above 90% of cases of celiac disease carry the *heterodimer* DQ2, either *in cis* or *in trans*, whereas the remaining patients carry the HLA-DQ8 heterodimer, encoded by DQA1*03 (α chain) and DQB1*0302 (β chain) *(Dubois PC et al., 2008).* However, HLA-DQ2 is present in 30%–35% of the Caucasian population (in which celiac disease has a high prevalence), but only 2%–5% of gene carriers develop celiac disease *(Romanos J et al., 2014).* Recent genetic studies in large numbers of patients with celiac disease, relatives, and matched controls revealed additional risk factors, most of which are genes related to T-cell regulation and inflammation *(Almeida R et al., 2014).* This implicates other genetic as well as environmental factors as contributors to the manifestation of the disease.

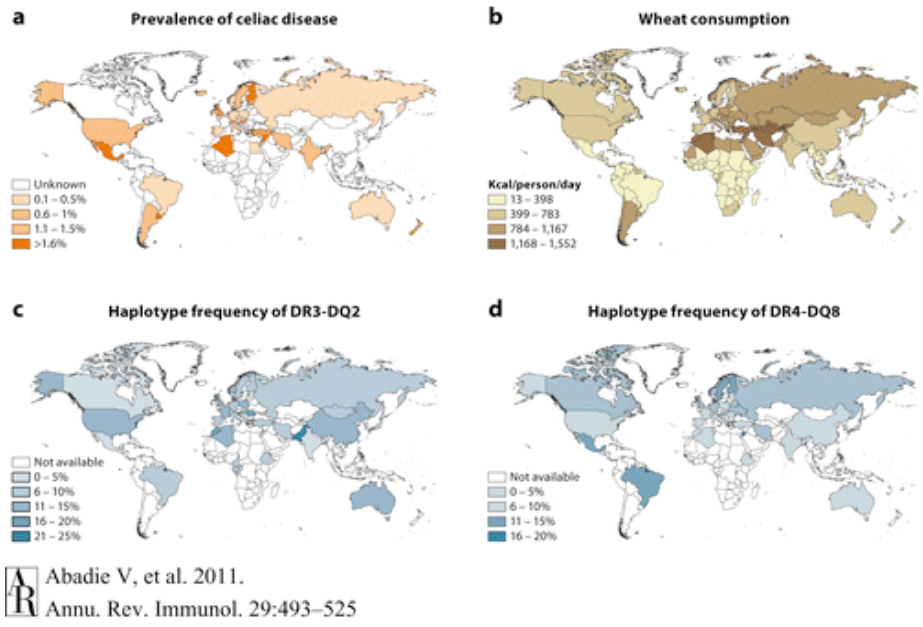


Fig 3. Distribution of prelvalence of celiac disease and aplotypes DQ2 and DQ8*. Abadie V., 2011.*

However, the activation of the adaptive immune response remains a pivotal event in the pathogenesis of celiac disease.

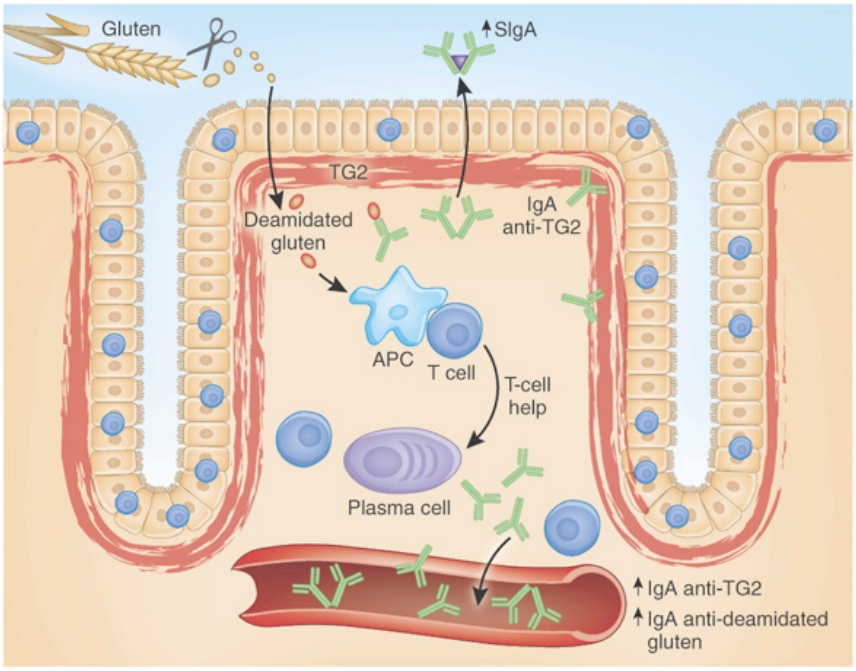
Generally, antigens presented by APCs are recognized as foreign-antigens by cells of the immune system, which active a cascade of events, including inflammatory processes. In the case of celiac disease, the antigen presented to T cells is derived from gluten proteins, in particular from one of its component.

Gluten is a blend of different proteins; among these, gliadin is the most abundant and it is subdivided in α, β and γ gliadins, composed of different peptides which differ the sequence of aminoacids. The most part of these peptides result resistant to digestion by gastrointestinal proteases; for example the 33mer peptide, from residues 57 to 89 of α-gliadin, is considered a celiac "superantigen" because it is a strong stimulating agent of T cells maturation *(Shan L et al., 2002).* 31-43 peptide (from residues 31 to 43 of α-gliadin), instead, is considered a direct toxic agent for intestinal mucosa since it can lead its cells to apoptosis *(Picarelli A et al., 1999).*

Once gluten peptides have crossed the intestinal barrier, they can be further processed in the submucosa; due to their high content in glutamine, proline and hydrophobic amino acid residues, gluten proteins are preferred substrates for transglutaminase 2 enzyme (TG2). When de-amidated, most of the resultant negatively charged gluten peptides bind more strongly to HLA-DQ2 (or HLA-DQ8), fact which leads to a more rigorous gluten-specific CD4+ Th1 T-cell activation in the lamina propria.

Thus T-cells act as central effector cells of the intestinal inflammation resulting in crypt hyperplasia, villus atrophy, loss of tight junctions of intestinal epithelium and release of auto-antibodies; for this reason, celiac disease is also considered an auto-immune disease *(Schuppan D et al., 2009).*

Fig 4. Pathogenesis of Celiac Disease. *Schuppan D., 2009.*



2.2. SYMPTHOMS OF CELIAC DISEASE

The prevalence and the incidence of celiac disease (CD) is continuously growing. A published international study investigated a wide population sample in different European countries: the overall prevalence of CD was 1% on average, with large variations between countries, for example in Italy it was estimated about 1,5%; similar rates have been reported from the US population and from developed countries as Australia and New Zealand, and also in the Far East, which is changing alimentary customs, the rate is increasing *(Catassi C et al., 2012).*

The epidemiology of CD is efficiently conceptualized by the “iceberg model”: the overall of cases is the head of the iceberg, which is influenced not only by the frequency of the predisposing genotypes in the population but also by the pattern of gluten consumption. In developed countries, however, for each diagnosed case of CD, an average of five cases remains undiagnosed (the submerged part of the iceberg), usually because of atypical, minimal, or even absent complaints. These undiagnosed cases remain untreated, leaving individuals exposed to the risk of long-term complications, such as enteropathy-associated lymphoma, bowel adenocarcinoma, and other cancers of the gastrointestinal tract *(Lionetti E et al., 2011).*

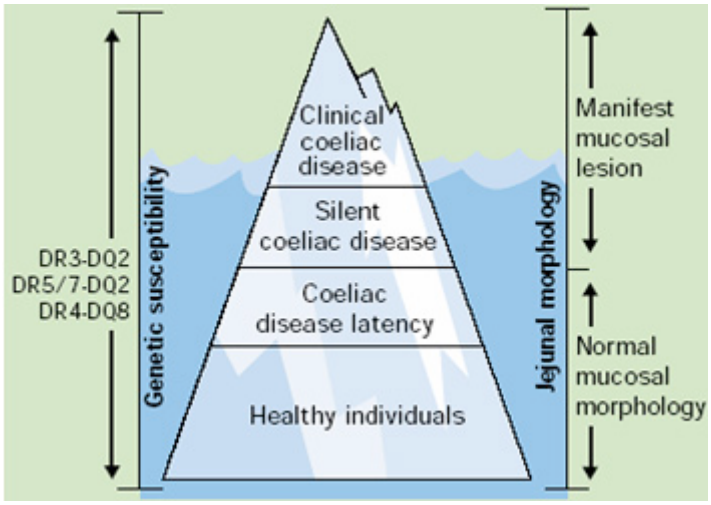


***Fig 5. Celiac Disease Iceberg**. Maki M., 2007.*

To describe the symptoms of CD is very complicated since clinical manifestations are different according to age of presentation: young children often present CD with "classic" symptoms including diarrhea, abdominal pain, malabsorption and growth retardation, whereas adults patients can have various clinical manifestations, intestinal and extraintestinal. Moreover, CD is frequently found in conjunction with other diseases, such as type 1 diabetes mellitus, autoimmune thyroiditis, dermatitis herpetiformis, osteoporosis and neurological disorders *(Diamanti A et al., 2014).* At histological level, CD patients display various degrees of intestinal inflammation, ranging from intraepithelial lymphocytosis to severe lamina propria mononuclear cell infiltration resulting in total villous atrophy coupled with crypts hyperplasia.

Patients can be classified according to the severity of the histological lesion using the Marsh-Oberhuber scale *(Oberhuber G, 2000).*

- MARSH 1: villus morphology is normal but there is an abnormal number of intraepithelial lymphocytes.
- MARSH 2: intraepithelial lymphocytosis is extended to lamina propria and cells of the intestinal crypts show an altered morphology and an increased mitotic activity.
- MARSH 3: this is the most severe grade of the disease; patients show an increase in apoptosis of intestinal cells, crypts hyperplasia and villus atrophy. These kind of patients could be divided in 3 sub-groups:

- 3A: villus show a soft atrophy (average height 300 micron) with a pathological increase of intraepithelial lymphocytes.
- 3B: villus show a moderate atrophy (average height between 150 and 300 micron) with a persistent increase of intraepithelial lymphocytes.
- 3C: villus are completely atrophic (average height less than 150 micron) with a severe increase of intraepithelial lymphocytes in the lamina propria.

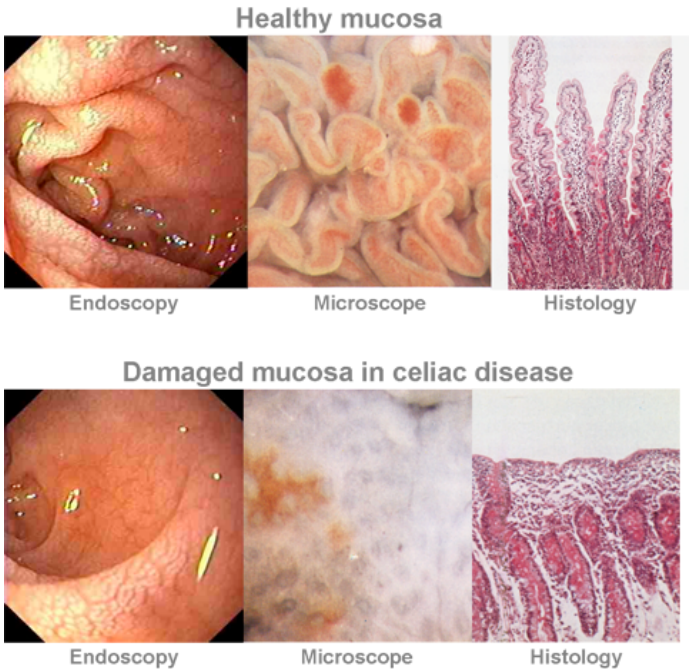


Fig 6. Endoscopy, microscopy and histology of duodenal mucosa. *Healthy subject compared to CD patient (Marsh 3C).*

2.3. IMMUNE SYSTEM

Small intestine has an important role in the immune mechanisms considering innate and adaptive immunity, as well as in the processes leading to the development of tolerance. Firstly, when pathogenic agents reach the intestine, immune cells of intestinal lymphoid tissues, such as Peyer's patches, activate a cascade of events that lead to an inflammatory response. Other structures involved in immune response are M cells, situated above the patches, which have clathrin-dependent receptors that link antigens and move them to the basolateral membrane where they are presented to macrophages and lymphocytes. In the intestinal mucosa, there are also isolated lymphoid follicles which increase their quantity during chronic inflammation *(Cheroutre H, 2006).*

Regarding the immune system involved in celiac disease, both innate and adaptive immunity should be considered.

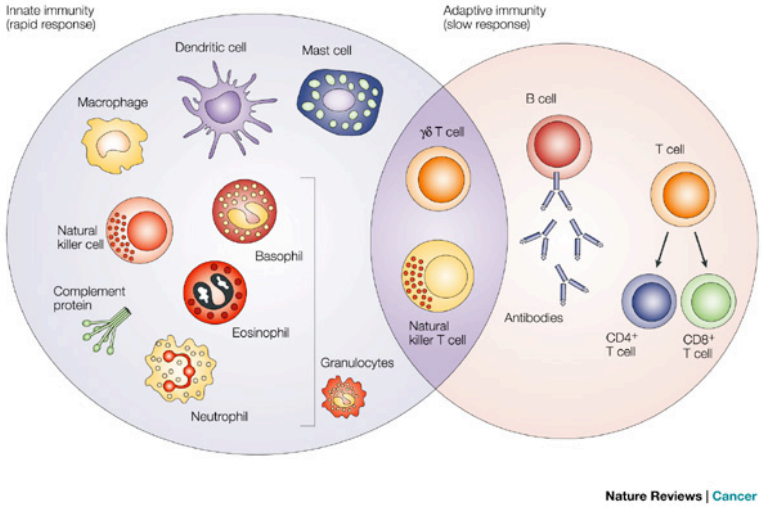


Fig 7. Innate and Adaptive Immunity. *Dranoff G., 2004.*

Innate immunity contributes to the pathogenesis of CD, mainly causing a damage in the tight junctions, fact that facilitates the passage of gliadin peptides through the intestinal barrier. The mechanism at the bottom is an immune process caused in particular by the 31-43 peptide of gluten, which stimulates macrophages and dendritic cells to secrete IL-15. This, in turn, leads to the activation of intraepithelial lymphocytes (IELs) and NK cells proliferation, causing IFNγ production, epithelial damage and cytotoxicity *(Maiuri L et al., 2007).* Other pivotal elements of innate immunity are pattern recognition receptors (PRRs), as Toll-Like Receptors (TLRs), which can induce the expression of various inflammatory genes in response to microbial components. These receptors have a role not only in gut immunity, but also in the control of barrier function and healing during intestinal inflammation, through the increased expression of pro-inflammatory cytokines and chemokines such as CXCL2 *(Walsh D et al., 2013).* Another group of PPRs are nucleotide-binding oligomerization domain-containing proteins (NODs) and among these a central role is played by NOD2, a cytosolic protein which recognizes peptidoglycan of the bacterial membrane and activates the NF-kB pathway, inducing the production of pro-inflammatory mediators such as Tumor Necrosis Factor (TNF), Interleukin 6 (IL-6) and Interleukin 8 (IL-8)

(Walsh D et al., 2012). These cytokines activate a cascade of events that leads to an excessive immunity response and to the demolition of the intestinal mucosa.

As already mentioned, adaptive immunity has been widely evaluated in particular considering the interactions between gliadin peptides and Human Leukocyte Antigen/ T-Cell Receptor (HLA/TCR). However, each immune response involving B cells, T cells and in particular Tregs (and the relationship between them) is crucial for the quality and the power of the response. T cell differentiation is carried out into the thymus where TCRs lead to the switch from peripheral Native T cells to Tregs for the highly specific identification of thymus antigens. However, this switch could be a consequence of a pro-inflammatory environment determined by high concentration of TGFβ and retinoic acid *(Picca CC et al., 2006).* After the differentiation, mature Tregs move to the lamina propria and to the lymphoid structures of the small intestine where their principal role is suppressing the reactivity of self T cells at the peripheral level, contributing to eliminate reactive T cells which escaped to the thymus control. In particular, they produce and release anti-inflammatory cytokines such as IL-10, which reduces the ability of monocytes and dendritic cells to present the antigens and inhibits the synthesis of pro-inflammatory agents. Moreover, Tregs produced TGF-β1, a cytokine with a double function in the immune response: it inhibits T cells response, promotes Th17 differentiation stimulating IL-22, which intervenes as regulator of intestinal epithelium re-shuffling and, on the other hand, activates Tregs to control the balance of immune response. Finally, Tregs induce the apoptosis of effector T cells through the expression on their surface of cytotoxic molecules such as CTLA-4, which limit the antigen presentation and the activation of self T cells *(Sakaguchi S et al., 2015).*

Tregs are characterized by the expression of FOXP3, a transcriptional factor essential for their development and functions. FOXP3 gene is situated on the chromosome X and it is involved in different pathways, many of them not completely clarified. It codifies for FOXP3 protein, necessary to the development and the maturation from lymphocytes T to Treg cells, determining the control of immune response, awarding to T cells all the regulatory features necessary to suppress Th1 (including IL-10 secretion) cells *(Zheng Y et al., 2007).* It has been demonstrated that FOXP3 interacts with different transcriptional factors such as NFAT, MAL1, RUNX1 and NF-kB. In

particular, the interaction with NFAT is necessary to carry out FOXP3 function in stimulating the expression of IL2, IL4, CTLA4 and the other gene involved in lymphocytes T activation. *(Wu Y et al., 2006)*. In CD pathogenesis, FOXP3 shows an uncontrolled expression that probably leads to a loss of Treg regulatory ability and an unbalance intestinal immunity, but a dysregulation of its expression could also have a role in the development of auto-immune diseases such as type 1 diabetes, Systemic Erythematous Lupus or Inflammatory Bowel Diseases.

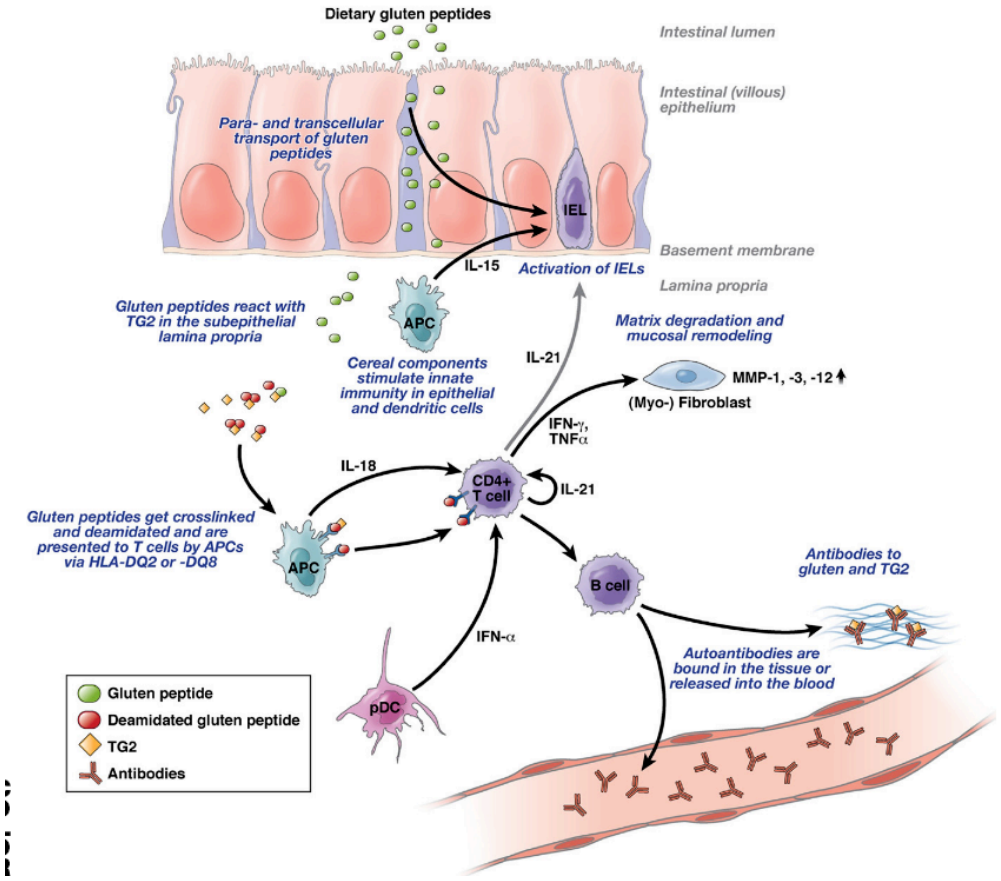


***Fig 8. Immunity in celiac disease**. Schuppan D., 2009.*

2.4. DIAGNOSIS AND THERAPY

In the past, CD diagnosis was entirely based on the detection of typical gastro-intestinal symptoms and confirmed by the small intestinal biopsy; later, the availability of sensitive and specific serological tools improved the possibility to identify asymptomatic cases *(Lionetti E et al., 2011).* Currently, the best approach to improve the diagnostic rate is a process of case finding focused on at-risk groups.

Diagnosis is currently based on serological test to detect:

- Anti-tissue-transglutaminase antibodies (tTG): this test identifies the presence of antibodies against transglutaminase 2, responsible for gliadin peptides deamidation, which increase their ability to trigger the immune response. It is the most sensitive blood test for celiac disease.
- Anti-endomysium antibodies (EMA): endomysium is the tissue which covers the muscular cells of intestine; the antibody are release dependent on the mucosal damage. The EMA test has a specificity of almost 100%, but is not as sensitive as the tTG-IgA test. About 5-10% of people with celiac disease do not have a positive EMA test.

However, the diagnosis of Celiac Disease is still based on endoscopy for a morphological analysis of intestinal biopsy, to detect the peculiar lesions of mucosa and identify, by Marsh' scale, the gravity of the intestinal damage.

All these diagnostic tools are employed also in pediatric patients, even though performing endoscopy in small children could be quite an invasive technique. For this reason, recent guidelines of ESPGHAN (European Society for Pediatric Gastroenterology Hepatology and Nutrition) declared that in the presence of high antibody levels (> 10 times upper limit of normal) the diagnosis of CD could be based on a combination of symptoms, antibodies, and HLA, thus omitting the duodenal biopsy *(Husby S et al., 2012).*

Considering the possible therapies for CD patients, the only currently available treatment is a lifelong gluten-free diet; although there has been an improvement of gluten-free food and restaurants, a strict adherence to the diet could compromise social well-being and the quality of life. Recent studies have suggested alternative methods for therapy, for example techniques to reduce gluten immunogenicity as OGM variants of wheats *(Kiyosaki T et al., 2007),* enzymes which cut immuno-toxic gluten regions, antibodies which neutralize toxic activity of gluten peptides *(Pinier M et al., 2009)* other studies proposed the inhibition of intestinal permeability by the using of an homologue peptide of ZOT toxin, produced by intestinal lesions, which competes for the link to the receptors, reducing its toxicity *(Watts T et al., 2005).* Finally, some studies suggested various methods to block the cascade of adaptive or innate immune response *(Bolin DR et al., 2000).*

2.5. miRNA AND INTESTINAL IMMUNITY

The dysregulation of molecules involved in the immune system represents a major event in the pathogenesis of various autoimmune diseases, and all the processes involved in its regulation could affect the clinical manifestation as well as the severity of these disorders. Investigations revealed that the expression of specific sets of miRNAs is dynamically regulated during immune cell development; for example miR-142 is involved in the development of T cell as demonstrated by Chen et al. (*Chen CZ et al., 2004), who* infected lineage-hematopoietic progenitor cells from mouse bone marrow with viral vectors expressing miR-142s, causing an altered differentiation with an increase in the T lymphoid lineage. Similar to that, a study analyzing systematically miRNA expression in cells of the murine hematopoietic system, identified miR-150 as a miRNA that was selectively expressed in mature cells, although it was up-regulated during B and T cell maturation and suppressed during the activation of $CD4^+$ and $CD8^+$ cells *(Monticeli S et al., 2005).* Furthermore, an intricate network of signaling facilitates the maturation of the adaptive immune system. The appropriate development and function of T and B cells is crucial in order to distinguish foreign antigens from self, and recent studies have shown that miRNAs are involved in various stages of their maturation and activation. For example, miR-181 family plays an important role in T cell lineages resulting in cytokine production with a marked bias towards Th1 development and IFN-γ production *(Galicia JC et al., 2014).* However, various miRNAs seem to contribute to Th1 and Th2 cell differentiation: overexpression of miR-155 influences CD4+ T cells to differentiate into Th1 cells while deficiency in miR-155 shows a bias towards Th2 differentiation *(Seddiki N et al., 2014).*

In addition, other authors, evaluating the effect of miRNAs on the differentiation and function of Th17 pathway, have identified direct and indirect regulatory mechanisms *(Honardoost MA et al., 2015).* This regulation involves also Tregs, the main mediators of adaptive tolerance, in which a signature composed of five miRNAs (21, 31, 125a, 181c and 374) that influenced the expression of Tregs-specific markers has been identified *(Rouas R et al., 2009).*

The innate immune system is the first defense against pathogens and relies primarily on early antigen recognition initiated by pathogen associated molecular patterns triggering extracellular

receptors (Toll-Like Receptors) or intracytoplasmic NOD-like receptors and downstream signaling cascades through diverse pathways including NFκB and IFN regulatory factors. Recent studies demonstrated that miRNAs actively regulate also these processes: for example the use of miR-23b induces immune tolerance through the inhibition of the Notch and NF-kB signaling pathways, with a role in improving the management of allergic diseases *(Zheng J et al., 2012).*

miRNA regulation does not involve just the immune system, but has a role in the development and differentiation of tissues and organs, including the intestine. In this case, some miRNAs seem to have a predominant role, as detected by McKenna et al. *(McKenna LB et al., 2010),* who analyzed the profile of expression of miRNAs in the intestinal mucosa, identifying 53 miRNA-families involved, including miR-192 that showed the predominant expression. The miRNA post-transcriptional control seems essential for the correct development of the intestine, since the analysis of mice knockout for Dicer, revealed that intestinal miRNAs had a role in determining epithelial architecture and cells migration *(Tili E et al., 2008).* Considering in particular intestinal immune system, a panel of miRNAs has been studied in inflammatory bowel diseases (IBD), disorders characterized by an altered activation of the immune system. Kalla et al. identified an IBD expression profile which included miR-192, miR-122, miR-29 and miR-146a; in particular, miR-192 could be regulating NOD2 expression *(Kalla R et al., 2015).* Another study, conducted on a pediatric population, identified a miRNA signature in ulcerative colitis, providing evidence that miR-4284 directly regulates CXCL5, a chemokine that resulted up-regulated in the affected tissues, further supporting the regulatory role of miRNAs in intestinal immune mechanisms *(Koukos G et al., 2015).*

3. HYPOXIA AND IRON METABOLISM

3.1. HYPOXIA

Hypoxia is commonly defined as a reduction in oxygen availability and it is considered a physiological stimulus that triggers both adaptive (homeostatic) and pathological situations. The duration of hypoxia can be acute or chronic: the first one induces fast but brief responses, conversely, chronic hypoxia induces delayed but more prolonged responses, but both of them are based on modifications of gene expression. Basically, hypoxic conditions trigger adaptation responses, which vary according to the type and duration of hypoxic stimulus, and that aim to limit the damage inflicted by low oxygen levels and to restore normal ones. Hypoxic condition may involve the entire organism, as in anemic patients or when the subjects ascend to high altitude, or it can be restricted to certain areas within a specific organ, as it happens in many ischemic cardiovascular diseases or in the inner region of most solid tumors. Systemic hypoxia increases blood pressure and breathing: kidneys and liver, triggering the production of erythropoietin (EPO), stimulate bone marrow erythropoiesis, increasing the blood oxygen transport capacity. Local response is characterized by the expression in tissues of high levels of angiogenic growth factors, such as vascular endothelial growth factors (VEGF), which stimulate the growth of new blood vessels. Moreover, also iron homeostasis is influenced by hypoxia conditions, since the request of more blood afflux determines the mobilization of stored iron and the increase of duodenal iron absorption, with consequent activation of molecules involved in these mechanisms as transferrin, ceruloplasmin, ecc*. (Piperno A et al., 2011).* Decreased oxygen availability also induces important changes at the cellular level, in particular inducing a shift from oxidative to glycolytic metabolism (anaerobic), also stimulating the transcription of related genes in order to improve the ATP generation; moreover it is required to maintain redox homeostasis, since mitochondria are the main source of reactive oxygen species (ROS), and this is paradoxically more true when oxygen tension is low *(Greco S et al., 2014).*

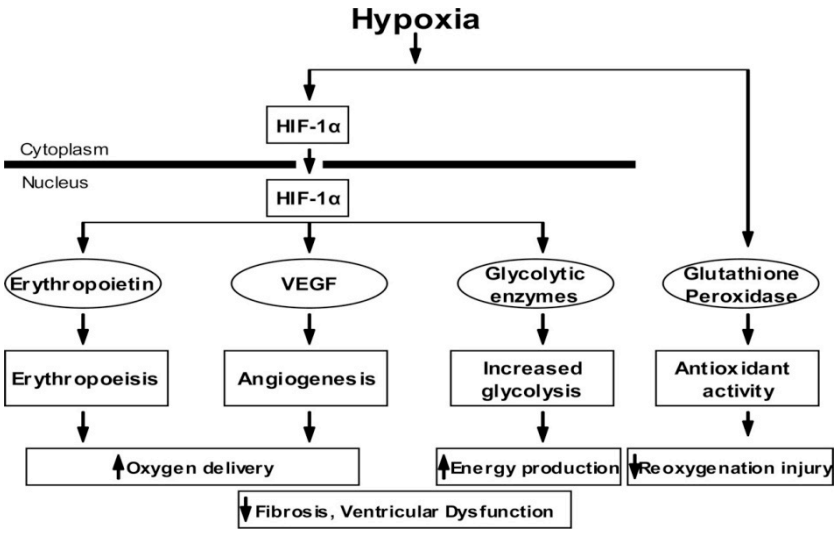


***Fig 9. Molecular effect of hypoxia**. Reddy S., 2006.*

In the control of all these hypoxia-dependent processes a pivotal role is played by HIFs (Hypoxia Inducible Factors); HIFs are transcription factors, composed by α and β subunits that bind to the hypoxia-response elements (HREs) on gene promoters, controlling their transcription. They are oxygen-dependent: under normoxia, HIF-subunits are targeted for proteasome degradation linking Von-Hippel Lindau (VHL) factor *(Peyssonaux C et al., 2007)* whereas under hypoxia, they regulate the transcription of different genes also involved in iron homeostasis.

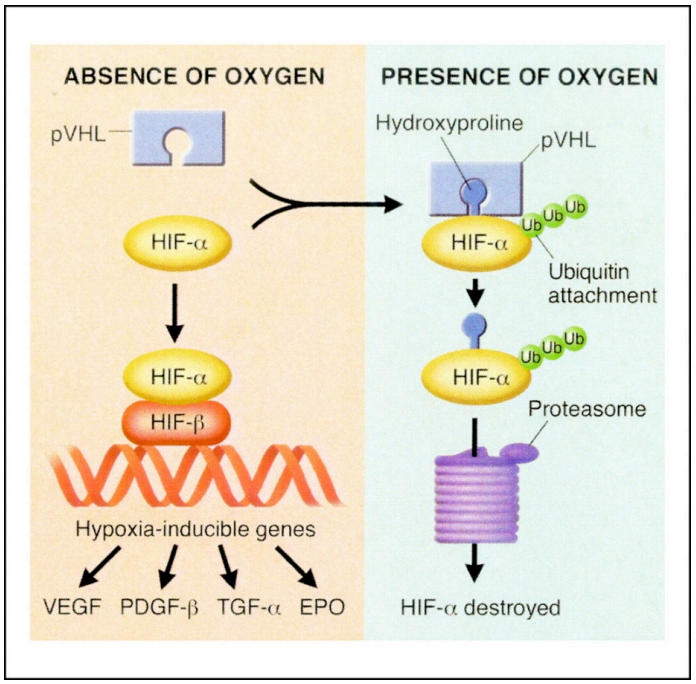


***Fig 10. HIF factors.** Brugarolas J., 2007.*

3.1.1. miRNA AND HYPOXIA

Considering their ability in the regulation of gene expression, miRNAs have a main role in the control of various processes, including those that take place in hypoxic conditions *(Ivan M et al., 2008).* In fact, experimental studies have identified several important mechanisms by which hypoxia regulates miRNA expression and activity *(Nallamshetty S et al., 2013).* Hypoxia-depended miRNAs could be divided into 2 groups: the first one is composed of a number of miRNAs called "hypoxamirs" that promote HIF expression and/or its activity through positive feedback circuits that support HIF-dependent hypoxic adaptation *(Loscalzo J et al., 2010).* Some of these miRNAs are direct transcriptional targets of HIF itself during hypoxia, due to the presence of HREs in their respective promoters. Indeed, in silico analysis of the putative promoter sequence of these hypoxamirs demonstrated a significant enrichment for the presence of HREs compared to the promoters of 23 randomly selected, unrelated miRNAs. Experimental validation was performed on a group of these hypoxamirs, including, miR-24–1, miR-26, miR-103, miR-181, miR-210, and miR-213, using genetic approaches, promoter-reporter assays, and chromatin immunoprecipitation (ChIP) *(Kulshreshtha R et al., 2010).*

The second group of miRNA regulated by hypoxia includes those not directly dependent on HIF regulation. In fact, although HIFs play a predominant role in the coordination of transcriptional changes during hypoxic stress, several other transcriptional factors execute important regulatory activities in the context of oxygen deprivation. For example, hypoxia can potently induce expression of p53, which can directly induce miR-210 expression as demonstrated in HIF-β knockout mouse embryonic fibroblasts under hypoxic conditions *(Cummins EC et al., 2005).* Hypoxic stress can also activate NF-κB, and it has been demonstrated by ChIP, promoter-reporter assays and gene knockdown studies, that NF-κB directly interacts with and transactivates the miR-210 promoter under hypoxic conditions *(Zhang Y et al., 2012).* As such, although early studies on hypoxia-mediated regulation of miRNA largely focused on transcription factors (TFs), subsequent works quickly recognized that transcriptional control of miRNA expression comprises only part of the narrative of hypoxamirs regulation. Hypoxia prompts specific changes in miRNA expression in a time frame that is too rapid to be explained solely by TF action; emerging evidences indicated

that hypoxia modulates several other phases of miRNA biogenesis, maturation, and function, including post-transcriptional modifications mediated by miRISC stability and activity.

For example, hypoxia regulates complex processes containing Drosha and Dicer, to mediate dynamic changes in hypoxamirs maturation and function.

The down-regulation of Dicer under chronic hypoxia is an adaptive mechanism that serves to maintain the cellular hypoxic response through HIF-α, providing an essential mechanistic insight into the oxygen-dependent microRNA regulatory pathways. Ho et al *(Ho JJ et al., 2012)* demonstrated that the down-regulation of miR-185 under chronic hypoxia is Dicer-dependent since mature miR-185 levels fell under chronic hypoxia, whereas its precursors were accumulated. Moreover, overexpression of miR-185 in hypoxic HUVEC resulted in the reduction of endogenous HIF-2α protein and mRNA levels; this interaction was verified by firefly luciferase-HIF-2α 3′-UTR chimeric reporters and it was abolished when wild-type 3′-UTR with mutations in both miR-185 binding sites was used.

Therefore, regulation of miRNA expression under hypoxia can be broadly categorized as HIF-dependent and HIF-independent.

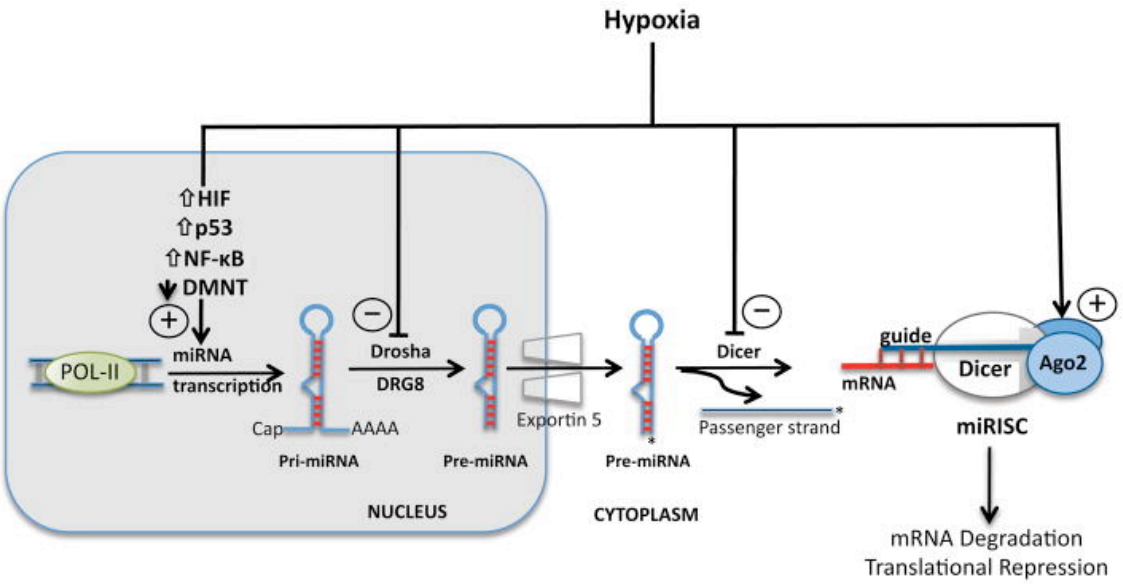


Fig 11. miRNA hypoxia-dependent. *Nallamshetty S., 2013.*

3.2. IRON

Iron in cells has a pivotal role: it is essential for metabolism, cellular processes, energy production, biosynthesis, replication and *l*ocomotion. In humans, erythropoiesis is the biological process with the highest demand for iron because of its requirement to heme synthesis and incorporation into hemoglobin molecules. The most common iron forms in the human body are ferrous (Fe2+) and ferric (Fe3+); under physiological O_2 concentrations the most stable form of iron is Fe3+ and the reduction of O_2 by Fe2+ results in the formation of superoxide radicals. All the mechanisms involving iron include the dietary iron absorption by the duodenum, transport in the circulation, cellular uptake and consumption, recycling by macrophages and storage in the liver *(Silva B et al., 2015).*

3.2.1. IRON UPTAKE, TRANSPORT AND STORAGE

Non-heme iron is mostly presented into the organism on its oxidized form (Fe3+).

In order to be absorbed by the enterocyte, it is reduced to Fe2+ by proteins present on the apical membrane, facing the gut lumen, that facilitate iron reduction such as duodenal cytochrome b (DcytB) *(McKie AT et al., 2001).*

Fe2+ is imported by Dmt1, a transmembrane protein that takes advantage of the proton gradient existing between the gut lumen and the enterocyte cytoplasm, and then is driven to the basolateral membrane of the enterocyte or stored in ferritin *(Sharp PA et al., 2010).* The export of iron to the circulation is a crucial step for the entrance of iron in the body: the enterocytes express on their basolateral membrane the protein Fpn1, the only known mammalian iron exporter. Fpn1 transports Fe2+ to the extracellular side of the basolateral membrane, where it is oxidized in Fe3+ by the ferroxidases, hephaestin (HEPH) and ceruloplasmin (Cp), in order to be associated with the circulatory transferrin (Tf). Tf is found in the plasma in three states: apo-transferrin (apo-Tf), when no iron is bound, monoferric transferrin (bounded to a single iron atom) and diferric transferrin, also known as holotransferrin (holo-Tf); the cellular uptake of iron-TF is mainly mediated by the transferrin receptor 1 (TfR1), located at the cell membrane, through a clathrin-dependent endocytosis. Then, the endosome pH decreases by the entry of H+ mediated by an ATP-

dependent proton pump, and Fe3+ is released *(Hentze MW et al., 2010).* The organism has a high requirement for iron, thus an effective iron storage methods is necessary; the major protein responsible for iron storage is ferritin (Ft*)* a 24-subunit multimer of heavy (Ft-H) and light (Ft-L) polypeptide chains that forming a spherical capsular structure that is able to store up to 4500 iron atoms on its core *(Liu X et al., 2005)*.

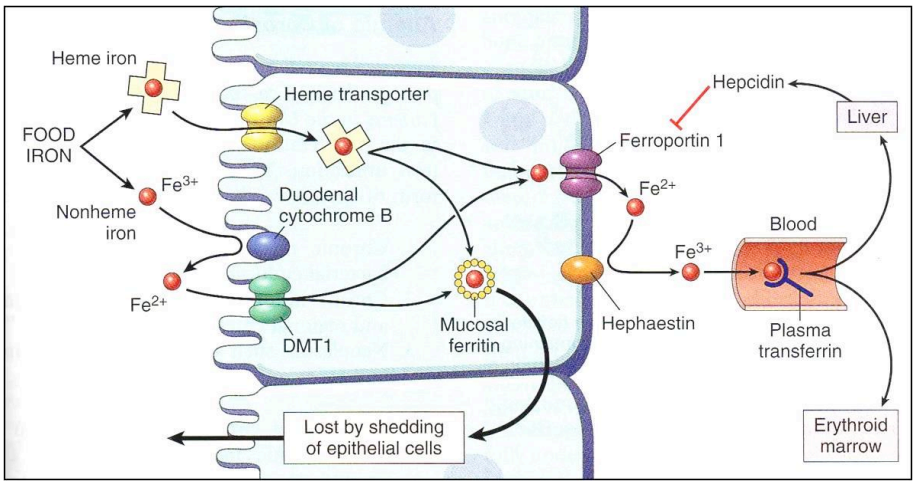


Fig 12. iron homeostasis. *Saunders D., 2006.*

3.2.2. IRON HOMEOSTASIS: IRE-IRP AND HAMP

The maintenance of iron homeostasis and the balance between iron released and stored is crucial for the control of all the processes of human organism; it could be carried out in two different ways: at cellular level, protein (IRP)/iron responsive element (IRE) system controls both mRNA stability and translation of transcripts coding for proteins involved in iron uptake, export and transport. IRP proteins have a [4Fe–4 S] cluster: if iron is abundant, the cluster forms its active site and it blocks the binding with the IRE regions present in the UTRs of gene involved in iron release. When iron is scarce, the cluster does not form and IRPs can bind to IREs, providing a block when the IRE is found in the 5′UTR and increased mRNA stability when found in the 3′UTR of the mRNA. An example are the ferritin and the transferrin (TfR) genes: the mRNAs of ferritin chains have one IRE in their 5′UTR, whereas the TfR1 gene has five IREs in its 3′UTR. The binding of IRPs to these IREs enhances TfR1 mRNA stability and blocks ferritin mRNA translation, facilitating iron uptake to an intracellular iron for metabolic use *(Chepelev NL et al., 2010).*

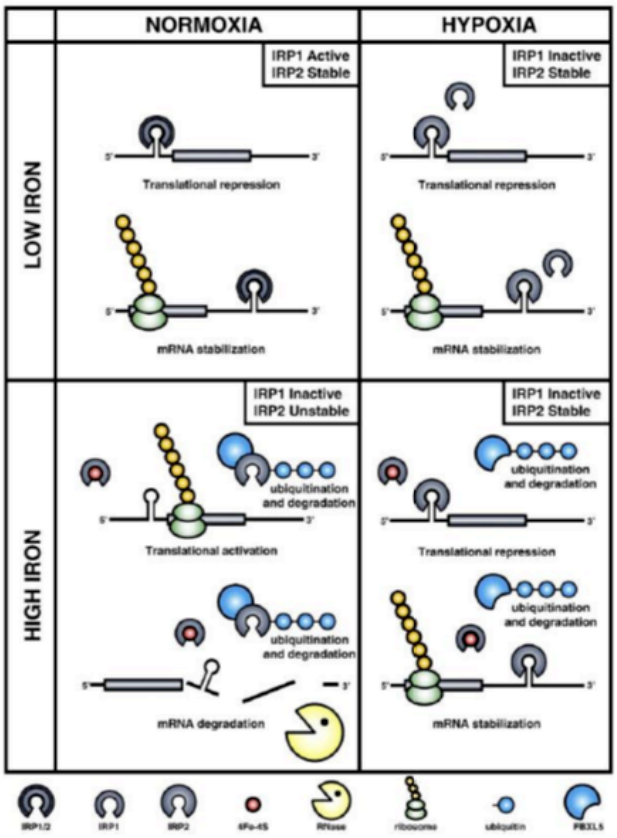


Fig 13. IRP/IRE mechanism. *Chepelev NL., 2010.*

The second mechanism for the systemic regulation of the iron, involves hepcidin, a 25 amino-acid peptide hormone mainly expressed by the liver *(Hentze MW et al., 2010).* Hepcidin is encoded by the HAMP gene located at the long arm of chromosome 19; it is firstly synthesized as a biologically inactive 84 aa pre-proprotein, and its maturation is performed by proteolytic cleavage mediated by the convertase furin. Hepcidin acts by triggering Fpn1 internalization and consequent degradation; therefore hepcidin regulates systemic iron metabolism by reducing Fpn1 levels and consequently, reducing dietary iron uptake and release from macrophages, hepatocytes and other cell types *(De Domenico I et al., 2011).*

HAMP transcription is up-regulated by high iron levels but also by other stimuli: iron overload, infection and inflammation up-regulate HAMP expression, whereas iron deficiency, hypoxia, anemia and erythropoiesis act on the opposite way *(Nicolas G et al., 2002).*

3.2.2.1. HAMP REGULATORS

IRON-DEPENDENT HAMP REGULATORS: BMPs and TGFβ PATHWAY

HAMP expression is finely controlled; one of the pathways involved in HAMP regulation depending by iron is the Bone Morphogenetic Protein - Hemojuvelin (BMP/HJV or BMP/SMAD) axis (*Kautz L et al., 2008)*. HJV is the co-receptor of BMP receptors, which are of different types; the activated BMPRs type I (BMPR1a or ALK3, BMPR1b or ALK6 and ALK2) make a dimerization with BMPR type II (BMPR2) and induce the phosphorylation of cytosolic Smad 1/5/8 which form complexes with Smad4. After its formation, this complex translocates into the nucleus, binds to the BMP responsive elements (BMPREs) present on HAMP promoter and induces its transcription. A negative regulators of this pathway is matriptase-2 (TRPRSS6) which, cutting HJV in its soluble form, inhibits the expression of HAMP *(Miyazono K et al., 2010).* The ligands of this pathway are the BMPs, in particular, those identifies as activators of HAMP expression are BMPs 2, 4, 5, 6, 7, and 9 *(Zhang AS et al., 2010).* The pivotal one, however, seems to be BMP6, since only the knockout in mice of BMP6 drives to an iron loading phenotype similar to the ones observed for the knockouts of HJV and HAMP, revealing its high importance in iron metabolism regulation. The expression of BMP6 occurs majorly in the liver; however, while hepcidin is synthesized by the hepatocytes, BMP6 expression in response to iron occurs preferentially at non-parenchymal liver cells, such as sinusoidal endothelial cells, hepatic stellate cells and Kupffer cells *(Andriopoulos B et al., 2009).* So, whenever iron levels increase in the body, BMP6 is produced by these cells, and secreted to the interstitial environment, where it binds to the HJV in hepatocyte, triggering HAMP expression.

There are also other molecules iron-dependent involved in HAMP regulation, for example the hereditary hemochromatosis protein HFE, as well as TfR1 e TfR2. HFE protein binds TfR1 competing for the link of transferrin-bound-iron (Tf-Fe) and diminishing iron entrance into the cells. In basal conditions, TfR1 abducts HFE blocking its ability to activate HAMP; when iron is abundant, HFE does not link TfR1 but TfR2, determining HAMP transcription *(Gao J et al., 2009).* This

pathway is not completely independent from the BMP/SMAD one, and it has been suggested a cross-talk between them: in fact TfR2 knockout mice failed to increase Smad 1,5,8 phosphorylation and HAMP expression despite iron overload and increased BMP6 expression. This result suggests that TfR2 interact with HFE and HJV forming a complex with ALK3 depending on iron status and regulating the downstream Smad and HAMP activity *(Wu XG et al., 2014).*

TGFβ pathway parallels the BMP/SMAD one, since its ligand, TGFβ, binds to its receptors TGFβr, activates through phosphorylation Smad 2/3, which make a complex with Smad 4, enters into the nucleus and stimulates the transcription of different genes, including HAMP *(Whang RH et al., 2005).* Furthermore, activation of Smad2/3 could also depend on Activin, a member of TGFβ family, which binds Activin Receptors type I and II, phosphorylating Smad 2/3 and activating all the cascade to HAMP transcription *(Besson-fournier C et a., 2012).*

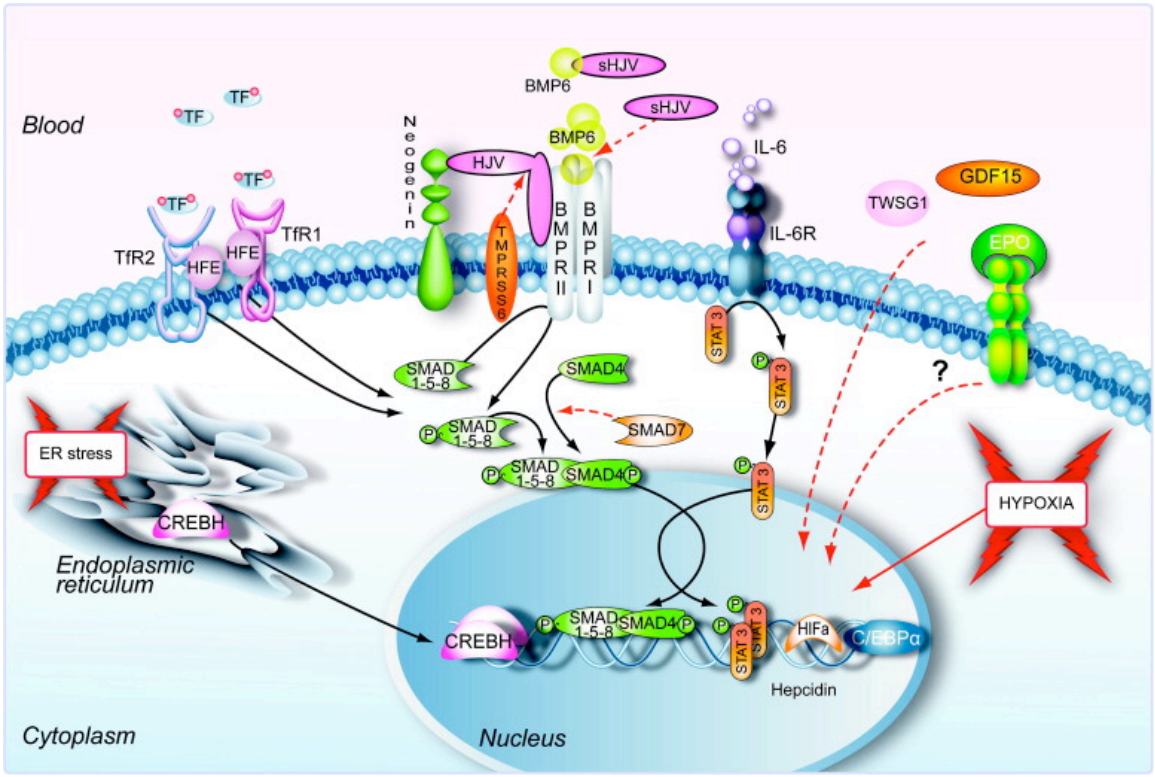


Fig 14. Regulation of iron metabolism. *Pietrangelo A., 2011.*

TRANSCRIPTIONAL FACTORS HAMP REGULATORS

HAMP transcriptional regulation involves also other transcriptional factors; for example it has been identified a binding site on HAMP promoter for CCAAT/Enhancer Binding Protein (C/EBP), belonging to a transcriptional factor family involved in the proliferation and differentiation of various cells, which induces HAMP transcription, and for Hepatocyte Nuclear Factor 4 alpha (HNF4α) which reduces its expression *(Trouksa J et al., 2007).* Patel et al. identified another interesting transcription factor involved in the modulation of HAMP transcription, ATOH8, which has a double role in iron balance: it binds the E-box region within HAMP promoter, stimulating its transcription, and in addition induces an increase in the phosphorylation of Smad 1/5/8, indirectly stimulating HAMP transcription *(Patel N et al., 2014).*

ERYTHROPOIETIC HAMP REGULATORS

There are various pathways EPO-dependent that are implicated in HAMP regulation; erythroid cells are the main consumers of body iron, for this reason the inhibition of HAMP by erythroid signals is crucial for the physiological homeostasis of the organism; nevertheless molecular mechanisms and proteins involved in this process are not completely clarified *(Ravasi G et al., 2014).* One of the hypothesis considers Growth Differentiation Factor 15 (GDF15) and Twisted Gastrulation (TWSG1), both released from erythroid precursors; the first is a member of TGFβ superfamily, secreted by the final phases of erythroid maturation; in vitro studies on primary hepatocytes demonstrated that the stimulation with high concentration of GFD15 determine a down-regulation of HAMP, even if the basis mechanisms are not completely understood *(Lakhal S. et al., 2009).* Moreover, a recent study showed that GFD15 expression is negatively regulated by intracellular levels of iron, with a HIF-independent process, suggesting the regulation of HAMP expression through a new pathway iron- and oxygen-dependent.

TWSG1, instead, is produced by immature erythroblast cells, during the first phases of the maturation process; the relationship between TWSG1 and HAMP has to be studied yet, but a study suggested its role in HAMP inhibition through the down-regulation of BMP/SMAD pathway *(Tanno T et al., 2009).*

INFLAMMATION-DEPENDENT HAMP REGULATORS

Whenever mechanisms for the control of iron homeostasis are perturbed, due to genetic or environmental factors, iron overload or iron deficiency pathologies may arise. For example, during infection, pathogens are recognized as foreign elements by several cell types, such as macrophages. This recognition triggers the expression and secretion of the pro-inflammatory cytokines interleukin IL-6, IL -22 and INF type 1 *(Lee P et al., 2005)*. They generate a defense mechanism that tends to reduce iron available to pathogens; in particular it has been demonstrated the ability of IL-6 to regulate HAMP transcription, acting on ALK3 (BMPr type 1) *(Mayeur C et al., 2014).* In this manner, IL-6 activates the transcription factor STAT3, its translocation to the nucleus and the link on the binding sites in the HAMP promoter triggering the expression of this gene and reducing iron mobilization *(Wrighting DM et al., 2006).*

HYPOXIA-DEPENDENT HAMP REGULATORS

Considering hypoxic conditions, the main process involved in the request of iron is erythropoiesis that needs iron to produce red blood cells and the oxygen transporter hemoglobin in order to overcome the low body oxygen levels *(Chepelev NL et al, 2014).* As previously described, the main modulator of both iron metabolism and erythropoiesis during hypoxia are HIFs. It has been suggested that in this contest, HIF-1 could play a role in the inhibition of HAMP expression in the liver; however, it remains controversial the way by which HIF-1 acts on HAMP: it could be through a HRE-mediated mechanism present on the promoter of the gene *(Nicolas G et al., 2002),* whereas other data supports an indirect regulation *(Volke M et al., 2009).* Piperno et al. had demonstrated that in human volunteers exposed to hypoxia at high altitude, a rapid decrease of serum ferritin preceded the down-regulation of HAMP expression, suggesting that hypoxia might directly induce ferroportin up-regulation. The timing of HAMP down-regulation contrasted with the hypothesis that hypoxia could be directly involved in HAMP suppression, and they suggested that other factors should be involved *(Piperno A et al., 2009)*. On the other hand, Sonnweber et al. showed that the platelet derived growth factor BB (PDGF-BB) which expression is up-regulated by HIF-1 during hypoxia, is responsible for the repression of HAMP mediated by the down-regulation

of both CREB and CREB-H transcription factors *(Sonnweber T et al., 2014).* Finally, Chaston et al. proposed an in vitro model of HuH-7/THP-1 exposed to hypoxia that showed HAMP mRNA repression through the inhibition of BMP/SMAD4 signaling pathway *(Chaston TB et al., 2011);* thus results are conforming to Ravasi et al study, that suggested an in vitro model of Huh-7 cell lines in which hypoxic serum, obtained from healthy volunteers exposed to high altitude, induced the down-regulation of HAMP expression *(Ravasi G.et al., 2014),* supporting the idea that iron-dependent HAMP activation occurs through the regulation BMP6-SMAD pathway *(Camaschella C, 2009).*

II.

Aim of the study

AIM OF THE STUDY

The main aim of this study was to understand the role of microRNAs as regulators of gene expression in the various processes underlying two different pathological conditions, namely celiac disease and hypoxia. The identification of their role could open the possibility of their use as biomarkers (to develop new diagnostic techniques) or as therapeutic tools, also considering miRNAs features of stability (in particular in the body fluids), ease of detection, and wide diffusion in the body.

Firstly, we focused our attention on celiac disease since it shows heterogeneous symptoms that could depend on variations in gene expression. We analyzed duodenal tissues, in order to identify a panel of miRNAs and target genes that could be implicated in the onset of the disease and which could be differently expressed in celiac patients compared to healthy subjects, according to the degree of mucosal damage. Then, considering the variety of clinical manifestations, in particular related to the age of presentations, we analyzed the same miRNAs and target genes in the duodenal tissues of pediatric patients (3-14 years old), comparing their expression with pediatric healthy subjects and also adult ones. Finally, we analyzed the levels of expression of these miRNAs in the plasma of same pediatric subjects in order to identify a panel of circulating molecules that could be specific for the diagnosis of celiac disease, improving current serological tools and possibly replacing invasive procedures.

The second issue on which we worked was the effect of hypoxia on iron metabolism and the regulatory mechanisms mediated by miRNAs. In order to evaluate this issue, we analyzed miRNA and gene target expression levels in the liver of mice undergoing hypoxia for different time-points and comparing it with mice in normoxia. To identify the mechanisms that triggered the hypoxia-dependent regulation of iron-related genes could be useful to better understand which molecules intervene in the processes of hypoxia-adjustment, for example at high altitude or in cancer tissues, and possibly employ them as pharmacological targets.

III. Materials and methods

MATERIALS AND METHODS

3.1. CELIAC DISEASE

3.1.1. PATIENTS COHORT AND SAMPLES

Duodenal biopsies were obtained from a total of 61 subjects (28 children, under 18 years old, followed by Pediatrics and Gastroenterology Unit (Fondazione MBBM), S.Gerardo Hospital - Monza, and 31 adults followed by Center for the Prevention and Diagnosis of Celiac Disease and UOC Gastroenterology ed Endoscopy, committee IRCCS Ospedale Maggiore, Milano) who underwent upper gastrointestinal endoscopy for diagnostic purposes, after having obtained their (or their parents') informed consent. Celiac patients were diagnosed according to the presence of anti-tTG and/or anti-endomysium antibodies and compatible duodenal histology (4 different duodenal biopsies obtained by standard pliers). Patients were subdivided into two groups, Marsh 3AB and Marsh 3C (based on the severity of the intestinal lesions).

Controls group consisted of subjects in whom celiac disease was excluded by histological and serological analyses and without other diseases, such as inflammatory bowel diseases. Duodenal biopsies were frozen for RNA isolation and proteins extraction, and maintained at -80°C until use. For experiments on plasma, we enrolled the same children who underwent endoscopy and additional 7 pediatric CD patients on GFD for at least 1 year; blood samples were immediately centrifuged at 3000g to obtain plasma fractions, which were aliquoted and stored at -80°C until use. For in vitro stimulation experiments, biopsies were obtained from additional 9 adult CD patients who had been on Gluten Free Diet (GFD) for at least 2 years and 5 control subjects, and incubated in vitro for 4 hours at 37°C and at 5% CO_2 with colture medium +/- peptic-tryptic digest of gliadin (PT gliadin, 1 mg/ml) and maintained at -80°C until use.

No statistically significant difference in demographic parameters was observed among all the group analyzed. The study was approved by the pertinent ethics committee of San Gerardo Hospital – Monza and of IRCCS Ospedale maggiore – Milano and it conformed to the standards set by the Declaration of Helsinki.

The characteristics of the enrolled patients are reported in the following tables.

ADULTS

	Controls (n. 10)	Marsh 3 AB (n. 9)	Marsh 3C (n. 12)	Controls (stimulated) (n. 5)	GFD (stimulated) (n. 9)
Age at enrollement (mean ± SD)	38.9 ± 12.45	31.55 ± 6.30	37.91 ± 10.23	36.76 ± 13.66	38.22 ± 14.36
Sex (F/M)	8 / 2	6 / 3	7 / 5	4 / 1	7 / 2
Subjects positive to Anti-TTG or EMA	0	9	12	0	0
Years on GFD	NA	NA	NA	NA	4.77 ± 1.92
Hb levels (g/L)	135.22 ± 11.38	110.00 ± 12.75	113.90 ± 14.40	132.10 ± 9.90	129.00 ± 9.00

CHILDREN

	Controls (biopsies and blood) (n. 8)	Marsh 3AB (biopsies and blood) (n. 8)	Marsh 3C (biopsies and blood) (n. 12)	GFD (only blood) (n. 7)
Age at enrollement (mean ± SD)	11 ± 3.8	8 ± 3.8	7 ± 3.6	9 ± 4.3
Sex (F/M)	4/4	7/1	4/8	4/3
Positivity ad Anti-TTG or EMA	0	8	12	0
Years on GFD	NA	NA	NA	2 ± 0.9
Hb Levels (g/L)	129.90 ± 11.03	120.93 ± 10.47	114.91 ± 13.37	127.19 ± 13.07

3.1.2. RNA EXTRACTION

Total RNA was extracted from biopsies using a MiRcury RNA Isolation Kit (Exiqon), whereas extraction from plasma was performed using miRNeasy Serum/Plasma Kit (Qiagen) following the manufacturer's instructions. Quality and quantification of extracted RNA was performed by NanoDrop 1000 spectrophotometer UV-visible (Thermo Scientific), and it showed a good quality of absorbance spectrum, without the presence of DNA or protein marks, as indicated by 260/280 nm and 260/230 nm ratio.

3.1.3. miRNA MICROARRAY ANALYSIS

To performe miRNA microarray analysis, estimation of RNA integrity number (RIN) was performed using 2100 Bioanalyzer (Agilent), and only samples with a RIN>7 were employed (5 controls and 6 Marsh 3C). miRNA expression profiling was performed using the miCHIP microarray platform. In brief, 500 ng of total RNA was labeled with a Cy3-conjugated RNA linker (Biospring) and hybridized on the microarray. miCHIP is based on a locked nucleic acid (LNA) technology a class of high-affinity RNA analogs in which the ribose ring is "locked" in the ideal conformation; as result, LNA oligonucleotides exhibit high thermal stability when hybridized to the complementary RNA strand. For each incorporated LNA monomer, the melting temperature ™ of the duplex increases by 2-8 °C, in addition oligonucleotides can be made shorter than traditional (important to detect small targets as miRNAs) and still retain a high Tm. LNA modified, Tm-normalized miRCURY capture probes (Exiqon), were printed on Codelink slides (GE Healthcare). miCHIP arrays were scanned in batches using the Genepix auto Photo Multiplayer (PMT) algorithm, with pixel saturation tolerance set to 0.2%. Tiff images generated by the Genepix 4200AL laser scanner were processed by the Genepix 6 microarray analysis software (Molecular Devices). Scanner output files were median normalized by using the miCHIP R-library running on Bioconductor (www.bioconductor.org). miRNAs with significant variations in expression were identified by Significance Analysis of Microarray (SAM) with a $p<0.01$.

3.1.4. miRNA qRT-PCR

For miRNA quantitative analyses, RNA was transcribed in cDNA using the commercial available kit TaqMan MicroRNA Reverse Transcription kit (Life Technologies) with the use of specific primers (Taqman MicroRNA assay, Life Tecnologies), following the manufacturer's instructions.

The hairpin design on these primers allow to extend the length of miRNA to obtain a better efficiency of the reaction.

qPCR was performed using 7900HT Fast Real-Time PCR System (Life Technologies) and Taqman probes TaqMan® MicroRNA Assays (Life Technologies) and Master Mix (GoTaq Probe Master Mix, Promega).

Each sample was examined in triplicate. All data were normalized to the amount of miR-Let-7b, selected analyzing the original microarray data due to the intensity and the similarity of the signal among different samples. The relative quantification with 2^(-ΔΔCt) method was employed to calculate relative changes in miRNA expression using an external reference sample (RNA obtained from duodenal biopsies of a control subject, not included in the analysis). Conversely, for each stimulation experiment, the reference sample was the untreated biopsy obtained from the same subject.

3.1.5. IDENTIFICATION OF TARGET GENES

Identification of miRNAs' possible targets was performed by softwares and databases considering the complementarity of miRNA SEED sequence and the 3'UTR of target genes and the positive energy of pairing between them.

The following softwares were employed to identify the possible target genes of miRNAs emerging from microarray analysis, considering their miR SVR score focusing in particular on those with an in vitro validation already published in literature.

MirTarBase (http://mirtarbase.mbc.nctu.edu.tw/), Targetscan (http://www.targetscan.org/), microRNA.org (http://www.microrna.org/microrna/home.do) and Mirò (http://ferrolab.dmi.unict.it/miro/).

3.1.6. TARGET GENES qRT-PCR

For gene expression, TaqMan Reverse Transcription Reagents kit (Life Technologies) were used with random primers to obtain cDNAs, and Sybr green GoTaq qPCR Master Mix (Promega) was employed for the detection of target mRNAs with the exception of FOXP3, IL18 and MAD2L1 (assessed by Taqman Gene Expression Assay, Life Technologies and GoTaq Probe Master Mix, Promega). Primers were designed intron spanning to avoid co-amplification of genomic DNA, and no signal was detected when un-retrotranscribed RNA was used as template:

NOD2 (*FORWARD:* 5'-GCTGCCTTCCTTCTACAACA-3', *REVERSE:* 5'-GCGTCTCTGCTCCATCATAG-3')

CXCL2 (*FORWARD:* 5'-CTCAAGAATGGGCAGAAAGC-3', *REVERSE:* 5'-CTCCTAAGTGATGCTCAAAC-3')

RUNX1 (*FORWARD:* 5'-GCAGCGTGGTAAAAGAAATC -3', *REVERSE:* 5'-GTGGAAGGCGGCGTGAAGCG -3')

STAT3 (*FORWARD:* 5'-TGGTGTTTCATAATCTCCTG -3', *REVERSE:* 5'-GGCTGCTGTGGGGTGGTTGG -3')

GAPDH (*FORWARD*: 5'-TGGTAAAGTGGATATTGTTGCC-3, *REVERSE*: 5'-GGTGAAGACGCCAGTGGAC-3')

qPCR was performed using 7900HT Fast Real-Time PCR System (Life Technologies).

For all the analysis, each sample was examined in triplicate. All data were normalized to GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase). The relative quantification with 2^(-ΔΔCt) method was employed as previously described.

3.1.7. WESTERN BLOT

Protein extraction was performed by RIPA buffer containing protease and phosphatase inhibitors (Sigma-Aldrich) after manual disruption of duodenal tissues, and the obtained protein quantified by Bradford method. Proteins were separated on NuPAGE Novel Bis-Tris (4-12%) gels (Life Technologies), transferred on nitrocellulose membrane, blocked with TTBS and 5% non-fat milk and hybridizations performed at 4°C overnight with the following antibodies: mouse anti-human

Nod2 (Abcam, 1:1000), goat anti-human Cxcl2 (Abcam, 1:2000), mouse anti-human Foxp3 (Abcam, 1:500), mouse anti-human Mad2l1 (Sigma-Aldrich, 1:1000 + 2% milk), rabbit anti-human phospho-Stat3 (ser727) (Cell Signalling, 1:1000) (Cell Signalling), and normalized on actin expression (rabbit Anti-human actin (Sigma, 1:1500). SuperSignal West Pico Chemioluminescent Substrate (Thermo Scientific) was used to visualize the signal. Quantification of signal was performed with a FluorS MultiImager using the Quantity One 4.1.1 Software Package, both from Bio-Rad (Milan, Italy).

3.1.8. IMMUNOISTOCHEMISTRY ASSAY

In order to identify Cxcl2 an Nod2 localization, duodenal biopsies were fixed in a 10% solution of paraformaldehyde in 0.1 M phosphate-buffered saline and embedded in paraffin. Sections were then mounted on polylysine- or silane-treated slides for hematoxylin and eosin staining. Slides were pretreated with 3% H_2O_2 for 15 min to inhibit endogenous peroxidase and with blocking agent for 60 min to prevent background staining. Antigen retrieval was performed by microwave treatment (60 sec at 800 watt, twice) in citrate buffer. Incubation with primary antibodies was performed for 1 h for Nod2 1:450 in Normal Goat Serum (NGS) 1% (Abcam) and Cxcl2 1:100 in Bovin Serum Albumine (BSA) 1% (Abcam). Detection of the hybridization signal was performed with biotinylated secondary antibodies (anti-mouse IgG for Nod2 and anti-goat for Cxcl2; Vector Elite Kit, Vector), followed by peroxidase-conjugated streptavidin and diaminobenzidine solution (ABC detection system, Vector). Counterstaining was performed with Harris hematoxylin.

3.1.9. LASER MICRODISSECTION

Serial cryosections of 10 μm of frozen duodenal tissue were mounted on clear polyethylene terephthalate (PET) membrane (MMI). The membrane was stained with Mayer's hematoxylin, placed onto a second glass slide and fixed in position on the microscope tray. The system used for the microdissection was MMI – Cell UVCut with the related software (laser parameters: depth 80%, power 80%, and speed 2%). We separated the epithelial from non-epithelial components and collected them in RNAse free tubes containing lysis buffer (miRCURY RNA Isolation Kit – Exiqon).

Total RNA from microdissected tissues was extracted using the miRCURY RNA Isolation Kit (Exiqon) following manufacturer's instructions.

3.1.10. CELLS TRANSFECTION

HEK293 cells were propagated in complete Dulbecco's modified Eagles medium (DMEM, Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum, 1x glutamine and 1x penicillin/streptomycin (Sigma-Aldrich). Plasmids containing the 3'UTR of the NOD2 gene downstream of Firefly luciferase gene (3'UTR Reporter Clone) and miR-192-5p or miR-23b (MicroRNA expression plasmids) were obtained from Origene whereas Renilla luciferase gene plasmid was purchased from Promega. Cells were co-transfected with different combination of plasmids using JetPrime® (Polyplus) following the manufacturer's instructions. After 48 hours, luciferase activity was measured using a Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol and normalized to Renilla luciferase activity.

3.1.11. STATISTICAL ANALYSIS

Data are reported as median/interquartile range. Comparison of the data between groups was performed using the ANOVA and Student t-test when appropriate. When data failed the equal variance test, the Kruskal-Wallis 1-way ANOVA on ranks was used followed by Dunn post-hoc test. Correlation coefficients were determined by Pearson correlation test. The significance level was set at $p\text{-value} < 0.05$. Statistical evaluation was performed with the SYSTAT software package.

3.2. HYPOXIA AND IRON METABOLISM

3.2.1. MICE SAMPLES

A total of 31 C57/B6 male mice of ten weeks were employed, and hypoxia was obtained placing the mice in an hypoxic chamber (with 10% of oxygen) that simulated 4500m of altitude. Analyses were performed on 7 control mice (in normoxia) and 24 mice exposed to different time-points of hypoxia: 8 mice for 12 hours, 8 mice for 24 hours, 8 mice for 48 hours. After killing, livers were extracted, frozen and maintained at -80°C until use. The study was approved by the pertinent ethics committee (Ministry of Health).

To differentiate the effects due to hypoxia from those caused by a change in iron status, we utilized additional 18 mice: 6 following a normal diet, 6 following an iron-rich diet (added with 2% carbonyl iron) and 6 following an iron-deplete diet (6 ppm).

3.2.2. RNA EXTRACTION

Total RNA was extracted using a MiRcury RNA Isolation Kit (Exiqon) following the manufacturer's instructions. Nuclear and cytoplasmic RNA were extracted separately by SurePrep Nuclear and Cytoplasmic RNA Extraction (Fisher Scientific). Quality and quantification of extracted RNA was performed by NanoDrop 1000 Spectrophotometer (Thermo Scientific).

3.2.3. miRNA MICROARRAY ANALYSIS

miRNA microarray analysis was performed using microarray miRCURY LNA ™ (Exiqon) as previously described.

RNA used for this experiment were divided into 4 pools: controls – 12 hours – 24 hours and 48 hours of exposure to hypoxia. Samples was double labelled in fluorescence and their expression compared to an external common reference (generated by assemblying a small quantity of RNA from each pool) in order to identify which miRNAs were up- (green) or down- (red) regulated in the various pools. Finally, statistical analysis and cluster analysis of the defined group was performed considering all the miRNAs included into miRBase database of Sanger Institute.

3.2.4. miRNA qRT-PCR

qRT-PCR for miRNA validation were performed as previously described (paragraph 3.1.4)

All data were normalized to the amount of SnU6.

3.2.5. TARGET GENE qRT-PCR

As previously described the identification of miRNAs' possible targets was performed by various softwares and databases (paragraph 3.1.5).

qRT-PCR for all target genes were performed by Taqman technology as indicated in paragraph 3.1.6. except for Bmp6 and Hprt on nuclear RNA, performed with Sybr Green methods, using GoTaq PCR Master Mix (Promega) and the primers described below. All data were normalized to the SnU6 for miRNA analysis and to Hprt for gene expression and the data analysis performed as previously indicated.

3.2.6. PCR FOR BMP6 ANALYSIS

BMP6 mRNA down-regulation could be depend on a transcriptional or post-transcriptional regulation. To evaluated if a transcriptional regulation could be involved, bioinformatic analyses were performed by Genomatix software (Genomatix Software GmbH), in order to identify if there were hypoxia-dipendent transcription factors acting on Bmp6 promoter. Moreover, on nuclear and cytoplasmic cDNA, PCR end-point was performed using PCR Master Mix (Promega) following manufacturer's instructions and the primers for Bmp6 and Hprt as described:

Bmp6 F: 5' CTACGCTGCCAACTACTGTG 3' R: 5' ACTCGGGATTCATAAGGTGG 3'

Primers were designed on exon 6 and 7, with an intron of 91 bp; cytoplasmic amplicons were 110 bp and nuclear amplicons were 201 bp.

Hprt F: 5' CTGGTGAAAAGGACCTCTCG 3' R: 5' GGGCATATCCAACAAAC 3'

Primers were designed on exon 6 and 7, with an intron of 160 bp; cytoplasmic amplicons were 88 bp of length and nuclear amplicons were 248 bp.

Amplifications was checked on 2% agarose gel, running in Tris-Borate-EDTA (TBE) buffer.

3.2.7. WESTERN BLOT

Total protein extraction was performed with RIPA buffer containing protease and phosphatase inhibitors (Sigma-Aldrich), after having disrupted manually liver tissues, except for phospho-Smad 1,5,8 analyzed on nuclear proteins extracted by NePER kit (Pierce). Proteins were separated on NuPAGE Novel Bis-Tris (4-12%) gels (Life Technologies) and transferred on nitrocellulose which was then blocked with TTBS and 5% non-fat milk. Hybridizations performed with the following antibodies: mouse anti-Fpn1 (1:1000, Abcam), rabbit anti-phospho-Smad1,5,8 (1:1000, Cell Signalling), mouse anti-Atoh8 (1:1000, Cell Signalling), goat anti-Bmp6 (1:500, Santa Cruz) and anti-phospho-Smad2 (1:1000, Cell Signalling). Values were normalized on actin expression except for nuclear proteins, normalized on histone H3 (mouse anti-H3, 1:1000, Cell signaling). SuperSignal West Pico Chemioluminescent Substrate (Thermo Scientific) was used to visualize the signal. Quantification of signal was performed with a FluorS MultiImager using the Quantity One 4.1.1 Software Package, both from Bio-Rad (Milan, Italy).

3.2.8. LIVER IRON CONCENTRATION (LIC) MEASUREMENT

Liver samples were stored at -80°C until analysis. Then, they were dried for 18 hours (O/N) at 65°C, weighed, transferred to a small iron-free vessel, acid-digested with 0.3 mL of a 1/1 (v/v) mixture of concentrated sulphuric and nitric acids in a 3 mL. A blank was treated similarly. The LIC (HIC) was measured by atomic absorption spectrophotometry and expressed in µmol/g dry weight. Wet weight was also taken before handling biopsy in order to have wet:dry ratio.

3.2.9. STATISTICAL ANALYSIS AND SOFTWARE

Data were analyzed as indicated in paragraph *3.1.11*

Genomatix software was used to analyzed the promoter of the different genes.

IV.

Results

RESULTS

4.1. CELIAC DISEASE

4.1.1. miRNA MICROARRAY

We performed a microarray analysis, comparing miRNA expression, on RNA extracted from 5 duodenal biopsies of adult control subjects and 6 of Marsh 3C adult CD patients. All the employed samples had a RNA integrity number (RIN) >7.

Hybridization signal was detected by laser scan and the differences of intensity of expression of each miRNA in each sample, between the two groups, was compared by Multi-Experiment-Viewer Software using Significance Analysis of Microarray (SAM).

The result of SAM analysis was a flow chart in which the control/CD ratio was reported: on Y axis the statistically observed values and on X axis the expected values; the dashed lines represent the range of standard deviations: on the bisector line and within this range are depicted the ratios that were not significantly different, on the contrary, red values, indicated miRNAs significantly differently expressed in the two groups (FIG 15).

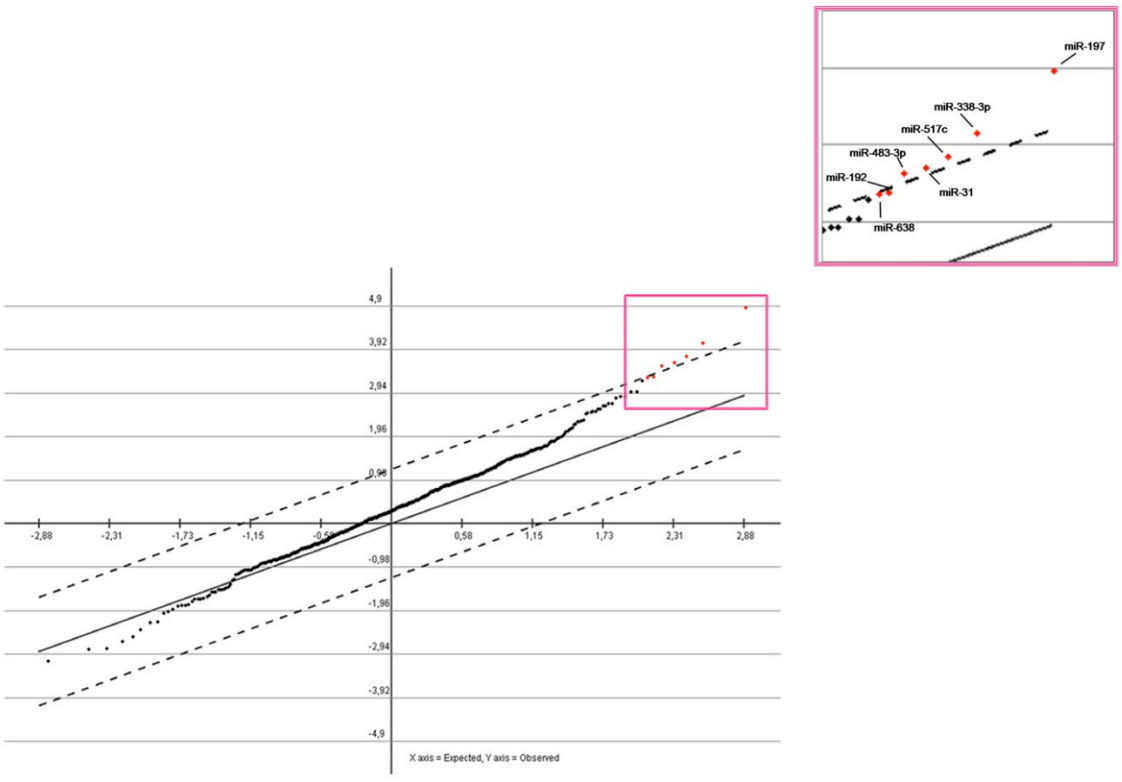


Fig 15. SAM analysis. *miRNAs outside the dashed lines are differently expressed in CD patients vs. controls .*

The result of this analysis were 7 miRNAs (miR-638, miR-192-5p, miR-483-3p, miR-31-5p, miR-517c, miR-338-3p and miR-197) significantly down-regulated in biopsies of CD subjects compared to controls ($p<0.01$) (FIG 2).

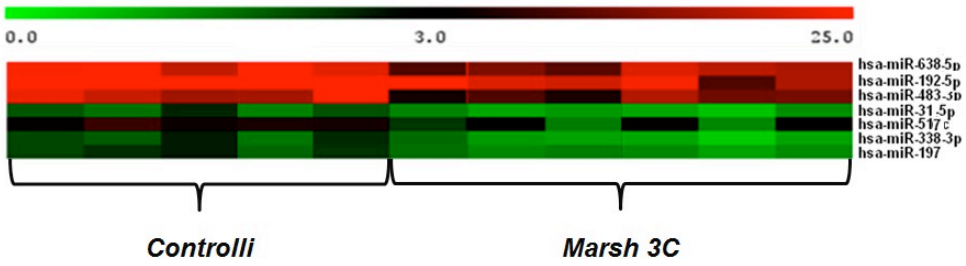


Fig 16. Cluster analysis. *miRNAs differently expressed in the group of CD patients (n=6) compared to controls (n=5). The colour represents the intensity of the signal observed for each sample; more abundant miRNAs are shown in red, less abundant in green.*

4.1.2. VALIDATION OF miR-192-5p EXPRESSION AND ITS TARGET GENES: CXCL2, NOD2, MAD2L1

We validated by qRT-PCR, miR-192-5p expression in duodenal biopsies of pediatric and adult CD patients (considering their Marsh' grade) compared to controls.

miR-192-5p expression was significantly reduced in biopsies obtained from adult CD patients, with a more profound decrease observed in Marsh 3C patients, in whom its levels were about 32% of those observed in controls (1.93 ± 0.70, 1.31 ± 0.29 and 0.62 ± 0.29 in controls, Marsh 3AB and Marsh 3C, respectively; ANOVA $p=0.007$ for Marsh 3AB and $p=0.0001$ for Marsh 3C, respectively vs controls). Moreover, we detected a similar trend of expression in biopsies obtained from pediatric CD patients, with a more profound decrease in Marsh 3C patients (0.90 ± 0.32 vs 0.59 ± 0.39 and 0.47 ± 0.34 in controls, Marsh 3AB and Marsh 3C, respectively, $p=0.013$ by ANOVA).

Then, we assessed the circulating levels of the miRNAs described above in the plasma of CD pediatric patients at diagnosis or on Gluten Free Diet (GFD); miR-192-5p expression showed a significant reduction in CD patients at diagnosis, Marsh 3AB (0.32 ± 0.14) and Marsh 3C patients (0.35 ± 0.19) compared to controls (1.14 ± 0.36) ($p<0.05$ with ANOVA test, both), but in GFD patients it did not return to a level comparable to controls (0.24 ± 0.14) (FIG 17).

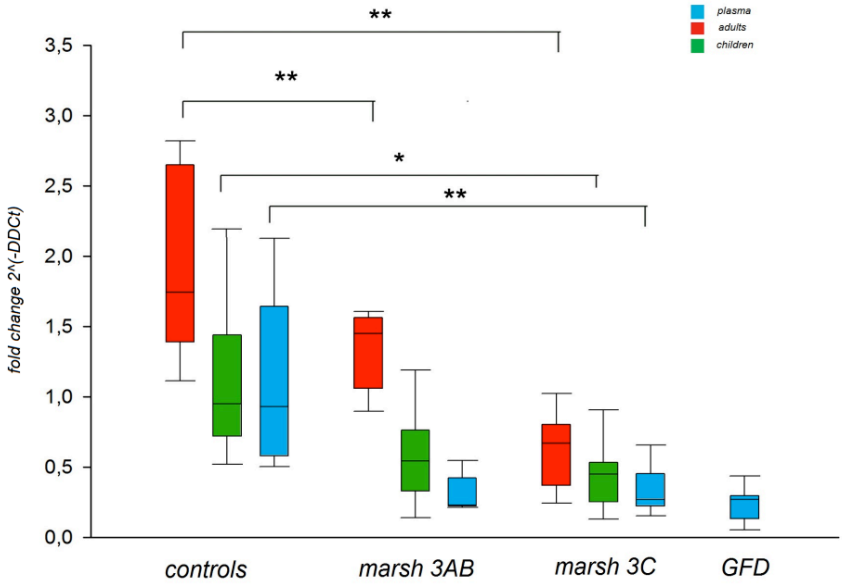
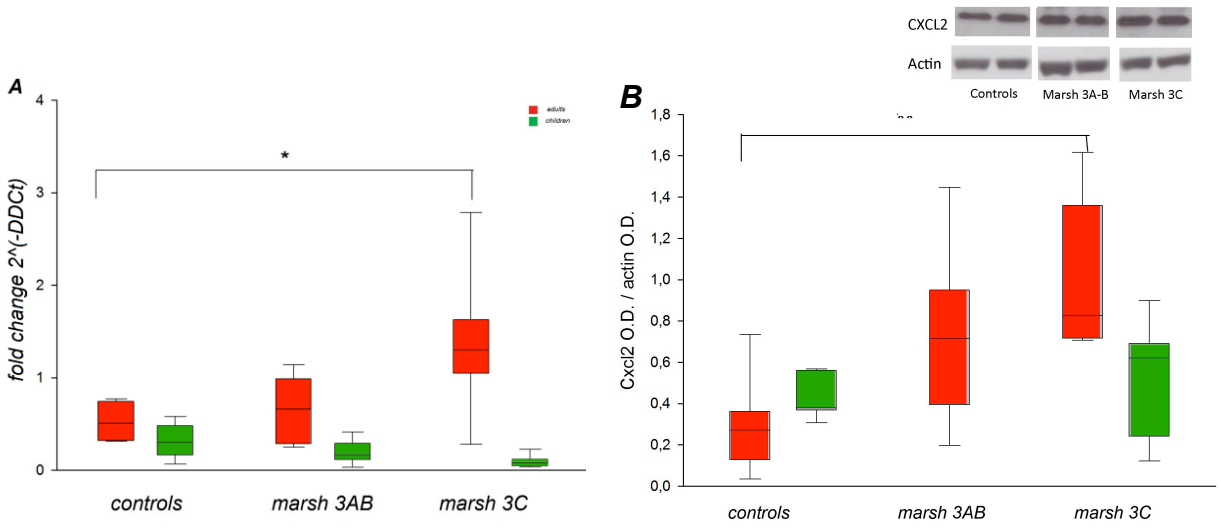


Fig 17. miR-192-5p expression. *miR-192-5p was down-regulated in adult and pediatric CD patients compared to controls, in duodenum as well as plasma samples. However, plasma expression in GFD patients did not return to levels comparable to controls. Red bars: adults, green bars: children, blue bars: plasma. * = p<0.05, ** = p<0.01. Biopsies: controls n=10 (adults) n=8 (children), Marsh 3AB n=9 (adults) n=8 (children), Marsh 3C n=12 (adults) n=12 (children). Plasma children: n=8 (controls), n=8 (Marsh 3AB), n=12 (Marsh 3C), n=7 (GFD).*

In silico analyses with different target prediction softwares were performed for all miRNAs that resulted significantly down-regulated. For miR-192-5p we identified two molecules involved in innate immunity, namely CXCL2 and NOD2. These analyses detected a binding region for miR-192-5p starting at nucleotide 255 of CXCL2 3'UTR, and in NOD2 3' UTR starting from nucleotide 129 and from nucleotide 1137 (FIG 18).

Fig 18. miR-192-5p and NOD2/CXCL2 alignment. *MicroRNA.org software for target prediction identified CXCL2 and NOD2 as targets of miR-192-5p.*

We analyzed the expression of CXCL2 and NOD2 in adult and pediatric CD patients; in adults, CXCL2 mRNA expression was about three times higher in the biopsies of Marsh 3C CD patients compared to controls (0.53 ± 0.20, 0.63 ± 0.37 and 1.44 ± 1.09 in controls, Marsh 3AB and Marsh 3C, respectively p=0.026, ANOVA) (FIG 19A). When the data of all adult subjects were analyzed, CXCL2 mRNA levels showed a significant negative correlation with miR-192-5p expression, with a r value of -0.47 and p=0.014, consistent with the possible regulatory effect of the miRNA (FIG 19C). The increase in the mRNA level was paralleled by an increase in Cxcl2 protein expression, as shown in the western blot image and by the densitometric analysis: after normalization of actin expression, an increase in Cxcl2 protein expression was observed both in patients with Marsh 3AB and Marsh 3C lesion, being about three and five times more abundant than in controls, although this difference was significant only in patients with a more severe lesion (0.23 ± 0.70, 0.71 ± 0.19 and 1.17 ± 0.39 in controls, Marsh 3AB and Marsh 3C, respectively p<0.05 by ANOVA) (FIG 19B). Differently from what expected, the expression of this molecule did not inversely correlate with miR-192-5p levels in pediatric patients; CXCL2 mRNA expression was reduced in biopsies obtained from CD children, with a more profound decrease observed in Marsh 3C patients (0.32 ± 0.20 vs 0.20 ± 0.15 and 0.10 ± 0.07 in controls, Marsh 3AB and Marsh 3C, respectively, p<0.05 by ANOVA) (FIG 19A) with a not significant Pearson correlation (r =0.35, p=0.09) (FIG 19D) moreover we did not detect any variations in Cxcl2 protein expression in controls and Marsh 3C (0.43 ± 0.11 vs 0.52 ± 0.30, respectively, p>0.05 by t-test) (FIG 19B).



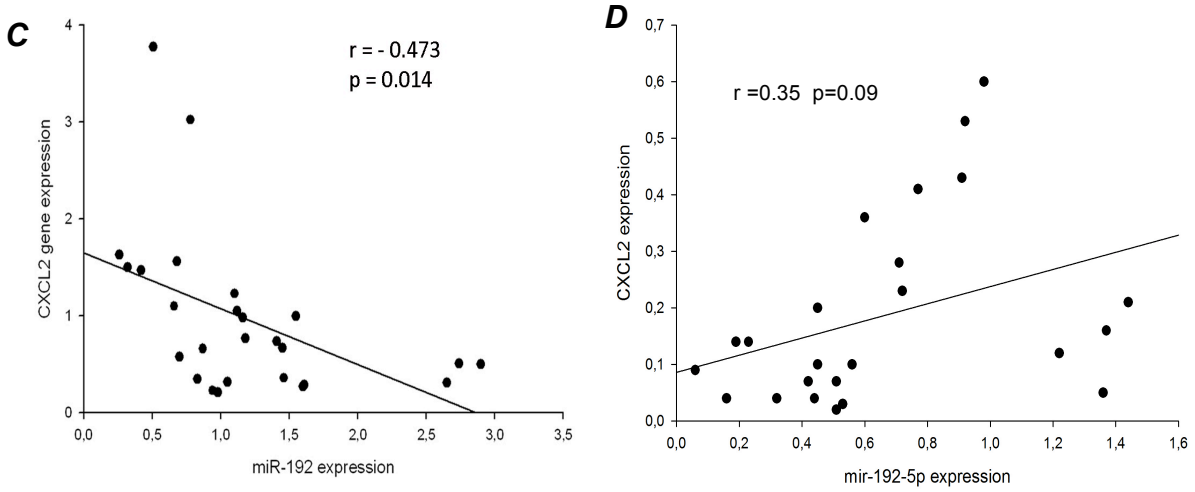


Fig 19. CXCL2 expression. *CXCL2 mRNA (A) and protein (B) expression are up-regulated in adult CD patients compared to controls. Western Blot is representative of adult samples. (C) Pearson correlation showed a negative correlation between miR-192-5p and CXCL2 in adult patients whereas no correlation is demonstrated in pediatric patients (D). Red bars: adults, green bars: children. * = p<0.05. Biopsies: controls n=10 (adults) n=8 (children), Marsh 3AB n=9 (adults) n=8 (children), Marsh 3C n=12 (adults) n=12 (children).*

A similar trend was detected for NOD2 expression; in the adult group NOD2 mRNA levels were increased in both CD patient groups, but a significant difference was detected only in Marsh 3C subjects in whom NOD2 was about three times higher than controls (1.03 ± 0.83, 2.43 ± 1.71 and 3.27 ± 2.01 in controls, Marsh 3AB and Marsh 3C, respectively p=0.005, ANOVA) (FIG 20A). A significant inverse correlation was detected between NOD2 and miR-192-5p expression (r = -0.57, p= 0.002) (FIG 20C). Nod2 protein expression was significantly increased in both categories of CD patients, being four times higher in Marsh 3C subjects (0.19 ± 0.70, 1.83 ± 0.88 and 2.88 ± 0.89 in controls, Marsh 3AB and Marsh 3C, respectively, p<0.05 for both, ANOVA) (FIG 20B).

Similarly to that previously report for CXCL2, NOD2 did not inversely correlated with miR-192-5p expression in children: its mRNA levels were unchanged in CD groups compared to controls (0.17 ± 0.15 in controls, 0.40 ± 0.45 in Marsh 3AB and 0.20 ± 0.19 in marsh 3C, respectively) (FIG 20A) and the Pearson correlation resulted not significant (r=0.06, p=0.87) (FIG 20D). Also Nod2 protein levels were comparable between groups (0.44 ± 0.20 vs 0.60 ± 0.59 in controls and Marsh 3C, respectively) (FIG 20B).

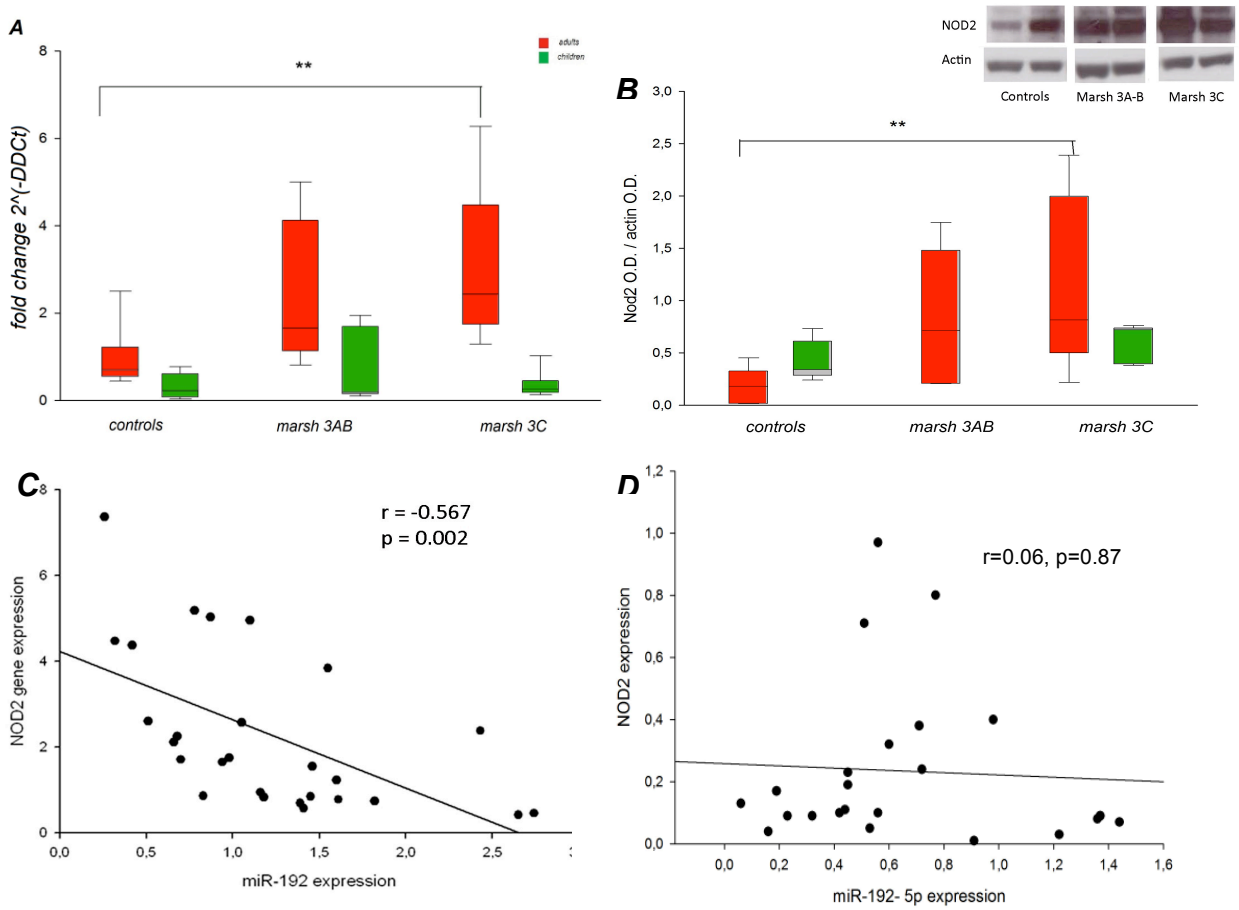


Fig 20. NOD2 expression. *NOD2 mRNA (A) and protein (B) expression are up-regulated in adult CD patients compared to controls. No changes of expression was detected in the duodenum of children subjects. Western Blot is representative of adult samples. (C) Pearson correlation showed a negative correlation between miR-192-5p and CXCL2 in adult patients (D) whereas no correlation is demonstrated in pediatric patients. Red bars: adults, green bars: children. ** = p<0.01. Biopsies: controls n=10 (adults) n=8 (children), Marsh 3AB n=9 (adults) n=8 (children), Marsh 3C n=12 (adults) n=12 (children).*

Since several miRNAs can contribute to the regulation of the expression of a single gene, the lack of effect of miR-192-5p on CXCL2 and NOD2 levels could be justified by the presence of other miRNAs acting on these targets and balancing their expression levels. We thus performed in silico analyses that identified the miR-486-5p seeding sequence within the 3' UTR of NOD2 and CXCL2 (FIG 21).

As hypothesized, this miRNA was significantly up-regulated in CD pediatric patients compared to controls (0.49 ± 0.23, 0.93 ± 0.31 and 1.17 ± 0.47 in controls, Marsh 3AB and Marsh 3C, p=0.030 and p=0.001, respectively, by ANOVA.

Furthermore, miR-486-5p levels were not increased in adult CD patients (1.13 ± 0.82 vs 1.05 ± 0.78 in controls and Marsh 3C patients, respectively, p>0.05, ANOVA) supporting the presence of a different miRNA-regulatory pattern (FIG 22).

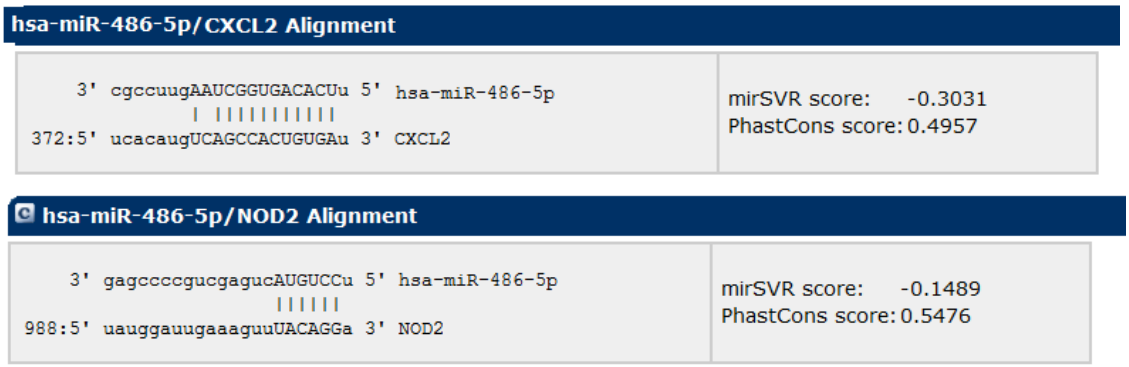


Fig 21. miR-486-5p and NOD2/CXCL2 alignment. *MicroRNA.org software for target prediction identified CXCL2 and NOD2 as targets of miR-486-5p*

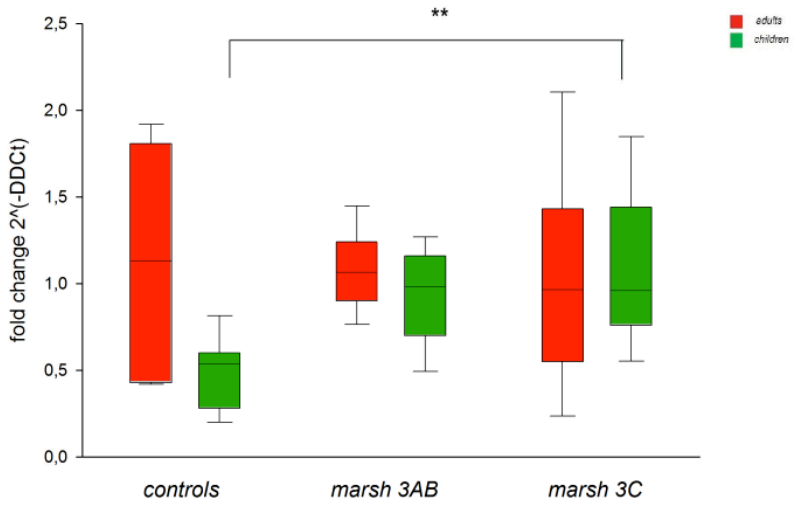


Fig 22. miR-486-5p expression. *miR-486-5p expression resulted up-regulated only in pediatric CD patients compared to controls. Red bars: adults, green bars: children. ** = p<0.01. Biopsies: controls n=10 (adults) n=8 (children), Marsh 3AB n=9 (adults) n=8 (children), Marsh 3C n=12 (adults) n=12 (children).*

To assess whether the miR-192-5p down-regulation observed in pediatric patients was able to influence the expression of other genes, we identified by in silico analysis MAD2 mitotic arrest deficient-like 1 (MAD2L1) mRNA as possible target of miR-192-5p, as already reported in the literature (Georges et al. 2008). We analyzed its expression in adult and children groups: MAD2L1 mRNA expression was up-regulated in children with CD, with a three-fold increase in Marsh 3C

patients compared to controls (0.72 ± 0.60, 1.72 ± 1.07 and 2.12 ± 1.33 in controls, Marsh 3AB and Marsh 3C respectively, p<0.05 by ANOVA) (FIG. 23A). The protein expression paralleled mRNA expression: its level was up-regulated in CD pediatric patients, in particular in Marsh 3C subjects (0.56 ± 0.32, 0.78 ± 0.34 and 2.55 ± 1.71, controls, Marsh 3AB and Marsh 3C, respectively, p<0.05 ANOVA) (FIG 23B). Confirming our hypotheses, this finding was present only in children, since in adult CD subjects mRNA levels were comparable to controls (1.56 ± 1.07, 1.86 ± 0.98 and 1.94 ± 1.05 in controls, Marsh 3AB and Marsh 3C, respectively, p> 0.05 ANOVA) (FIG 23A) and the same trend was shown in protein expression, since we didn't detect any change of expression between controls and CD patients (0.82 ± 0.25 in controls and 1.04 ± 0.13 in Marsh 3C, p>0.05 ANOVA) (FIG 23B).

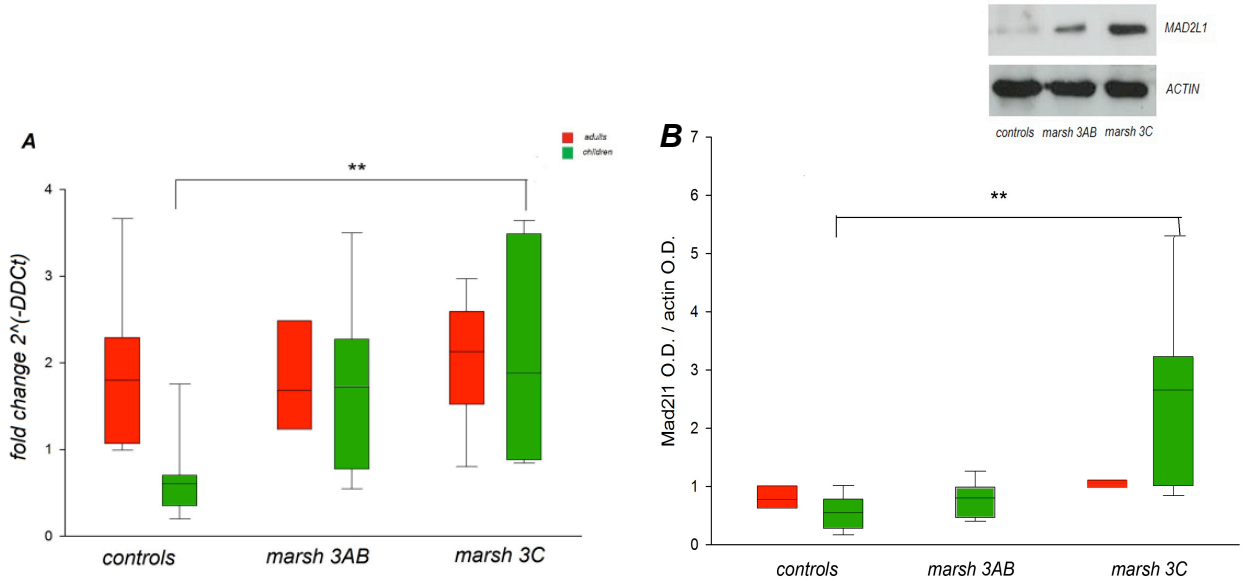


Fig 23. MAD2L1 expression. *MAD2L1 mRNA (A) and protein (B) expression are up-regulated in CD children patients compared to controls. No changes in expression are shown in the duodenum adult subjects. Western Blot is representative of pediatric samples. Red bars: adults, green bars: children. ** = p<0.01. Biopsies: controls n=10 (adults) n=8 (children), Marsh 3AB n=9 (adults) n=8 (children), Marsh 3C n=12 (adults) n=12 (children).*

4.1.2.1 TARGET LOCALIZATION

Due to the presence of various cell types in duodenal biopsies, we wanted to assess whether the expression of miR-192-5p and its possible targets overlapped in the same cell types. For this reason we performed laser microdissection in adult control samples, separating epithelial cells from other cell types, and qRT-PCR to determine the presence of both miR-192-5p and its possible targets. miR-192-5p was detected mainly in the epithelium (with levels about 250 times higher than in non epithelial cells), whereas CXCL2 and NOD2 mRNA were about 120 times more abundant in the epithelium compared to other cell types (data not shown).

Localization of Cxcl2 and Nod2 protein was also confirmed by immunohistochemistry. In adult controls both proteins were localized in the villus epithelium, showing a modest expression, whereas an increased expression was detected in CD patients, both in those with Marsh 3AB and Marsh 3C lesion. Even in these cases the localization of the two proteins was mainly confined in the epithelium covering villi remnants, although some faint staining could be detected in the submucosa (FIG 24).

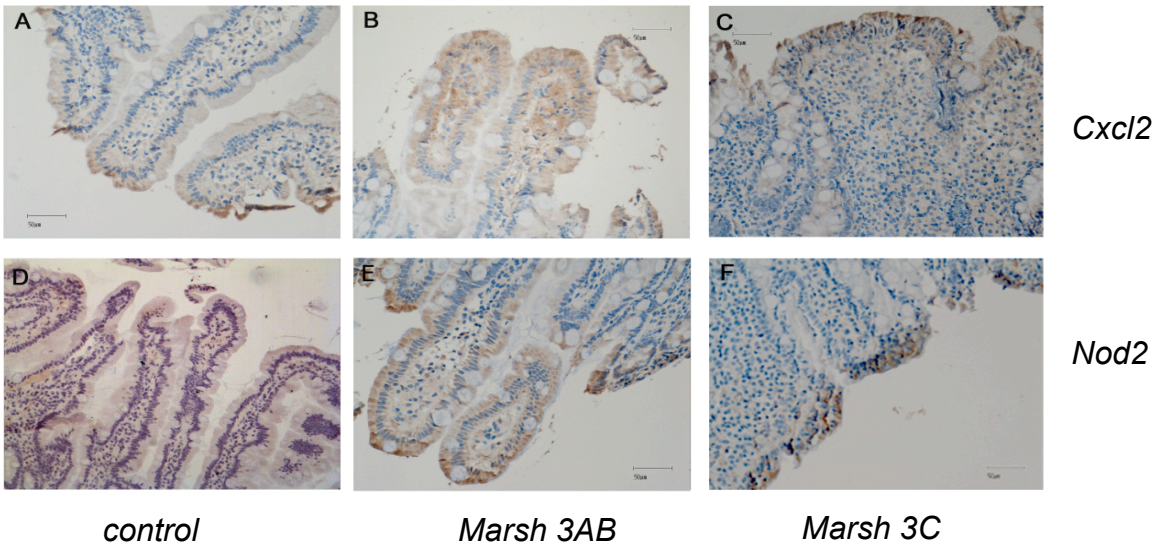


Fig 24. Immunohistochemistry analysis of Cxcl2 and Nod2. Immunohistochemistry was performed on paraffinized sections after antigen retrieval. In control sections both Cxcl2 (A) and Nod2 (D) were detectable in the apical epithelium of the villi, with only a modest staining. In Marsh 3AB the staining became more intense for Cxcl2 (B) and Nod2 (E). In Marsh 3C staining resulted very intense in the remnants of the villi (Cxcl2, C) (Nod2, F), although some staining could be detected in the submucosa.

4.1.2.2 CELLS TRANSFECTION

The co-localization of miR-192-5p and the possible targets was suggestive of a direct interaction between miR-192 and CXCL2 and NOD2; this interaction had already been demonstrated by transfection studies for CXCL2 *(Wu F et al., 2008)* and MAD2L1 *(Georges SA, et al., 2008)* thus we investigated the possible effect on NOD2 by transfection experiments of cultured HEK293 cells using a reporter plasmid containing the 3'UTR of NOD2. The co-transfection of miR-192 containing plasmid reduced NOD2 expression in a dose-dependent manner, also at 1:3 and 1:6 NOD2:miRNA ratio, and reaching a 70% decrease with a 1:9 ratio. This effect was related to the presence of this specific miRNA, since co-transfection of NOD2 with miR-23b (which did not have any matching sequence in NOD2 mRNA) did not show a significant decrease in the luciferase activity (FIG 25).

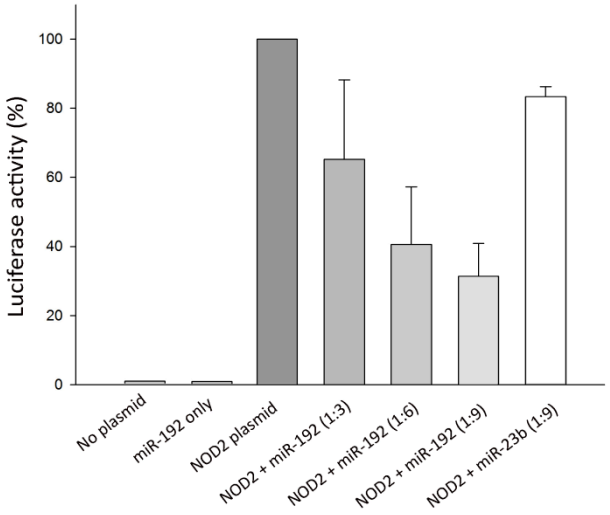


Fig 25. Transfection assay. Luciferase activity of plasmid containing Firefly luciferase associated with 3'UTR of NOD2 co-transfected with miR-192 plasmid (1:3, 1:6 and 1:9 ratio) is reduced in a dose-dependent manner. Co-transfection of NOD2 with miR-23b did not cause a significant decrease in the Firefly luciferase activity. In all cases normalization of the data was performed co-transfecting a Renilla luciferase containing plasmid and measuring its activity.

4.1.3. VALIDATION OF miR-31-5p EXPRESSION AND ITS TARGET GENE: FOXP3

We further analyzed the expression of the other miRNAs detected as down-regulated by microarray analysis. qRT-PCR confirmed that miR-31-5p in adults was significantly down-regulated in Marsh 3C biopsies (1.66 ± 0.74 vs 1.34 ± 0.45 and 0.79 ± 0.38 in controls, Marsh 3AB and Marsh 3C, respectively, p=0.001 by ANOVA) and we detected the same trend in children group where miR-31-5p was significantly down-regulated in both CD groups (1.02 ± 0.38 in controls, 0.19 ± 0.08 in Marsh 3AB and 0.40 ± 0.16 in Marsh 3C, ANOVA vs controls p<0.05 both). miR-31-5p in plasma of CD patients at diagnosis showed a significantly decrease in Marsh 3AB (0.27 ± 0.12) and 3C patients (0.43 ± 0.25) compared to controls (0.71 ± 0.26, p=0.004 and 0.003 for Marsh 3AB and 3C, respectively); GFD patients showed an increase of the miRNA levels compared to CD patients (0.59 ± 0.35), but its level did not return to control values (FIG 26).

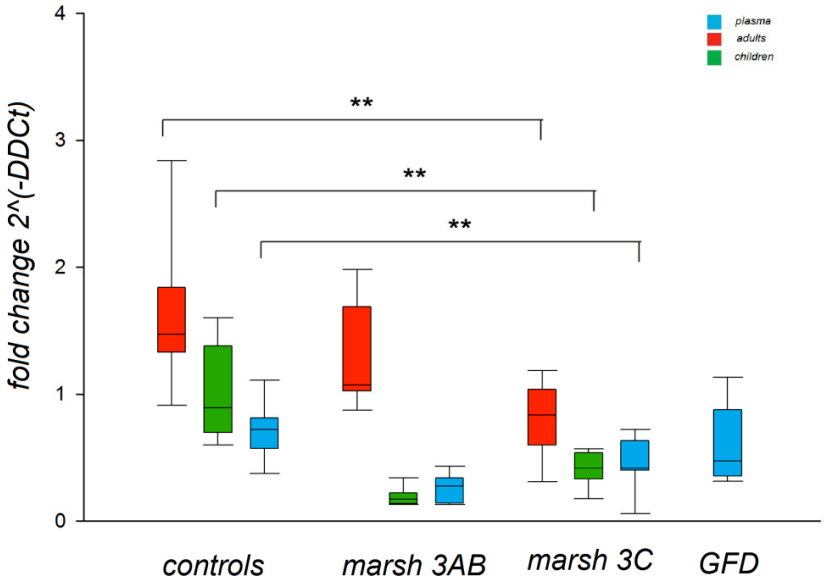


Fig 26. miR-31-5p expression. miR-31-5p was down-regulated in adult and pediatric CD patients compared to controls, in duodenum and plasma samples. Treatment with GFD increased its plasma levels, not reaching control values. *Red bars: adults, green bars: children, blue bars: plasma. ** = p<0.01. Biopsies: controls n=10 (adults) n=8 (children), Marsh 3AB n=9 (adults) n=8 (children), Marsh 3C n=12 (adults) n=12 (children). Plasma children: n=8 (controls), n=8 (Marsh 3AB), n=12 (Marsh 3C), n=7 (GFD).*

For miR-31-5p the analysis in silico identified, among other targets, FOXP3 (gene involved in the regulation of the adaptive immune system) (FIG 27), interaction already validated by transfection studies *(Rouas R et al., 2009).* Analysis by qRT-PCR showed a significant increase in its expression in Marsh 3C adult patients (0.93 ± 0.42 vs 2.23 ± 2.02 and 2.76 ± 1.22 in controls, Marsh 3AB and Marsh 3C respectively, p=0.006) (FIG 28A); moreover, miR-31-5p and FOXP3 mRNA expression showed a significant inverse correlation (r = -0.56, p = 0.005) (FIG 28C). Increased Foxp3 expression was confirmed also at the protein level, as shown by representative western blots and their densitometric analysis (1.88 ± 0.49 vs 1.58 ± 0.39 and 3.94 ± 0.89 in controls, Marsh 3AB and Marsh 3C, respectively, p=0.009, ANOVA) (FIG 28B). FOXP3 increased mRNA expression was detected also in pediatric CD patients: in Marsh 3C pediatric CD patients, its expression was 4.3 times higher levels compared to controls (4.37 ± 2.48 vs 7.46 ± 7.55 and 18.92 ± 21.75 in controls, Marsh 3AB and Marsh 3C, respectively, p=0.005 by ANOVA) (FIG 28A). Pearson correlation showed an inverse correlation even though it wasn't statistically significant (r =-0.16, p=0.51) (FIG 28D). Moreover, Foxp3 protein expression paralleled mRNA expression, being up-regulated in Marsh 3C pediatric patients compared to controls (1.88 ± 0.49, 1.58 ± 0.38 and 3.94 ± 0.89, controls, Marsh 3AB and Marsh 3C respectively, p=0.012 by ANOVA) (FIG 28B).

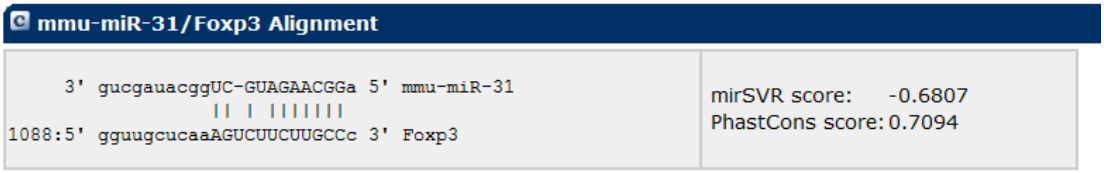


Fig 27. miR-31-5p and FOXP3 alignment. MicroRNA.org software for target prediction identified FOXP3 as target of miR-31-5p.

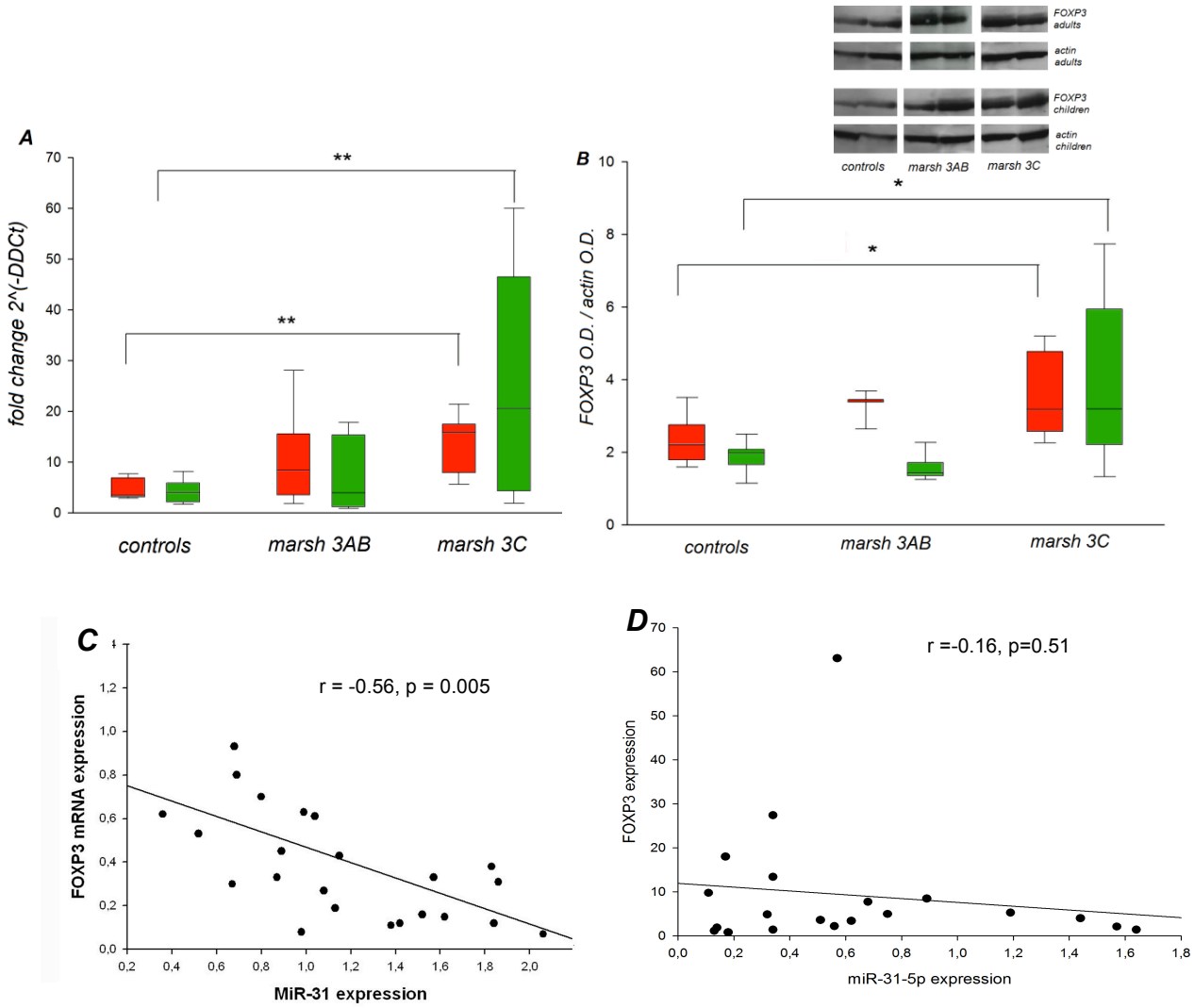


Fig. 28. FOXP3 expression. *FOXP3, target of miR-31-5p, was up-regulated at mRNA (A) and protein level (B) in adult and pediatric CD patients, thus suggesting the same regulatory mechanism independently of age. Pearson correlation showed a negative correlation between miR-31-5p and FOXP3 in adult patients (C) and pediatric ones (D). Red bars: adults, green bars: children. * = p<0.05 ** = p<0.01. Biopsies: controls n=10 (adults) n=8 (children), Marsh 3AB n=9 (adults) n=8 (children), Marsh 3C n=12 (adults) n=12 (children).*

4.1.4. VALIDATION OF miR-338-3p EXPRESSION AND ITS TARGET GENE: RUNX1

We continued with the validation of miRNAs resulted down-regulated in the microarray analysis analyzing miR-338-3p, which was significantly diminished in CD adult patients according to the severity of the mucosal damage (4.79 ± 2.08 vs 2.58 ± 1.75 and 1.90 ± 1.01 in controls, Marsh 3AB and Marsh 3C, respectively, p=0.002 in Marsh 3C by ANOVA). This trend was maintained in pediatric CD patients, where miR-338-3p was significantly down-regulated in CD patients compared to controls, in particular in Marsh 3C patients (0.62 ± 0.28, 0.37 ± 0.31 and 0.29 ± 0.21, in controls, Marsh 3AB and Marsh 3C, respectively. $p < 0.05$, ANOVA). We attempted to analyze miR-338-3p in plasma or serum of pediatric patients, but the obtained data were inconsistent (non-reproducibility even for the same patient) and no further evaluated; this fact is probably due to the limits of qPCR method, unable to detect the low expression of this miRNA (FIG 29).

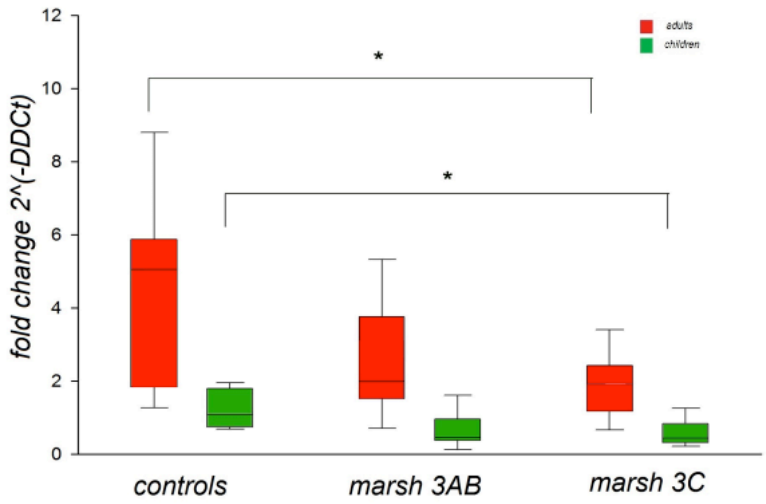


Fig 29. miR-338-3p expression. qRT-PCR validated the down-regulation of miR-338-3p in both adult and pediatric CD patients, compared to controls. It was not detectable in plasma. *Red bars: adults, green bars: children. * = p<0.05. Biopsies: controls n=10 (adults) n=8 (children), Marsh 3AB n=9 (adults) n=8 (children), Marsh 3C n=12 (adults) n=12 (children).*

Softwares of target prediction, identified Run-related transcription factor 1 (RUNX1) as target of miR-338-3p (FIG 30) and in the analyzed biopsies we observed a significant increase in its mRNA expression in Marsh 3C patients (0.70 ± 0.54 vs 2.65 ± 2.19 and 4.34 ± 2.17 in controls, Marsh 3AB and Marsh 3C, p=0.010) (FIG 31A). We also observed a significant inverse correlation

between miR-338-3p and RUNX1 mRNA levels (r = -0.49, p = 0.030) (FIG 31B). Also in pediatric CD patients it was significantly up-regulated in Marsh 3C patients compared to controls, with a 4 fold increase (0.51 ± 0.42 in controls, 0.77 ± 0.17 in Marsh 3AB and 2.01 ± 1.42 in Marsh 3C, p<0.05) (FIG 31A), with a Pearson correlation inverse but not statistically significant (r =-0.24, p= 0.35) (FIG 31C).

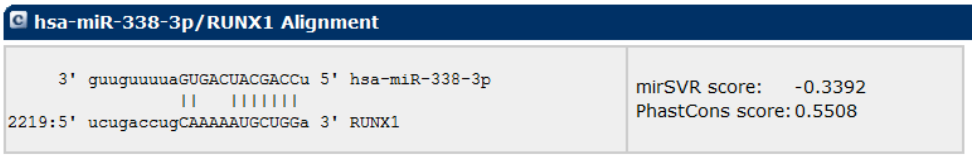


Fig 30. miR-338-3p and RUNX1 alignment. *MicroRNA.org software for target prediction identified RUNX1 as target of miR-338-3p*

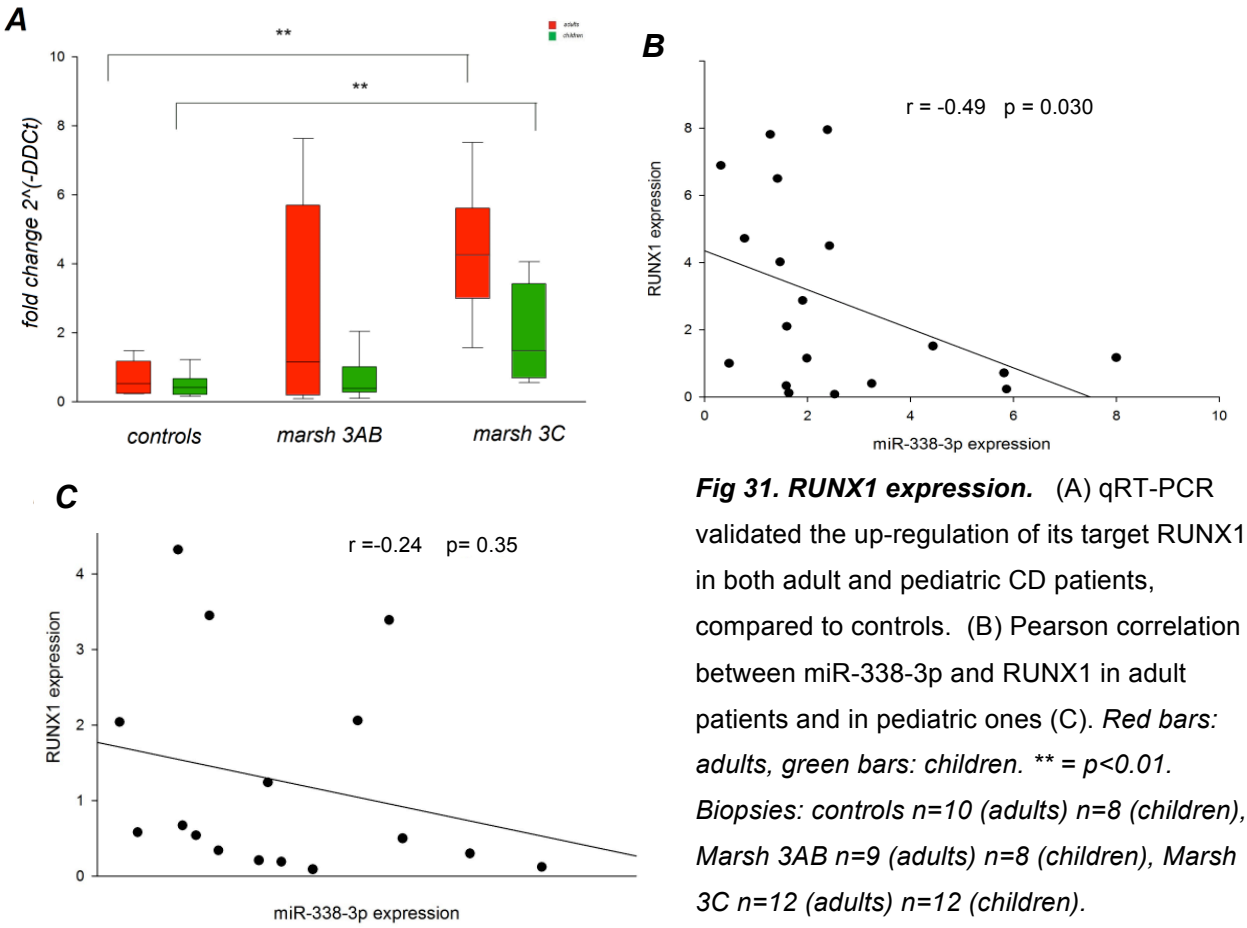


Fig 31. RUNX1 expression. (A) qRT-PCR validated the up-regulation of its target RUNX1 in both adult and pediatric CD patients, compared to controls. (B) Pearson correlation between miR-338-3p and RUNX1 in adult patients and in pediatric ones (C). *Red bars: adults, green bars: children. ** = p<0.01. Biopsies: controls n=10 (adults) n=8 (children), Marsh 3AB n=9 (adults) n=8 (children), Marsh 3C n=12 (adults) n=12 (children).*

4.1.5. VALIDATION OF miR-197 EXPRESSION AND ITS TARGET GENE: IL18

We lastly verified the expression of miR-197 in duodenal biopsies of adult CD patients; also in this case, the mRNA expression was down-regulated in Marsh 3C patients compared to controls (5.87 ± 1.70 vs 2.82 ± 1.17 in controls and Marsh 3C, p=0.049, ANOVA). This down-regulation was also detected in CD pediatric patients (1.95 ± 0.49 and 0.44 ± 0.12 in controls and Marsh 3C, respectively, p=0.049, ANOVA) (FIG 32).

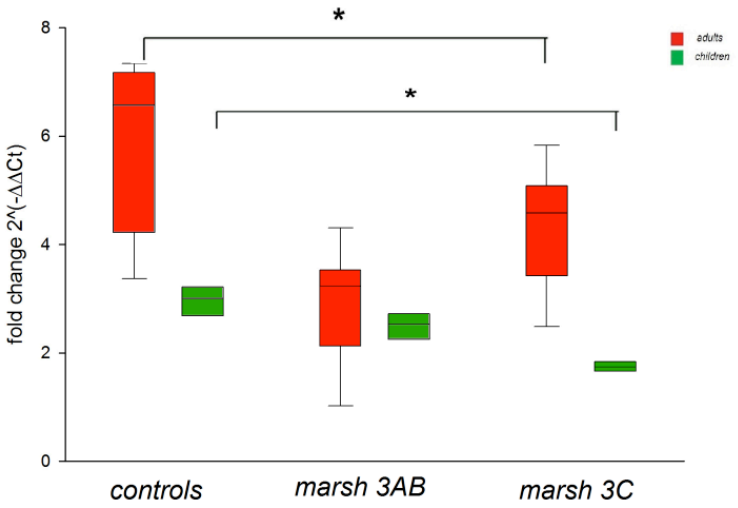


Fig 32. miR-197 expression. qRT-PCR validated the down-regulation of miR-197 in both adult and pediatric CD patients, compared to controls. It was not detectable in plasma. *Red bars: adults, green bars: children. * = p<0.05. Biopsies: controls n=10 (adults) n=8 (children), Marsh 3AB n=9 (adults) n=8 (children), Marsh 3C n=12 (adults) n=12 (children).*

In silico analysis revealed IL18 as target of miR-197 (FIG 33), cytokine involved in the innate immune response. IL18 mRNA levels were significantly up-regulated in Marsh 3C adult patients (0.82 ± 0.41 vs 2.15 ± 1.59 in controls and Marsh 3C, p=0.010, by ANOVA), data consistent with an activation of innate immunity. We observed the same trend considering CD pediatric patients, in whom we noticed a significant up-regulation of IL18 mRNA (6.04 ± 5.00 vs 1.37 ± 0.95 in Marsh 3C and controls, p=0.030). In both cases we were unable to observe a significant correlation with miR-197 expression, possibly due to the small number of the analyzed samples (FIG 34).

hsa-miR-197/IL18 Alignment

```
    3' cgacCCACCUCUUCCACCACUu 5' hsa-miR-197
          ||| |||:  |||||||
223:5' cggaGGUAGAGGUUGUGGUGAg 3' IL18
```

mirSVR score: -0.1912
PhastCons score: 0.5082

Fig 33. miR-197 and IL18 alignment. MicroRNA.org software for target prediction identified IL18 as target of miR-197.

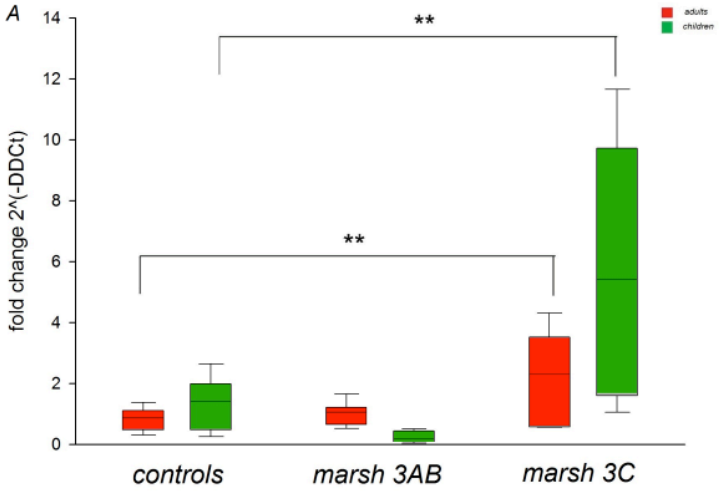


Fig 34. IL18 expression. qRT-PCR validated the up-regulation of IL18 in both adult and pediatric CD patients, compared to controls. *Red bars: adults, green bars: children. ** = p<0.01. Biopsies: controls n=10 (adults) n=8 (children), Marsh 3AB n=9 (adults) n=8 (children), Marsh 3C n=12 (adults) n=12 (children).*

4.1.6. VALIDATION OF miR-517c, miR-638 AND miR-483-3p

On the other hand, for the last 3 miRNAs identified by microarray analysis, we could not validate differences in their expression in adult CD patients compared to controls, i.e. miR-638 (2.84 ± 0.65 in controls vs. 3.00 ± 0.96 in Marsh 3C, FIG 35A), miR-483-3p (0.68 ± 0.48 vs. 0.45 ± 0.18 in controls and Marsh 3C, respectively, FIG 35B) and miR-517c (0.96 ± 0.57 vs. 0.70 ± 0.27 in controls and Marsh 3C, respectively, FIG 35C). Anova, p>0.05 for all of them.

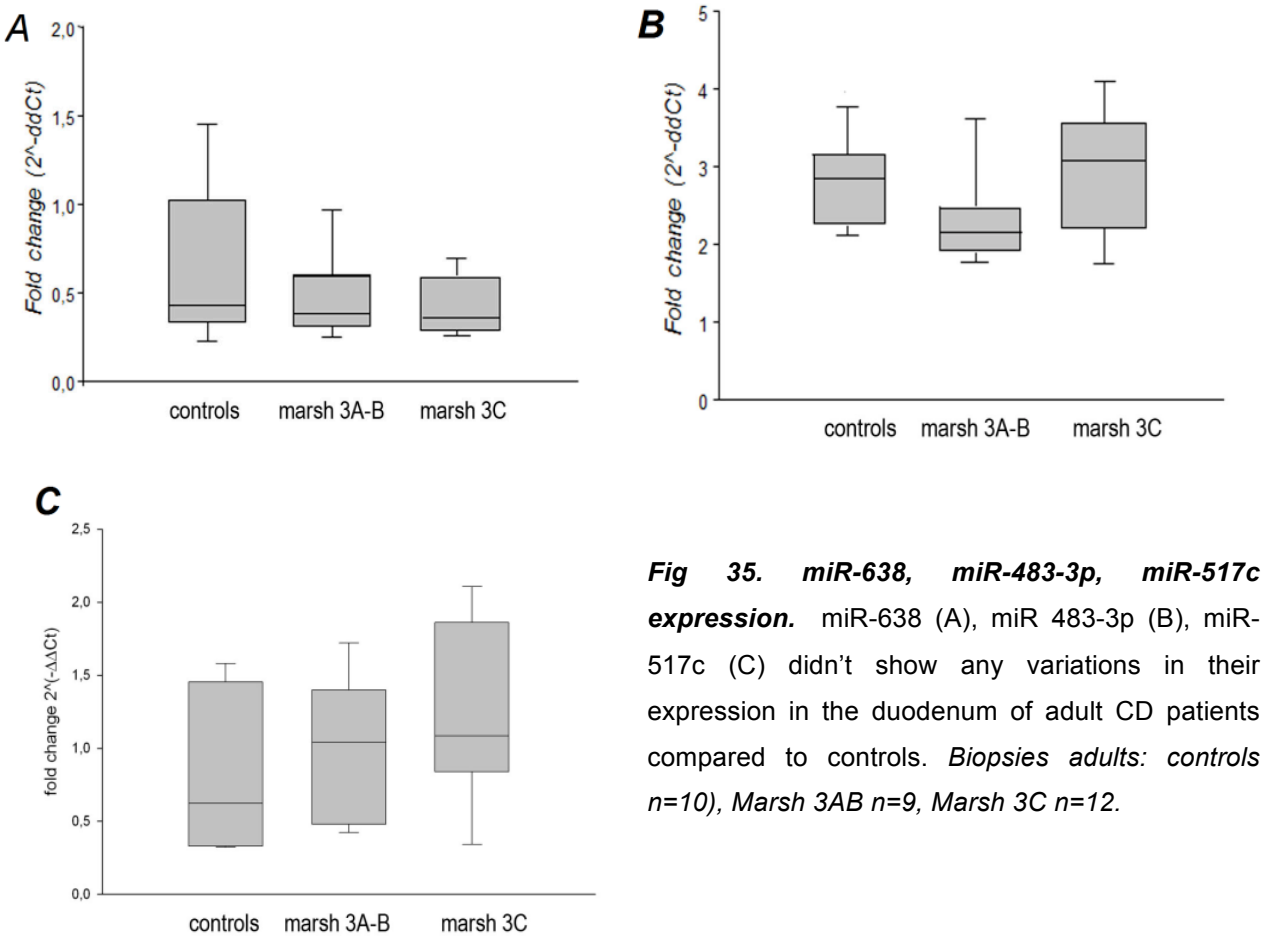


Fig 35. miR-638, miR-483-3p, miR-517c expression. miR-638 (A), miR 483-3p (B), miR-517c (C) didn't show any variations in their expression in the duodenum of adult CD patients compared to controls. *Biopsies adults: controls n=10), Marsh 3AB n=9, Marsh 3C n=12.*

4.1.7. miR-21-5p and miR-21-3p EXPRESSION AND STAT3

The performed microarray analysis returned only seven miRNAs, all down-regulated, and this could be due also to the stringent conditions employed for the analysis. To evaluate other possible variations we selected miR-21-5p, which had been previously reported as involved in inflammatory and immune response, in particular being a stimulating factor for cytokines expression (*Schetter AJ et al, 2009)*. Thus, in theory, this miRNA should be up-regulated due to the presence of an activated inflammatory/immune response in celiac biopsies. Nevertheless, in adult patients, we didn't detect any variation in its expression in CD patients compared to controls (2.08 ± 0.60 vs 2.38 ± 0.62 in controls and Marsh 3C, respectively, $p>0.05$, ANOVA). On the contrary, we detected a significant up-regulation of miR-21-5p expression in Marsh 3C pediatric patients (1.30 ± 0.41, 1.65 ± 1.41 and 2.41 ± 0,73 in controls, Marsh 3AB and Marsh 3C, respectively, $p<0.05$ by ANOVA).

miR-21-5p trend in plasma was comparable to biopsies, and in Marsh 3C patients its levels were 3 times higher than controls (1.18 ± 0.64, 1.24 ± 0.15 and 3.90 ± 1.79 in controls, Marsh 3AB and 3C respectively, $p<0.05$ by ANOVA). GFD patients showed a decrease in miR-21-5p and it reached levels similar to controls (1.18 ± 0.64 vs 1.94 ± 1.40, in controls and GFD respectively) with a difference that was not statistically significant compared to controls ($p>0.05$, ANOVA) (FIG 36A). Since the processing of miRNAs leads to the generation of two mature forms, we also wanted to assess if miR-21-3p had the same trend. In adult CD patients, also this miRNA didn't show any variations in its expression (4.59 ± 3.02 (controls), 6.05 ± 2.53 (Marsh 3AB), 5.55 ± 1.18 (Marsh 3C), $p>0.05$ by ANOVA); on the other hand, we observed an up-regulation in Marsh 3C pediatric patients, with a two-fold increase compared to controls (2.54 ± 0.51, 3.15 ± 2.66 and 5.59 ± 1.77 in controls, Marsh 3AB and Marsh 3C, respectively, $p<0.05$ by ANOVA). Also in plasma, miR-21-3p showed a trend similar to that detected in biopsies: 0.82 ± 0.38 (controls), 1.80 ± 1.60 (Marsh 3AB), 1.98 ± 2.03 (Marsh 3C) and 0.52 ± 0.12 (GFD) but the results were not statistically significant for any group (FIG 36B).

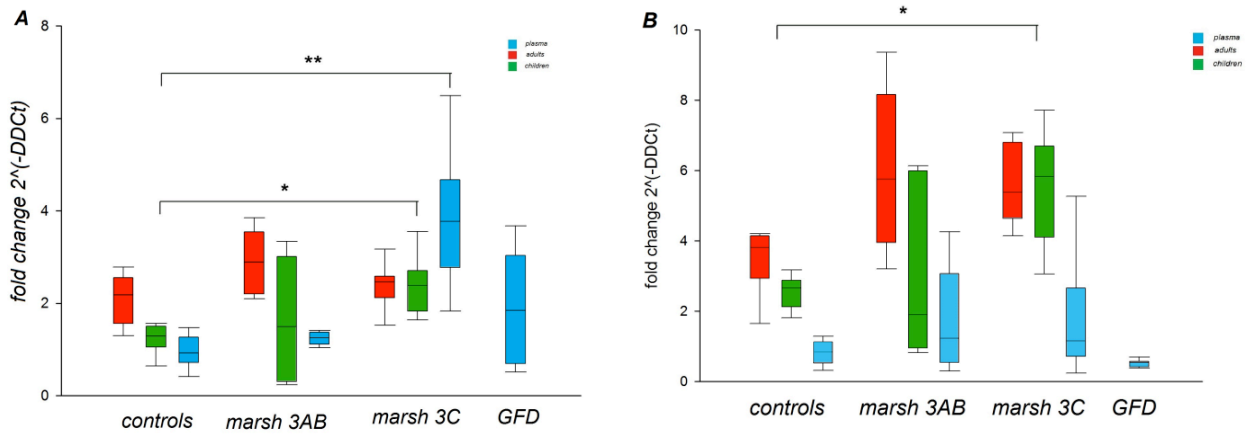


Fig 36. miR-21-5p and miR-21-3p expression. (A) miR-21-5p resulted up-regulated only in pediatric CD patients compared to controls and in the plasma we detected the same trend of expression. (B) also miR-21-3p resulted up-regulated only in pediatric CD patients and in the plasma showed a similar trend to biopsies but it didn't reach a significant statistically result. *Red bars: adults, green bars: children, blue bars: plasma. * = p<0.05, ** = p<0.01. Biopsies: controls n=10 (adults) n=8 (children), Marsh 3AB n=9 (adults) n=8 (children), Marsh 3C n=12 (adults) n=12 (children). Plasma children: n=8 (controls), n=8 (Marsh 3AB), n=12 (Marsh 3C), n=7 (GFD).*

In silico analysis of possible targets of miR-21-5p revealed a binding site for this miRNA on 3'UTR of signal transducer and activator of transcription 3 (STAT3) *(Kim et al., 2012).* We analyzed its expression in pediatric patients and we observed a significant increase in mRNA levels in CD patients, with 2.4 times higher expression in Marsh 3C patients (1.23 ± 1.30, 2.04 ± 0.79 and 3.36 ± 1.54 in controls, Marsh 3AB and Marsh 3C, respectively, $p<0.05$ by ANOVA, FIG. 23A). The relationship between miR-21 and STAT3 appears controversial, since STAT3 can cause the transcriptional activation of miR-21 *(Kohanbash et al., 2012).* We thus evaluated Stat3 activation by western blot on nuclear extracts, detecting an increase in serine 727 phosphorylation in CD patients (0.86 ± 0.36 vs 1.42 ± 1.08 in controls and Marsh 3C, respectively, $p<0.05$ by ANOVA) (FIG 37B)

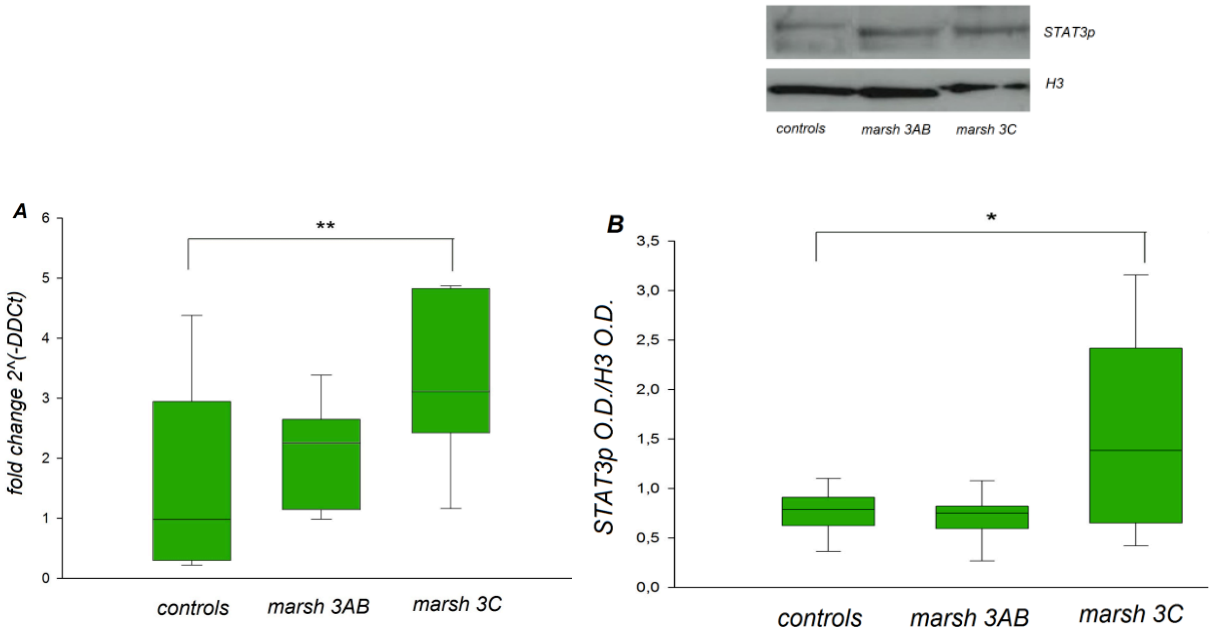


Fig 37. STAT3 expression. *STAT3 mRNA (A) and Stat3 phosphorylation on Serine 727 (B) are up-regulated in CD children patients compared to controls. Western Blot is representative of pediatric samples. * = p<0.05, ** = p<0.01. Children biopsies : controls n=8, Marsh 3AB n=8, Marsh 3C n=12.*

4.1.8 IN VITRO STIMULATION

To assess whether the observed expression variations were due to gluten, we exposed duodenal biopsies of controls and CD adult patients on Gluten Free Diet (GFD) to peptic-tryptic digest of gliadin for 4 hours, in ex-vivo stimulation experiments. PT gliadin was able to elicit a significant decrease in miR-192-5p expression in CD patients' biopsies, with a reduction of 42% in stimulated GFD biopsies as compared to unstimulated ones (0.77 ± 0.48 vs 0.45 ± 0.33, p=0.002, by t-test) (FIG 38A). Consistently, we observed a significant increase in the mRNA level of CXCL2 (p=0.048) (FIG 38E) and NOD2 (p=0.049) (FIG 38F) in stimulated GFD biopsies as compared to unstimulated ones, whereas no difference was observed in biopsies obtained from control subjects.

In vitro stimulation studies revealed also a down-regulation in miR-31-5p (1.23 ± 1.03 vs 0.99 ± 0.95 in unstimulated and stimulated GFD biopsies, respectively) (FIG 38B) and increased levels for FOXP3 mRNA (22.00 ± 6.06 vs 30.03 ± 10.50 in unstimulated and stimulated GFD biopsies) (FIG 238G) in the biopsies of CD patients stimulated with gliadin, thus showing expression variations similar to those observed in CD patients at diagnosis, although these changes did not reach statistical significance. We also detected a decreased level of miR-338-3p (FIG 38C) in GFD biopsies treated with PT gliadin as compared to untreated ones, although even this reduction did not reach statistical significance (11.14 ± 5.51 vs. 7.92 ± 3.97 in unstimulated and stimulated GFD biopsies, respectively). On the other hand, we could not observe any reduction in miR-197 levels (FIG 38D) after PT treatment (2.35 ± 1,00 vs. 3.71 ± 2.86 in unstimulated and stimulated GFD biopsies, respectively). The lack of statistically significant data could depend on the brief time of stimulation; further experiments were performed stimulating the biopsies for a longer time but this procedure caused the degradation of the mRNA.

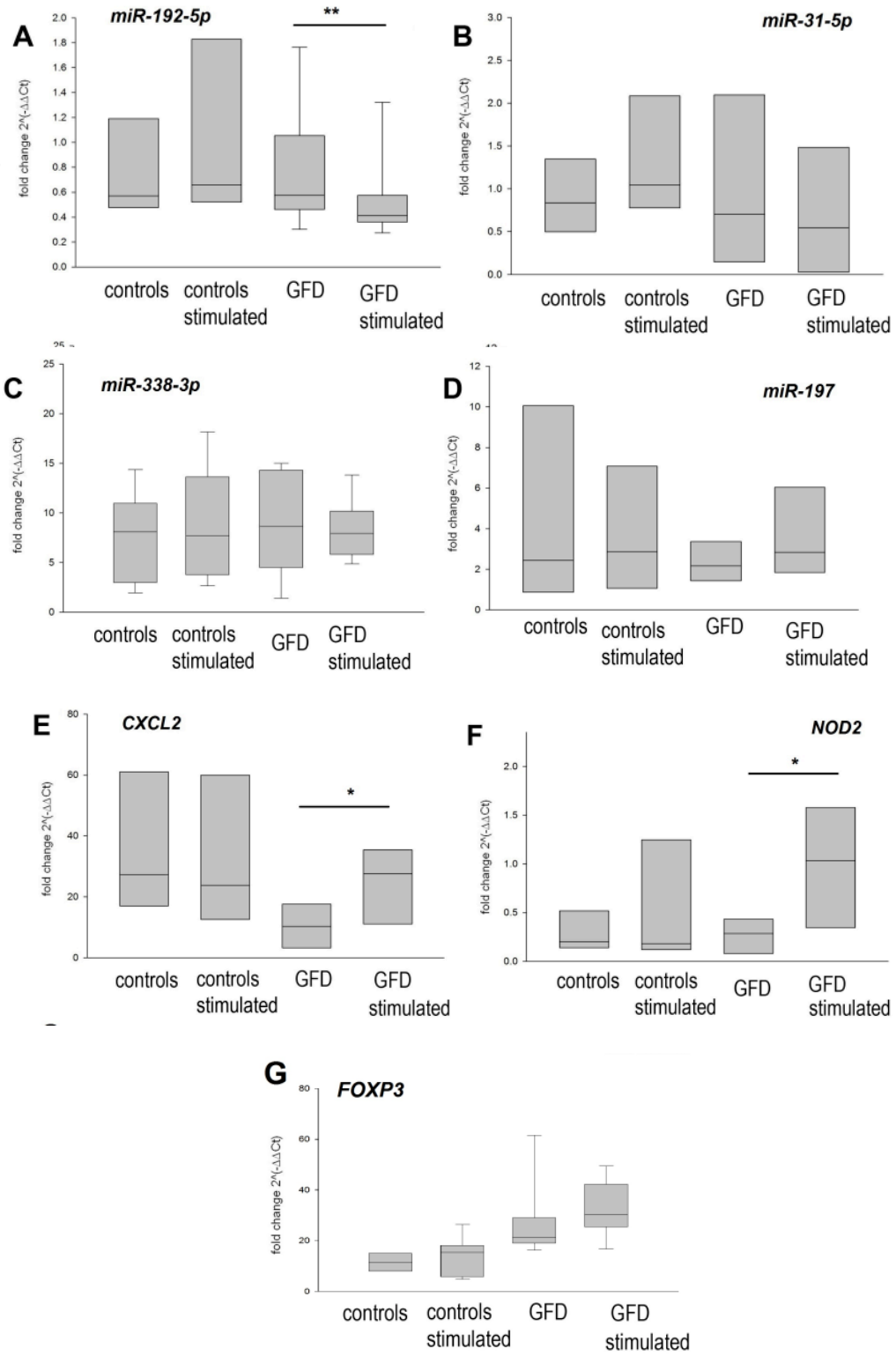


Fig 38. In vitro stimulation. *miR-192 (A) showed a down-regulation of expression in GFD stimulated biopsies and its targets CXCL2 (E) and NOD2 (F) resulted up-regulated. miR-31-5p (B) showed a reduced trend and its target FOXP3 (G) showed an up-regulation in stimulated GFD biopsies but they did not reach significant results. miR-197 (D) and miR-338-3p(C) expression, didn't show any variation. * = p<0.05, ** = p<0.01. Biopsies: controls n=5, GFD n=9.*

4.2. HYPOXIA AND IRON METABOLISM

4.2.1. miRNA MICROARRAY

We performed a microarray analysis, comparing miRNAs expression, on RNA extracted from the liver of mice exposed to different time points of hypoxia, compared to mice in normoxia. The cluster analysis showed 50 miRNAs that resulted differently expressed in the 4 groups of mice analyzed: red signal indicated miRNA down-regulated and green signal miRNA up-regulated, considering as common reference a sample generated by pooling all the analyzed groups (FIG 39).

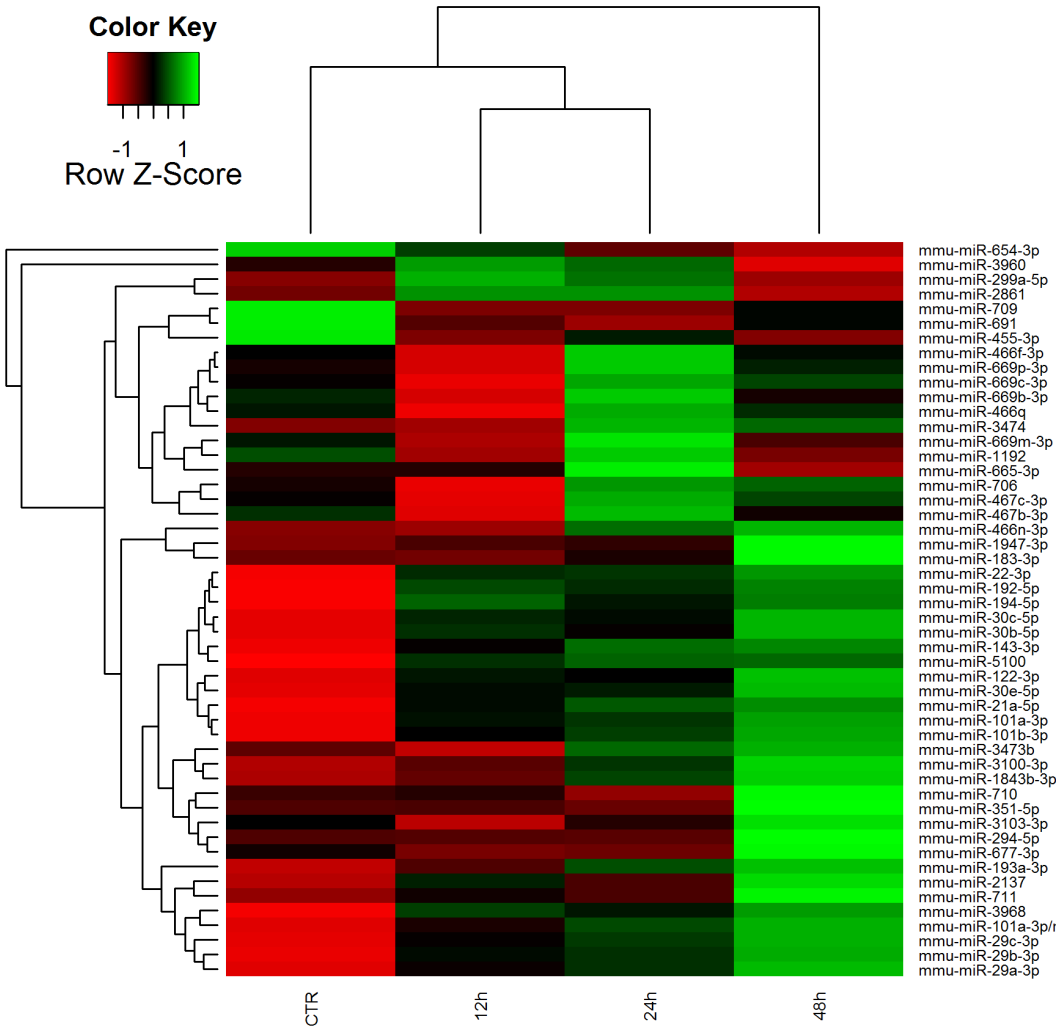


Fig 39. miRNA microarray. *Cluster analysis of the 50 miRNAs differently expressed in the groups of mice exposed to hypoxia compared to controls.*

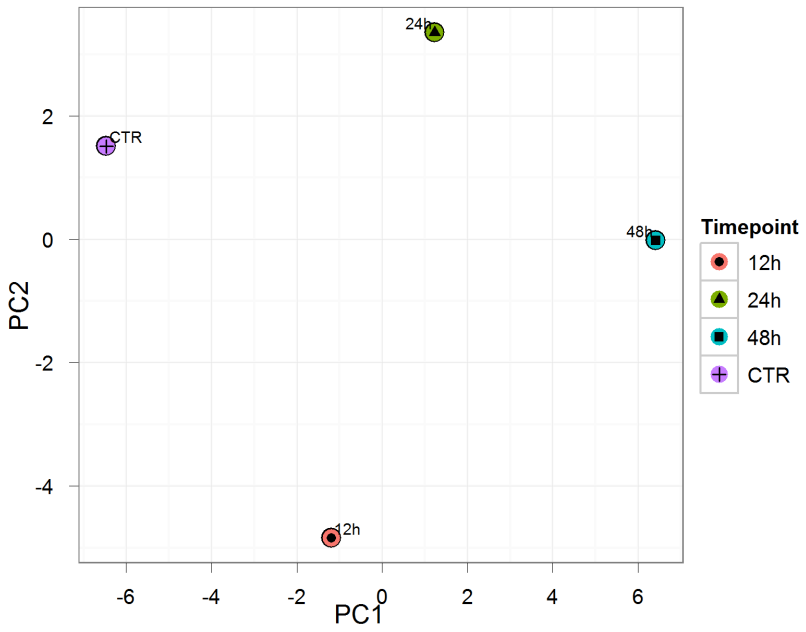


Fig 40. microarray PCA *Traditional PCA plot. The principal component analysis was performed on all samples, and on the top 50 microRNAs with the highest deviation from the standard. The normalized log ratio values have been used for the analysis.*

Although the PCA analysis was performed on the different pools, it provided a good separation of the samples, indication a different pattern of expression according to the time of hypoxia.

4.2.2. VALIDATION OF miR-22-3p EXPRESSION AND ITS TARGET GENE: BMP6

Among the others, we focus our attention on miRNAs identified from microarray which could have a role in iron homeostasis, as assessed by data in the literature or software target prediction. Firstly, we verified by qRT-PCR the expression of miR-22-3p; the results showed a significant up-regulation of this miRNA in the liver of mice exposed to different time-points of hypoxia compared to mice in normoxia, with an increase of 97% after 48 hours (0.08 ± 0.10 in controls, 0.43 ± 0.41 at 12 hours, 1.60 ± 1.24 at 24 hours (p=0.034), 2.72 ± 1.87 at 48 hours (p=0.001) by ANOVA compared to controls) (FIG 41).

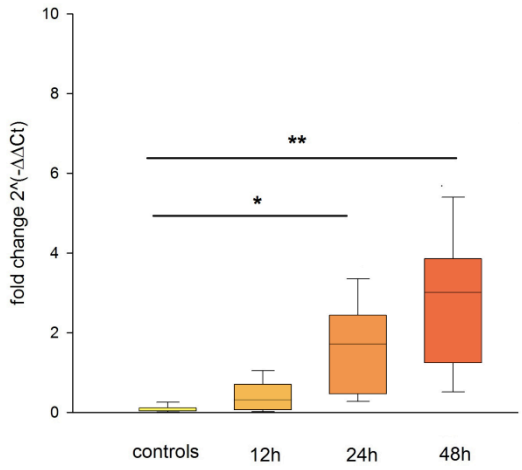


Fig 41. miR-22-3p expression. *miR-22-3p was significantly up-regulated in mice exposed to hypoxia at all the time-points considered. * = p<0.05, ** = p< 0.01. Controls n=7, 12h n=8, 24h n=8, 48h n=8.*

In silico analyses were performed to identify the target genes of this miRNA; data of literature *(Long J et al., 2013)* and target prediction softwares identified Bmp6 as target gene of miR-22-3p (FIG 42).

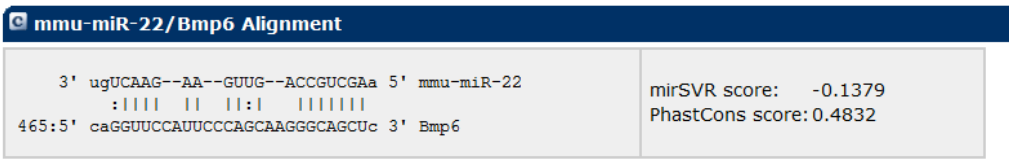


Fig 42. miR-22-3p and Bmp6 alignment. *MicroRNA.org software for target prediction identified Bmp6 as target of miR-22-3p*

We analyzed the expression of Bmp6 mRNA in the liver of mice exposed to hypoxia compared to mice on normoxia, and the result of qRT-PCR showed that Bmp6 mRNA expression was significantly reduced: in fact it decreased to 50% at 12 hours and 25% after 48 hours of exposure (2.04 ± 1.03 in controls, 1.01 ± 0.43 at 12 hours, 0.56 ± 0.34 at 24 hours, 0.51 ± 0.23 at 48 hours p<0.001 for all groups compared to controls, by ANOVA, FIG 43A). Moreover, Bmp6 mRNA expression resulted significantly inversely correlated with miR-22-3p (p=0.004, R= -0.53, Pearson

Correlation, (FIG 43B). Furthermore, Bmp6 protein expression paralleled mRNA expression: as shown in the western blot image and by the densitometric analysis, protein levels in mice undergoing hypoxia were significantly reduced compared to controls, with a strong and immediate decrease after 12 hours of exposure (60% reduction) which endured for the other time points (2.12 ± 0.73 in controls, 0.82 ± 0.09 at 12 hours, 0.66 ± 0.23 at 24 hours, 0.71 ± 0.25 at 48 hours, $p<0.001$ by ANOVA, for all groups compared to controls) (FIG 43C).

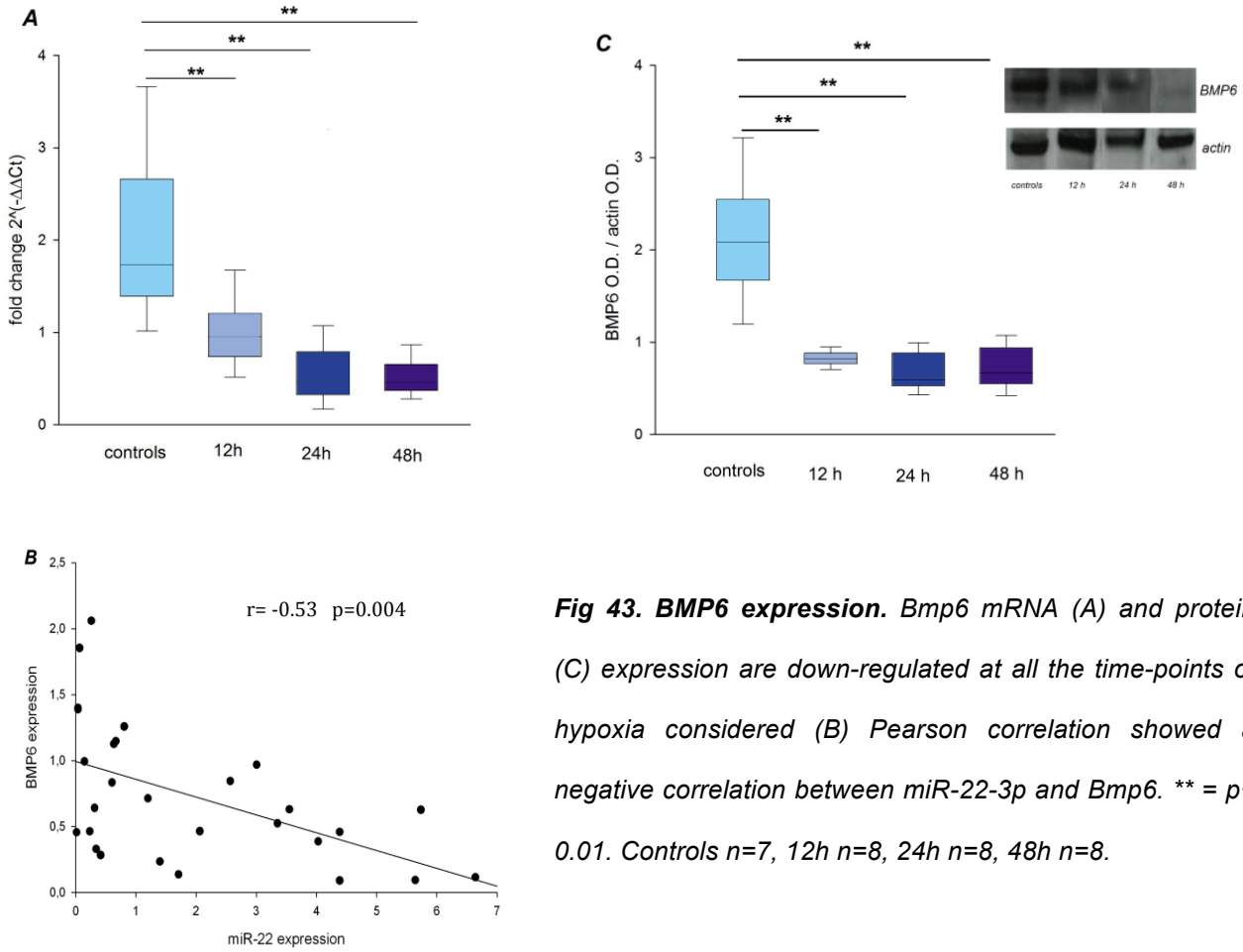


Fig 43. BMP6 expression. *Bmp6 mRNA (A) and protein (C) expression are down-regulated at all the time-points of hypoxia considered (B) Pearson correlation showed a negative correlation between miR-22-3p and Bmp6. ** = p< 0.01. Controls n=7, 12h n=8, 24h n=8, 48h n=8.*

To clarify if Bmp6 mRNA down-regulation was caused by transcriptional or post-transcriptional processes, we used both an in silico and an experimental approach. Firstly we evaluated the Bmp6 promoter by Genomatix Software, in order to identify the presence of some binding sites for Hypoxic Responsive Elements (HRE). The software identified several possible binding sites; however, all of them were able to bind hypoxia-induced transcriptional activators.

This, in theory, could result in an increased expression of BMP6, which is the opposite of what was observed in the liver of hypoxic mice. To assess whether the observed down-regulation on stady-state mRNA was due to a post-transcriptional mechanism, we also designed a PCR assay able to distinguish the spliced (cytosolic) or unspliced (nuclear) forms of BMP6 and HPRT mRNAs. FIG 44A represent the obtained amplification products, showing a good separation of nuclear and cytosolic RNAs. qRT-PCR for Bmp6 on nuclear RNA extracted from liver of mice exposed to 48 hours of hypoxia, compared on mice on normoxia, did not show any altered expression, confirming the idea that the down-regulation of Bmp6 did not depend on transcriptional regulation, but rather on the post-transcriptional mechanism mediated by miR-22 (FIG 44B).

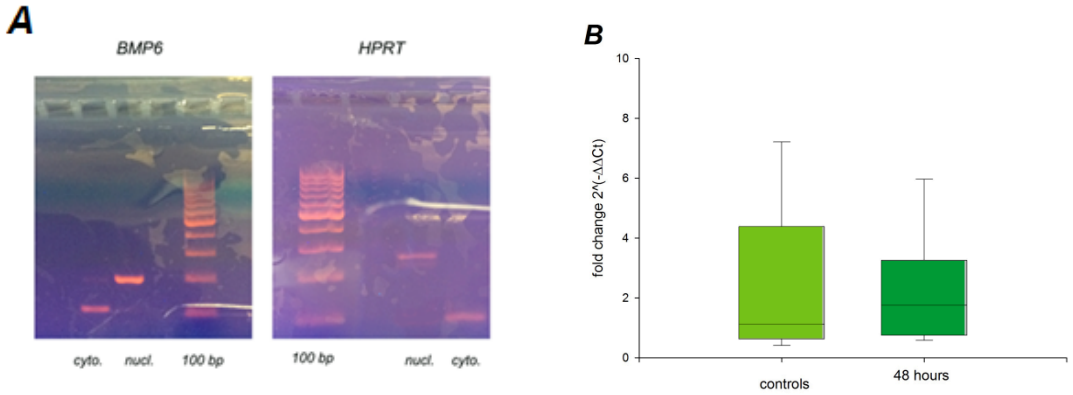


Fig 44. Bmp6 nuclear and cytoplasmic expression. *(A) PCR end-point for Bmp6 analysis in nuclear and cytoplasmic RNA extracted from mice liver; the image showed the good separation of the extracted RNAs. (B) qRT-PCR for Bmp6 mRNA expression in the nucleus of mice exposed for 48 hours to hypoxia compared to normoxia. The results didn't show any change of expression between the two groups. Controls n=7, 48h n=8.*

4.2.2.1 LIVER IRON CONTENT AND miR-22-3p EXPRESSION

Variations in miR-22-3p could in theory depend on variation in liver iron content that could occur in hypoxia due to iron redistribution. We firstly assessed whether LIC (liver iron content) was different in mice undergoing hypoxia compared to normoxic ones, but no significant variation was observed among groups (0.38 ± 0.11 in controls, 0.41 ± 0.10 at 12 hours, 0.45 ± 0.17 at 24 hours, 0.42 ± 0.20 at 48 hours $p>0.05$ for all groups compared to controls, by ANOVA).

We also analyzed the liver of mice treated with either an iron-rich or an iron-deficient diet, compared to mice on normal diet.

Firstly, we analyzed the Bmp6 mRNA levels in the liver of these mice; the results showed an increase of expression in mice on iron-rich diet and a down-regulation in mice iron-deficient, confirming the goodness of the mouse model (0.75 ± 0.31 in controls, 2.37 ± 1.05 in diet Fe +, 0.33 ± 0.31 in diet Fe -, ANOVA $p<0.05$, both) (FIG 45A).

Therefore, we analyzed the levels miR-22-3p, and either in mice following an iron-rich and iron-deficient diet, miRNA expression was up-regulated compared to mice following normal diet (1.28 ± 0.44 in controls, 3.74 ± 2.80 in diet Fe +, 3.04 ± 1.72 in diet Fe -, ANOVA $p<0.05$, both). These data suggest that in case of iron unbalances BMP6 regulation does not depend upon miR-22-3p action, but also that miR-22-3p does not linearly respond to iron, differently from what observed for hypoxia (FIG 45B).

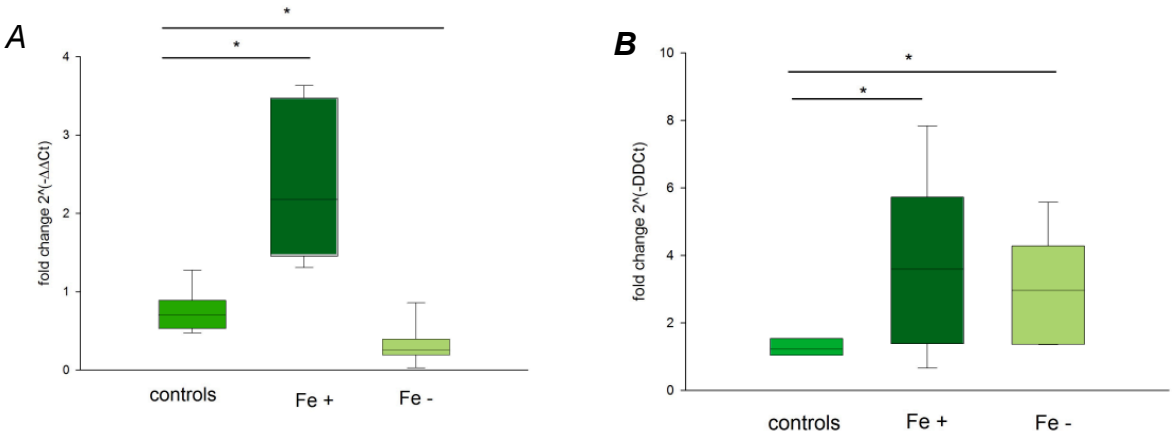


Fig 45. Bmp6 and miR-22-3p expression on mice on diet. (A) *Bmp6 expression was up-regulated in mice following an iron-rich diet and down-regulated in mice following an iron-deficient diet. (B) miR-22-3p expression was up-regulated both in mice following an iron-rich diet and an iron-deficient diet. * = p<0.05. mice controls n=6, Fe+ n= 6, Fe- n=6.*

4.2.3. BMP RECEPTOR COMPLEX, MIRNAS AND HYPOXIA

BMPs interact on the cell surface with heterodimeric receptors formed by Bmpr1 and Bmpr2; in the pathway that regulates iron metabolism, the heterodimer needs a co-receptor called hemojuvelin.

Due to the effect of hypoxia on BMP6, we wanted to assess whether other hypoxia-induced miRNAs were targeting the BMP receptor complex.

4.2.3.1 VALIDATION OF miR-101a-3p EXPRESSION AND ITS TARGET GENE: BMPR1a

Analysis in silico revealed Bmpr1a as target of miR-101a-3p and this miRNA resulted differently expressed in hypoxic conditions by miRNA microarray (FIG 46).

Fig 46. miR-101a-3p and Bmpr1a alignment. *MicroRNA.org software for target prediction identified Bmpr1a as target of miR-101a-3p.*

We verified by qRT-PCR the expression of miR-101a-3p, and we detected an up-regulation in mice exposed to hypoxia compared to mice in normoxia, in particular we observed a more profound increase after 48 hours of exposure with significant statistic values at 24 and 48 hours (0.80 ± 0.82 in controls, 1.07 ± 0.60 at 12 hours, 4.21 ± 3.14 at 24 hours (p=0.008) and 5.19 ± 3.78 at 48 hours (p=0.005), ANOVA compared to controls) (FIG 47).

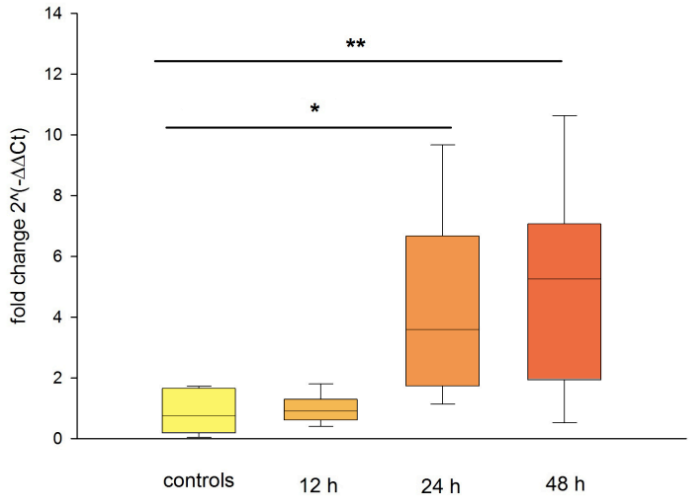


Fig 47. miR-101a-3p expression. *miR-101a-3p expression was significantly up-regulated in mice exposed to hypoxia. * = p<0.05, ** = p< 0.01. Controls n=7, 12h n=8, 24h n=8, 48h n=8.*

Then, we analyzed the expression of Bmpr1a, receptor of type I for Bmp6, and in our mice we detected a down-regulation of this gene that resulted statistically significant at 24 and 48 hours of exposure to hypoxia (0.70 ± 0.34 in controls, 0.81 ± 0.33 at 12 hours, 0.38 ± 0.16 at 24 hours (p=0.037) and 0.36 ± 0.10 at 48 hours (p=0.038), ANOVA compared to controls) (FIG 48A).

The idea that iron homeostasis, regulated by Hamp, is influenced by hypoxia is further supported by these data, nevertheless Pearson statistic test didn't detect a negative correlation between miR-101a-3p and Bmpr1a expression, probably due to the standard deviation of the values (p=0.13 r= -0.38) (FIG 48B).

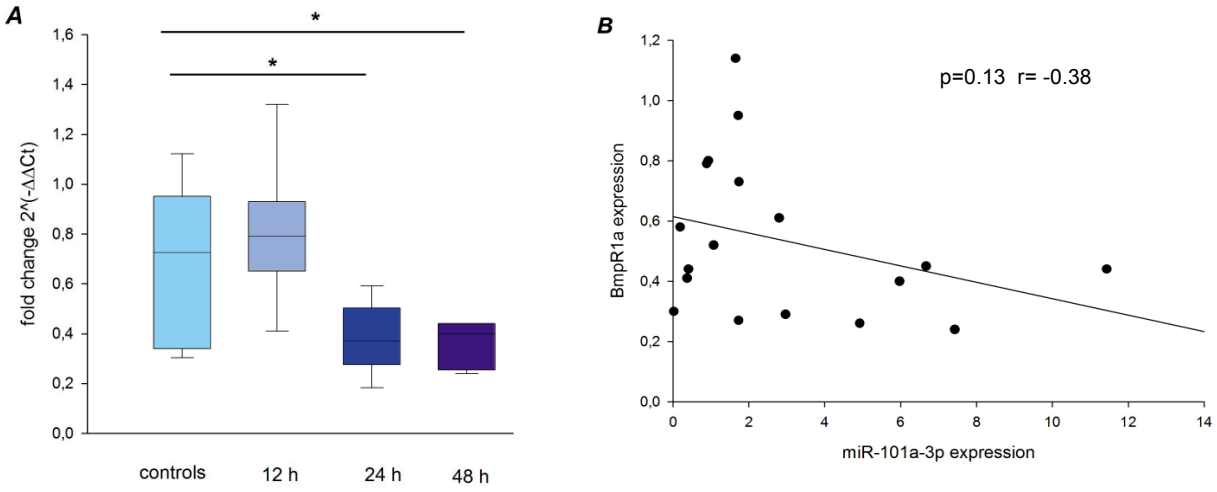


Fig 48. Bmpr1a mRNA expression. *(A) Bmpr1a mRNA expression resulted down-regulated in mice exposed to hypoxia. (B)Bmpr1a / miR-101a-3p Pearson Correlation.* Although we observed a negative correlation *trend, the analysis did not reached significance level. * = p<0.05. Controls n=7, 12h n=8, 24h n=8, 48h n=8.*

4.2.3.2. VALIDATION OF miR-192-5p EXPRESSION AND ITS TARGET GENE: BMPR2

In their activation, Bmp receptors type I form a dimer with Bmp receptor type 2, thus we assessed whether Bmpr2 was a target of one of the hypoxia-regulated miRNAs. In silico prediction identified a match between the 3'UTR of the mRNA and miR-192-5p.

Fig 49. miR-192-5p and Bmpr2 alignment. *MicroRNA.org software for target prediction identified Bmpr2 as target of miR-192-5p.*

miR-192-5p was detected as up-regulated by microarray after hypoxia exposure, data confirmed by qPCR, with a more profound increase at 24 hours of exposure and a significant statistically result after 24 and 48 hours (1.45 ± 1.40 in controls, 2.28 ± 1.90 at 12 hours, 5.50 ± 2.67 at 24 hours (p=0.016), 4.60 ± 2.57 at 48 hours (p=0.018), ANOVA compared to controls) (FIG 50).

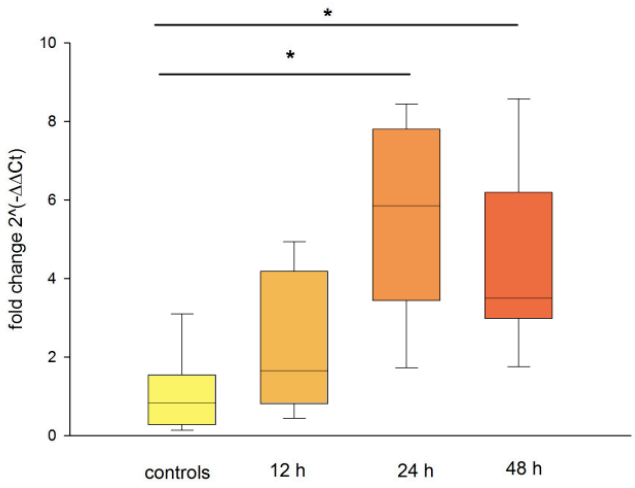


Fig 50. miR-192-5p expression. *miR-192-5p was up-regulated in mice exposed to hypoxia at different time-points considered. * = p<0.05. Controls n=7, 12h n=8, 24h n=8, 48h n=8.*

Thus we analyzed Bmpr2 mRNA levels; unexpectedly in hypoxic mice this gene did not show any change of expression compared to mice in normoxia (0.69 ± 0.73, 0.74 ± 0.65, 0.45 ± 0.18 and 0.57 ± 0.21, respectively in controls, 12, 24, 48 hours of exposure to hypoxia, p>0.05, ANOVA) (FIG 51).

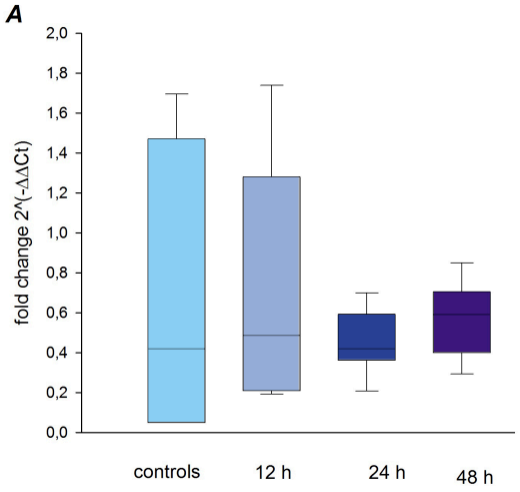


Fig 51. Bmpr2 mRNA expression. *Bmpr2 mRNA expression didn't show any change in mice exposed to hypoxia. Controls n=7, 12h n=8, 24h n=8, 48h n=8.*

4.2.3.3. ANALYSIS OF miR-122-5p EXPRESSION AND ITS TARGET GENE: HJV (HFE2)

Another important actor of Bmp/Smad pathway is Hemojuvelin (Hjv or Hfe2) which is the co-receptor of Bmp6. We performed in silico analysis but we didn't find any match between the 3'UTR of the hemojuvelin mRNA and miRNAs regulated by hypoxia in our experimental model.

However, we verified its expression in our mice model, and the result showed that this gene in hypoxic conditions is down-regulated, even if this decrease is significant only up to 24 hours of exposure (5.08 ± 1.67 in controls, 2.68 ± 1.52 at 12 hours (p=0.028), 1.62 ± 0.74 at 24 hours (p=0.004), 3.76 ± 3.26 at 48 hours, by ANOVA compared to control group) (FIG 52).

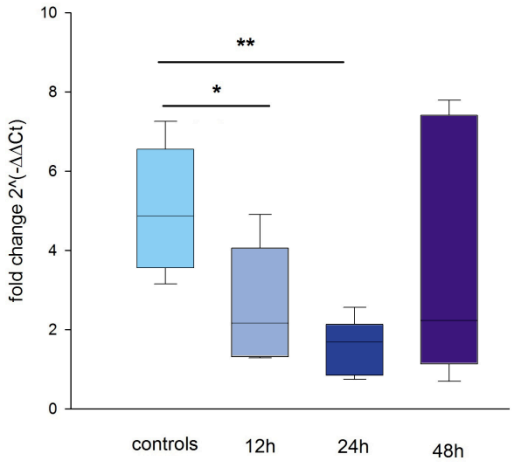


***Fig 52. Hjv expression**. Hjv was significantly down-regulated in hypoxic conditions. * = p<0.05, ** = p< 0.01. Controls n=7, 12h n=8, 24h n=8, 48h n=8.*

Analysis in silico and data of literature *(Castoldi M et al., 2012)* revealed a target site for miR-122-5p on Hfe2 3'UTR. Interestingly, qRT-PCR analysis of miR-122-5p in mice liver did not show significant changes related to hypoxia when all the time points were considered (0.38 ± 0.33, 1.02 ± 0.63, 1.83 ± 1.20 and 0.86 ± 0.85, respectively in controls, 12, 24 and 48 hours) (FIG 53), and no significant Pearson correlation was detected (data not shown). Nevertheless, if we considered data up to 24 hours of exposure to hypoxia, we detected a significant increase in miR-122-5p (p=0.03), and a significant inverse correlation between miR-122-5p and Hfe2 (p=0.04, R= -0.48).

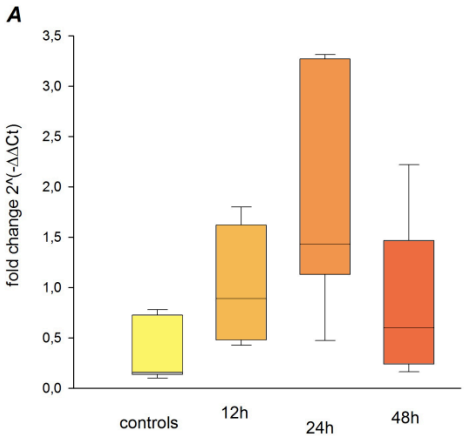


***Fig 53. miR-122-5p expression**. miR-122-5p expression didn't show significant changes in mice exposed to hypoxia when all the time-points were considered, but only if we considered values up to 24h of exposure (p=0.03). Controls n=7, 12h n=8, 24h n=8, 48h n=8.*

4.2.4. EXPRESSION OF MOLECULES INVOLVED IN BMP/HJV PATHWAY: SMAD 1/5/8p, HEPCIDIN, ID1 and FPN1

The down-regulation of Bmp6 and of part of the receptor complex suggested that a reduced activation of the pathway was present. We thus analyzed the intracellular portion of the pathway to verify its decrease in hypoxic conditions.

Firstly, we analyzed the role of Smad proteins, in particular the phosphorylation of Smad 1/5/8 required for the activation of the complex. Our results showed, in nuclear extracts, a significantly decrease of the phosphorylation at every time-points of hypoxia, up to a decrease of 57% at 48 hours, highlighting the involvement of this pathway in the down-regulation of Hamp in hypoxic conditions [1.09 ± 0.27, 0.72 ± 0.23 (p=0.009), 0.78 ± 0.12 (p=0.019) and 0.51 ± 0.12 (p=0.001) in controls, 12 hours, 24 hours and 48 hours of hypoxia, respectively, by ANOVA compared to control group] (FIG 54).

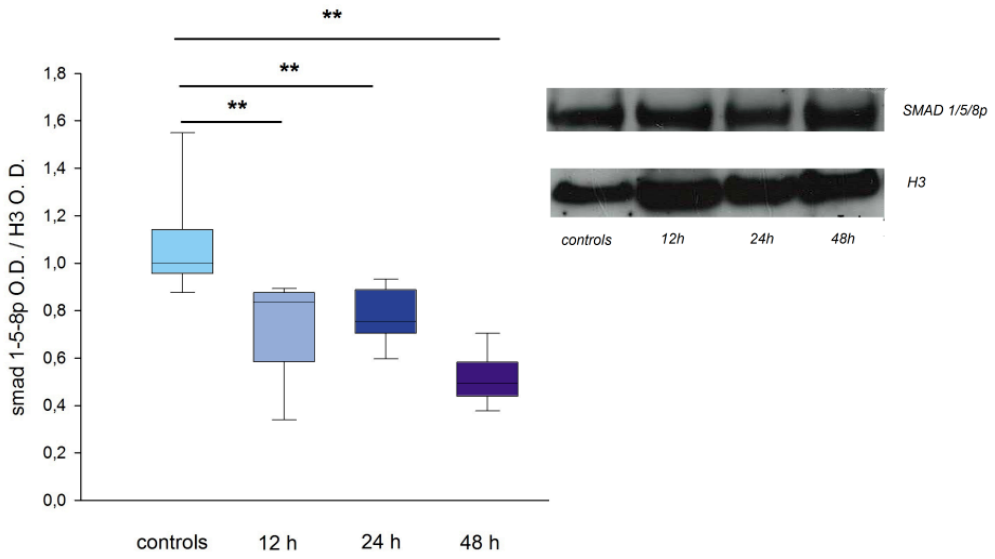


Fig 54. Smad 1/5/8p expression. *Smad 1/5/8 phosphorylation was significantly reduced in hypoxic conditions in nuclear proteic extracts; depicted values were normalized on histone H3 expression. ** = p< 0.01. Controls n=7, 12h n=8, 24h n=8, 48h n=8.*

Then, we evaluated the expression of Hamp mRNA in the liver of mice undergoing hypoxia compared to mice on normoxia. Hamp expression was significantly reduced, with a profound decrease of 50% immediately after 12 hours of exposure (0.80 ± 0.36 in controls, 0.39 ± 0.24 at 12 hours (p=0.022), 0.43 ± 0.24 at 24 hours (p=0.022) and 0.32 ± 0.28 at 48 hours (p=0.003) by ANOVA compared to control group) (FIG 55).

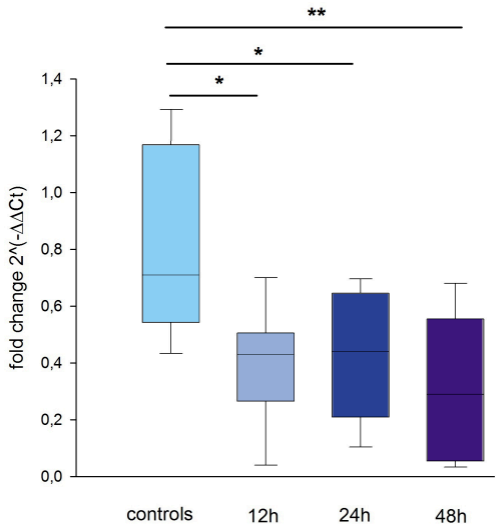


Fig 55. Hamp expression. *Hamp mRNA is significantly down-regulated at all the considered time-points of hypoxia. * = p<0.05, ** = p< 0.01. Controls n=7, 12h n=8, 24h n=8, 48h n=8.*

To confirm the effect of the reduced SMAD phosphorylation on gene expression, we also analyzed the expression of Id1 mRNA, another target of the pathway not directly involved in iron metabolism. Also this gene showed a significant mRNA down-regulation in hypoxic conditions, starting from 24 hours of exposure (2.01 ± 1.20 in controls, 1.69 ± 0.64 at 12 hours, 0.85 ± 0.48 at 24 hours (p=0.009), 1.22 ± 0.35 at 48 hours (p=0.049) by ANOVA compared to control group) (FIG 56).

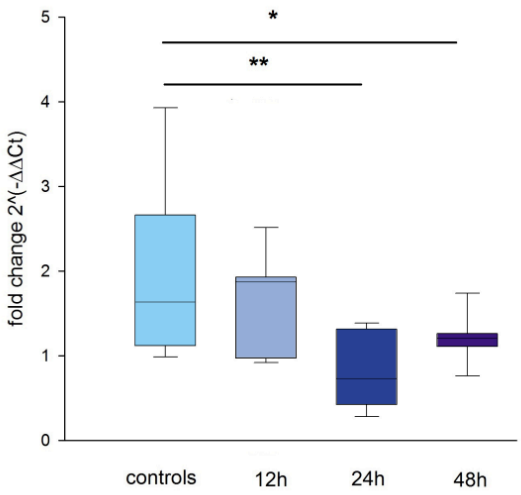


Fig 56. Id1 expression. *Id1 mRNA was significantly down-regulated in hypoxic conditions. * = p<0.05, ** = p< 0.01. Controls n=7, 12h n=8, 24h n=8, 48h n=8.*

Finally, considering the role of Hamp in iron homeostasis, we also evaluated the expression of ferroportin (Fpn1), the only known iron-exporter from cells, whose expression is regulated by Hamp. Fpn1 mRNA in the liver of our mice did not show any variation in hypoxic conditions, (1.91 ± 0.54, 2.16 ± 1.65, 1.72 ± 0.69 and 1.88 ± 0.38, in controls, 12 hours, 24 hours and 48 hours of hypoxia, respectively) (FIG 57A). However, ferroportin protein expression in liver resulted significantly increased in hypoxic conditions compared to controls, demonstrating the inverse correlation with Hamp expression and its post-transcriptional regulation; in particular it was significantly up-regulated at all the time points of hypoxia, with a more profound increase after 48 hours of exposure (0.56 ± 0.30 in controls, 1.07 ± 0.33 at 12 hours (p=0,033), 1.15 ± 0.55 at 24 hours (p=0.002), 1.38 ± 0.31 at 48 hours of hypoxia (p=0.002), ANOVA compared to control group) (FIG 57B).

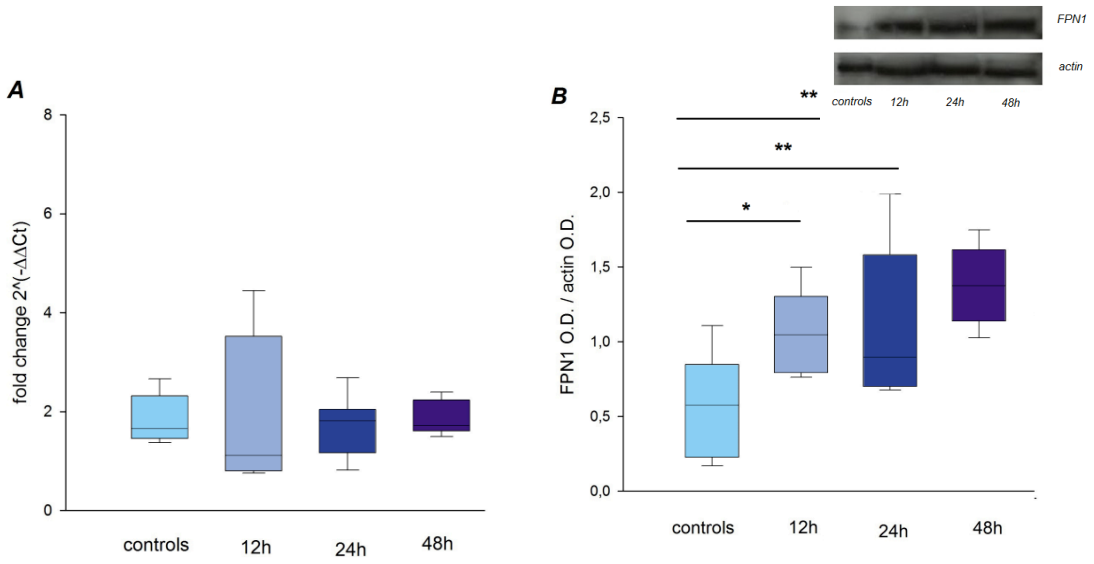


Fig 57. Fpn1 expression. *(A) Fpn1 mRNA expression didn't show any change in hypoxic conditions (B) Fpn1 protein expression showed a significant up-regulation at all the time-point considered. * = p<0.05, ** = p< 0.01. Controls n=7, 12h n=8, 24h n=8, 48h n=8.*

4.2.5. ANALYSIS OF TGFβ PATHWAY

Hepcidin expression is regulated by several pathways; among those the TGFβ pathway is similar to the BMP one, also using a common SMAD (SMAD4) to form the active transcription factor complex. In order to verify whether this molecular pathway was dependent on hypoxia, we studied the phosphorylation of Smad2 in nuclear extracts obtained from the liver of mice exposed to hypoxic conditions.

The results showed that Smad2-p did not show any change in its phosphorylation status in mice exposed to hypoxia compared to mice on normoxia (0.32 ± 0.17, 0.24 ± 0.12, 0.42 ± 0.24, 0.48 ± 0.26, respectively in controls, 12, 24 and 48 hours of exposure to hypoxia, p>0.05 by ANOVA). These data thus support the idea that this pathway wasn't influenced by hypoxia (FIG 58).

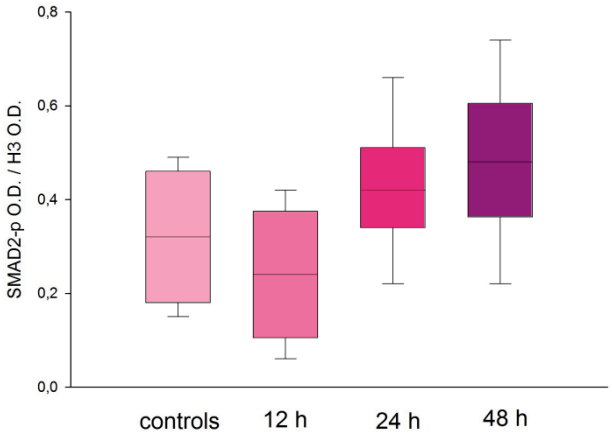


Fig 58. Smad2-p protein expression. *Phosphorylation of Smad2 didn't show any significant change in hypoxic conditions. Controls n=7, 12h n=8, 24h n=8, 48h n=8.*

4.2.6. VALIDATION OF miR-351-5p EXPRESSION AND ITS TARGET GENE: ATOH8

Among the other pathways that could influence Hamp expression, an important transcriptional factor is Atoh8, which increases hepcidin expression *(Patel, N et al., 2014).* The identification of a miRNA-dependent decrease of its mRNA could be another element able to assure the down-regulation of Hamp in hypoxic conditions. In silico analyses identified, on the 3'UTR of this gene, a binding site for miR-351-5p, miRNA detected as up-regulated by the microarray analysis (FIG 59).

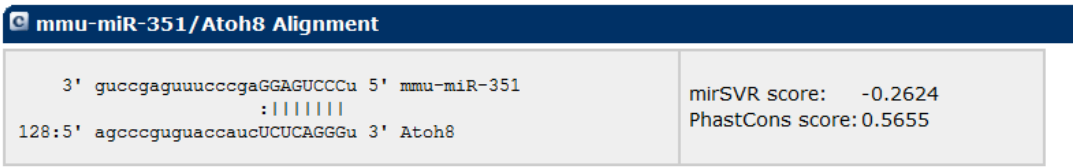


Fig 59. miR-351-5p and Atoh8 alignment. *MicroRNA.org software for target prediction identified Atoh8 as target of miR-351-5p.*

Firstly, we verified miR-351-5p expression by qRT-PCR and we detected an up-regulation with a significant and more profound increase after 48 hours of exposure to hypoxia (1.22 ± 0.93 in

controls, 1.07 ± 0.46 at 12 hours, 1.69 ± 0.92 at 24 hours and 3.06 ± 1.37 at 48 hours (p=0.002), ANOVA compared to controls in normoxia) (FIG 60).

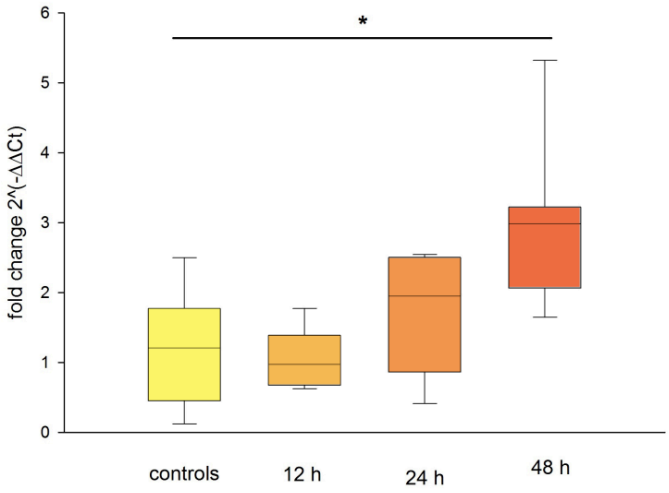


Fig 60. miR-351-3p expression. *miR-351-3p mRNA expression was significantly up-regulated after 48 hours of exposure to hypoxia. * = p<0.05. Controls n=7, 12h n=8, 24h n=8, 48h n=8.*

Regarding its target, we didn't detect any change of mRNA expression in mice exposed to hypoxia compared to mice in normoxia (1.54 ± 1.30, 1.23 ± 1.12, 0.38 ± 0.66 and 0.89 ± 0.20, respectively in controls, 12, 24, 48 hours of exposure to hypoxia, p>0.05 ANOVA compared to controls) (FIG 61A). Nevertheless, if we considered Atoh8 protein expression, the results obtained by western blot showed a significant down-regulation of this protein at all the time points considered (1.76 ± 0.79 in controls, 1.08 ± 0.57 at 12 hours (p=0.027), 1.18 ± 0.24 at 24 hours (p=0.040) and 0.93 ± 0.33 at 48 hours, p=0,011) (FIG 61B) indicating its post-transcriptional regulation by miR-351-5p action and supporting the hypothesis that Hamp down-regulation in hypoxic conditions is influenced by miRNAs involved in different pathways.

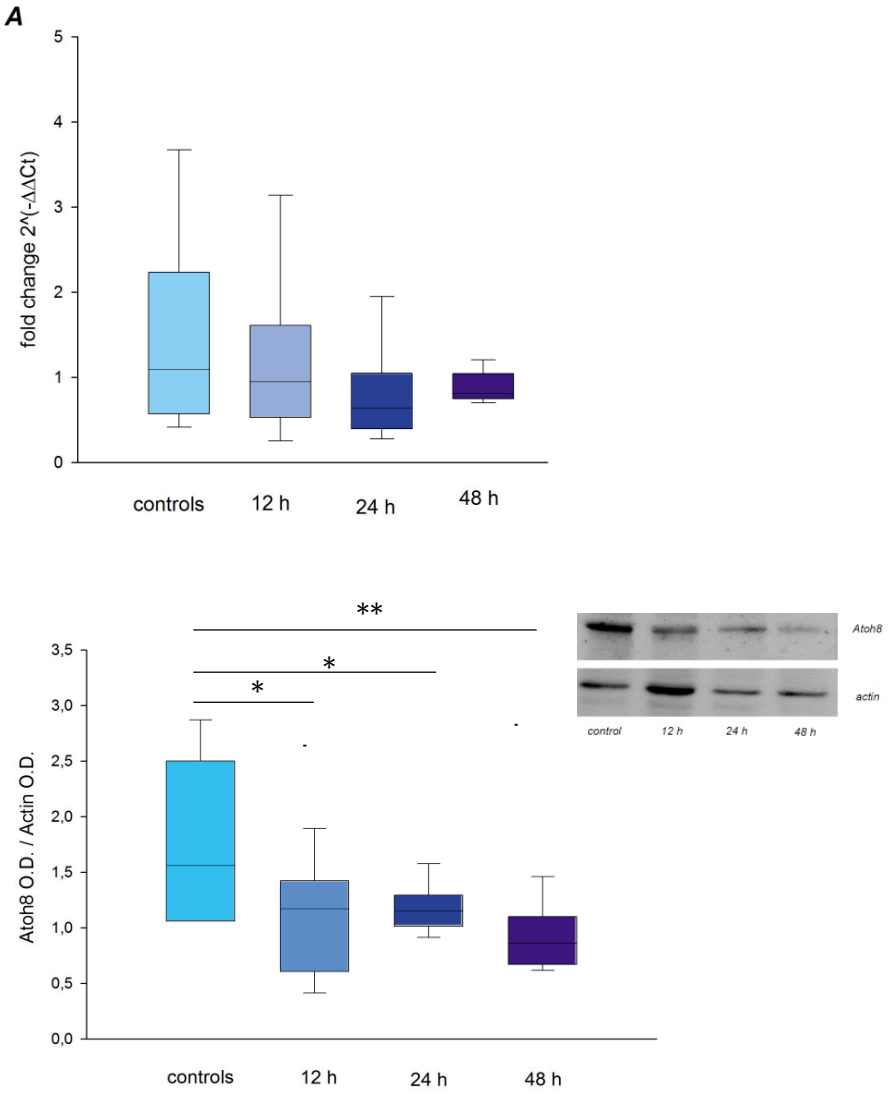


Fig 61. Atoh8 expression. *(A) Atoh8 mRNA expression didn't show any change in mice exposed to hypoxia (B) Atoh8 protein expression showed a significant down-regulation after 48 hours of exposure to hypoxia. * = p<0.05, ** = p< 0.01. Controls n=7, 12h n=8, 24h n=8, 48h n=8.*

V.

Discussion

DISCUSSION

The study showed the pivotal role of microRNAs as regulators of gene expression, underlining their ability to regulate several processes implicated in various pathological conditions. A lot of data support their role as major regulators, however it is becoming clearer that they do not work as an on/off mechanisms, but rather as a continuum, and that miRNAs levels, and consequently the expression of their target genes, could trigger the diseases.

miRNAs are expressed in various tissues, and the circulating ones are released by various organs in connection with a lot of different stimuli; nevertheless, there are also miRNAs expressed by particular tissues or peculiar conditions. Furthermore, data have demonstrated their stability in the body fluids even if the processes about their turn-over have to be clarified yet *(Kym YK, 2015).* These features could thus be very important in order to employ miRNAs as possible pathological biomarkers which could be useful either in diagnosis or follow-up.

However, the first step is represented by the identification of specific miRNAs for a particular disease, as well as by an evaluation of their levels both in the involved tissue and in plasma. We performed this analysis in two different disorders, namely celiac disease and hypoxia.

5.1. CELIAC DISEASE

Celiac disease is a human disorder characterized by various clinical manifestations that could differ according to the age of presentation, and according to the severity of the intestinal lesion. The only current therapy is gluten-free diet and adherence to it is usually assessed by auto-antibodies evaluation. However their levels do not always correspond to residual damage of the intestinal mucosa, which can only be assessed by invasive procedures. In this case miRNAs could represent an optimal alternative marker. In the first part of the present study we showed that the pattern of expression of miRNAs and related target genes in the duodenal mucosa of patients with celiac disease (CD) differs from that observed in healthy control subjects; moreover we observed changes in expression that correlated with the severity of the lesion, but also differed according to the age of presentation.

By miRNA microarray we identified 7 miRNAs significantly down-regulated in CD adult patients with a Marsh 3C lesion compared to controls; in the literature only two other studies approached the same issue, one in adults *(Vaira V et al., 2014),* and one on a children cohort *(Capuano M et al., 2011).* Compared to the adult study, four out of seven miRNAs showed a similar pattern of expression also in our cohort (miR-31-5p, miR-338-3p, miR-192-5p and miR-638-5p), whereas our data differed from those observed by Capuano et al. in the duodenal mucosa of children with CD, who detected several up- or down-regulated miRNAs but none in common with those identified in our study on adults. This fact could be explained by the different technique employed, but also hypothesizing the presence of differences due to the age of the analyzed subjects. For this reason we evaluated the expression of microarray-identified miRNAs in separate sets of adult patients but also in children ones. For 3 of the identified miRNAs (mir-468, miR-517c and miR-483-3p) we were not able to confirm the results obtained by microarray, probably because of the small number of the samples analyzed either in the original array or in the replication cohort. However we confirmed 4 of them (miR-197, miR-31-5p, miR-338-3p and miR-192-5p) as significantly reduced in biopsies of celiac patients both in children and adults; the decrease of expression of all these miRNAs paralleled the degree of intestinal damage, as assessed by Marsh's classification, and it was associated with the up-regulation of their target genes, mostly molecules involved in the immune system.

In particular, we detected miR-31-5p reduced in CD biopsies of both adult and pediatric patients, with increased mRNA and protein levels of its target, Foxp3, a transcription factor essential for Treg development, which represents a pivotal event in the regulation of the immune response, in particular regarding tolerance *(Zanzi D et al., 2011).* In CD patients, data of literature underlined the presence of an increased number of Foxp3 positive cells in the intestinal mucosa *(Brazowski E et al., 2010)* but data on their correct functionality are still conflicting, and it has been hypothesized that the ability of Treg to regulate the immune response in CD is actually defective *(Hmida NB et al., 2012).* Moreover, miR-31-5p has been reported upregulated in Crohn's disease, whereas different levels of expression have been reported in various tumors of the gastrointestinal tract (*Zhang Y et al., 2010)*, thus suggesting its importance in the homeostasis of the intestinal mucosa.

miR-197 was detected as significantly down-regulated in the biopsies of both adult and pediatric CDs, whereas its target, IL18, was significantly up-regulated. The role of IL18 in the intestine remains has not been completely defined yet; interestingly, polymorphisms of this gene have been associated with an increased risk of Crohn's disease development *(Tamura K, et al., 2002),* and increased level of IL18 have been detected in the mucosa of IBD (Inflammatory Bowel Disease) as well as in CD patients *(Salvati VM et al., 2002).* Finally, also the down-regulation of miR-338-3p and the increased expression of the transcription factor Runx1 was detected in adult and pediatric CD patients. Runx1 has been demonstrated to physically interact with Foxp3, as well as to be able to regulate its transcription *(Li L, et al., 2012),* and its up-regulation could potentiate Foxp3 effect on T cell commitment. Unfortunately, the data we generated are only based on RNA expression, since we were not able to obtain reproducible data regarding IL18 and RUNX1 protein expression, due to the lack of antibodies with sufficient sensibility and specificity.

For the miRNAs and targets described up to now, the same trend was observed in both groups, namely pediatric and adult CD patients, compared to controls. However, in the case of miR-192-5p, in particular regarding its targets, this pattern started to differ. miR-192-5p belongs to a miRNA cluster (miR-194-2-192) localized at 11q13.1 and highly expressed in the epithelium of the intestine *(McKenna LB et al., 2010).* In the gastrointestinal tract, its expression has been reported to be down-regulated in colon cancer *(Chiang Y, et al., 2012)* as compared to normal colonic tissue, as well as in the colon of patients affected by active ulcerative colitis *(Wu F et al., 2008).* We detected its significant down-regulation both in adult and pediatric CD patients, data that paralleled those obtained in CD patients subdivided according to the clinical manifestation *(Vaira V et al., 2014)* Bioinformatic analyses identified as possible targets of miR-192-5p, among others, CXCL2 and NOD2, molecules involved in innate immunity. The chemokine CXCL2 is expressed by epithelial cells of the intestine and involved in the pathogenesis of ulcerative colitis (UC) *(Lawrance IC, et al., 2001).* NOD2 is an intracellular sensor of peptides derived from bacterial peptoglycan *(Strober W et al., 2011),* and several studies suggest its involvement in different functions correlated with the immune system (in particular TLRs), (*Fritz JH, 2005),* with the tolerance to antigens *(Hsu LC, 2008*) and with the role of the intestine as a barrier *(Hiemstra IH et al., 2012).* Polymorphisms in the

CARD15 gene (which encodes for the NOD2 protein) have been identified as able to confer an increased risk of developing Crohn's disease *(Baumgart DC et al., 2013),* probably because of an altered NOD2 protein function which generates an excessive response to normal component of gut microflora. A direct interaction between miR-192 and CXCL2 had previously been shown in cultured cells *(Chiang Y, et al., 2012),* and we demonstrated a direct interaction between miR-192-5p and NOD2 by luciferase reporter assay, utilizing a construct that contains the 3'UTR of the NOD2 mRNA, co-transfected with miR-192. Moreover, we verified that the two targets and miR-192 co- localized in the intestinal epithelium, both in controls and CD biopsies.

We then analyzed NOD2 and CXCL2 expression in adult patients and we showed an up-regulation at mRNA and protein levels, in particular in Marsh 3C compared to controls, demonstrating that also in this case, the expression of immune system can be up-regulated by gluten in adult CD subjects. Surprisingly, for both targets, we could not detect any significant variation in pediatric CD patients compared to healthy subjects. This fact could be explained by the action of other miRNAs, since several binding sites for different miRNAs are present in the 3' UTR of mRNAs. In silico analyses revealed NOD2 and CXCl2 as target genes of another miRNA (miR-486-5p) involved in gastrointestinal diseases *(Zhu C et al., 2014)* and in the development of the gut *(Liang G et al., 2014).* Our results showed that miR-486-5p was up-regulated in pediatric CD patients compared to controls, but it didn't show any variations in adult patients. Thus, we could speculate that the expression of miR-486-5p could contrast the action of miR-192-5p on NOD2 and CXCL2 in the pediatric gut, determining their unchanged level of expression, possibly because of the modifications that take place in the immune system during early years of life *(Steege JC et al., 2012; Steegenga WT et al., 2012).* The processes involved in the maturation of the immune system are, in fact, extremely complex and modulated also by the interaction with the external environment. Hornef et al. showed the presence of modifications of the intestinal innate immune system according to the mucosal microbial homeostasis, as well as the differential expression of genes related to immunity in the neonate and adult murine intestine *(Hornef MW et al., 2014).* Moreover, also adaptive immunity seems affected, since differentiation of CD4(+) and CD8(+) T cells resulted age-dependent *(Howie D et al., 1998).*

The significant down-regulation in miR-192-5p expression observed also in pediatric CD patients should, in theory, affect the expression of other targets. In silico analyses identified MAD2L1, whose mRNA had already been validated as a target of miR-192-5p *(Georges SA et al., 2008).* In our samples, MAD2L1 resulted up-regulated in pediatric CD patients but not in adult ones, confirming data previously reported *(Bragde H et al., 2011).* This protein is implicated in the checkpoint mechanism that controls proper chromosome attachment to spindle microtubules during cell division *(Skinner JJ et al., 2008),* and in presence of an abnormal metaphase alignment of the chromosomes, it converts its inactive open form in the active close one and links BubR1 and Cdc20 proteins, inhibiting the progression of the cell cycle *(Han JS et al., 2013).*

Clinical manifestations of celiac disease are notoriously different between adult and children patients *(Guandalini S. et al., 2014),* and we could speculate that this could also be related to the various maturation processes involved in intestinal growth. The major changes that take place during gut development require a more precise control of the mitotic processes, and this might explain the increased MAD2L1 expression observed in pediatric CD patients but not in adult ones, due to the need to control anomalous mechanisms and unregulated processes of cell replication.

All the mechanisms of re-shuffling of intestinal mucosa that take place in the celiac patients, could be intensified in children patients who were physiologically already susceptible to intestinal development processes. This might increase that need of extremely tight regulations, also considering the fact that, during the proliferation and differentiation processes, as well as intestinal epithelial renewal, stem cells are playing an essential role, as recently demonstrated in vivo *(Watson CL et al., 2014)* and ex-vivo *(Leushacke M et al., 2014).*

The performed microarray analysis returned only seven miRNAs, all down-regulated, and this could be due also to the stringent conditions employed for the analysis. To evaluate other possible variations we selected miR-21-5p, that we expected to be up-regulated since it is involved in inflammatory and immune response (*Schetter AJ et al, 2009)*. Nevertheless, our analysis showed no significantly variation of miR-21-5p levels in adult CD patients whereas it resulted significantly increased in the duodenal biopsies of pediatric Marsh 3C patients; thus, we could speculate that not only targets, but also miRNAs could have a trend of expression related to the age of

presentation. miR-21-5p expression paralleled that of its verified target, STAT3 *(Kim YJ. et al., 2012);* This fact does not agrees with a direct effect of miR-21-5p on STAT3 as negative regulator of its expression, but it has been demonstrated that the connection between these two molecules could be complex, since STAT3 is a transcriptional factor that acts on miR-21-5p itself *(Loffler D et al., 2007; Han L et al., 2012).* We hypothesized that in celiac disease, the interaction between miR-21-5p and STAT3 could evolve in a complex regulatory loop *(Kohanbash G et al., 2012):* STAT3 is targeted by miR-21-5p, but its role as transcription factor prevails on the miRNA inhibitory effect, enhancing miR-21-5p transcription. In fact, we also noticed, in CD patients, an increase in STAT3 phosphorylation on serine 727, which is the post-translational modification responsible for STAT3 activity as transcriptional factor *(Yokogami K et al., 2000).* Moreover, STAT3 plays a major role in inflammation processes, and Musso at al. *(Musso A et al., 2005)* described an involvement of STAT3 in inflammatory bowel disease, thus it is reasonable to hypothesize a similar role in celiac disease.

To complete our study we verified that all the changes observed in miRNA and gene expression were triggered by exposure to gluten; to assess this, we evaluated their expression in duodenal biopsies of GFD patients stimulated in vitro for 4 hours with PT-gliadin and compared to biopsies of controls, stimulated and unstimulated. The results showed that miR-192-5p and miR-31-5p were down-regulated and their respective targets (Cxcl2, Nod2 and Foxp3) were up-regulated in GFD stimulated biopsies. These data reflected the same trend of expression observed in biopsies obtained from CD patients at diagnosis, and thus we could speculated that variations observed could directly depend on exposure to gliadin. Nevertheless, we did not detect variations in miR-338-3p and miR-197 expression; this could be due to the short time of exposure to gluten employed in our in vitro experiments, which was necessary to maintain RNA integrity. Therefore further studies will be required, also evaluating the role of the single gliadin peptides in inducing these variations.

Finally, the identification of a panel of circulating miRNAs that could be used as diagnostic / follow-up tools surely could be extremely useful, in particular in pediatric patients, to enforce the panel of serological tools currently used for the diagnosis. For this reason we analyzed, in the plasma of

pediatric patients, the expression of the same miRNAs up- or down-regulated in the duodenal biopsies. These miRNAs had in plasma the same trend of expression observed in duodenal biopsies, except for miR-338-3p and miR-197 that were below the detection threshold of our assay, and may thus need more sensitive methods. However, when we evaluated in the whole cohort the results obtained for the biopsy – plasma pairs, we were not able to detect a significant positive correlation, thus suggesting that the presence of other factors (or sources) contributes to determine the circulating level of each miRNA. It must be noted that, in GFD patients, all analyzed miRNAs, except miR-192-5p, were inclined to reach expression levels similar to controls. Taken together, these data suggest that a wider panel of plasmatic miRNAs should be analyzed in order to provide reliable information on the intestinal mucosa status and, possibly, reduce the need for invasive procedures, even though we are aware about the true criticalities.

5.2. HYPOXIA AND IRON METABOLISM

We then evaluated the role of hypoxia-dependent microRNAs in the regulation of pathways involved in iron metabolism. Briefly, the study showed that the expression of genes involved in iron homeostasis in the liver of mouse models undergoing hypoxia (10% oxygen) differs from what we observed in mice on normoxia, and that a pivotal role in these conditions could be played by miRNAs. Iron is strongly associated with oxygen sensing in cells and, as a consequence, hypoxia triggers molecular processes which act as compensatory mechanisms to increase erythropoiesis and maintain iron homeostasis *(Chepelev NL et al., 2011).* Hypothesizing that miRNAs could play a main role in the control of these processes, we performed a microarray to detect which miRNAs were differently expressed in the liver of mice undergoing hypoxia, and selected those that could have as targets genes involved in iron homeostasis. One of the most important pathways that regulates iron metabolism is the BMP/SMAD pathway. Microarray cluster analysis identified, as up-regulated, miR-22-3p which has a binding sites on Bmp6 3'UTR, the ligand and activator of this pathway, interaction that had already been validated by in vitro studies *(Long J et al., 2013).* In our samples miR-22-3p expression was significantly inversely correlated with that of Bmp6, which showed a reduction of about 50% already at 12 hours of hypoxia, reduction that was further

increased with longer hypoxia exposure; this fact suggests that the down-regulation of Bmp6 in hypoxic conditions could be caused by the up-regulation of miR-22-3p. However, the inverse correlation between miR-22-3p and Bmp6 in our samples *per se* could not exclude the possibility of a transcriptional down-regulation. To further validate the presence of the miRNA regulation, we employed an in silico approach which did not identify any binding site for HRE in Bmp6 promoter, as well as the analysis of Bmp6 nuclear mRNA obtained from mice in normoxia and after 48 hours of hypoxia exposure. The unchanged levels of Bmp6 nuclear mRNA allowed us to say that its down-regulation was not caused by transcriptional processes, but it could be attributed to post-transcriptional mechanisms that act on cytoplasmic mRNA. miR-22-3p expression could, however, be directly hypoxia-dependent or depend on variation in iron levels caused by mobilization of iron deposits due to hypoxia. To sort this issue, we firstly analyzed LIC in our samples, that was unchanged after hypoxia exposure. We also evaluated miR-22-3p expression in the liver of mice treated with an iron poor/rich diet, and interestingly miR-22-3p resulted up-regulated both in iron-rich and iron-deficient diet, differently from Bmp6 that increased significantly only in iron overload. These data, that need further investigation, indicate that in presence of variation in liver iron content the mechanisms involved in the up-regulation of Bmp6 do not rely on miRNA regulation but rather on a direct activation of its promoter.

It is intesting to note that previous data about hypoxia have demonstrated the down-regulation of miR-22-3p in in vitro experiments using colon cancer cells *(Yamakuchi M et al., 2011).* The different data observed in our setting could be attributable not only to the different experimental conditions but also to the different analyzed cells. In fact, analyzing miR-22-3p promoter, we detected a binding site for HNF4α (Hepatocyte Nuclear Factor 4 Alpha), a transcription factor which is highly expressed in hepatic tissue and positively influenced by hypoxia *(Hamdan FH et al., 2014).* This hypothesis will require further validation, and electrophoresis mobility assays are currently ongoing to assess HNF4α binding to miR-22-3p promoter. Considering all these data we could thus speculate the role of miR-22-3p as regulators of Bmp6 expression in hypoxic conditions. Other important actors of this pathway are the Bmp6 receptors *(Miyazono K et a., 2010);* BMP6 interact on the cell surface with these heterodimeric receptors formed by Bmpr1 and Bmpr2 that

need a co-receptor called hemojuvelin (HJV or Hfe2). Due to the effect of hypoxia on BMP6, we wanted to assess whether other hypoxia-induced miRNAs were targeting the BMP receptor complex. Analyzing them in our mice, we detected a significant Bmpr1a mRNA down-regulation starting from 24 hours of exposure, whereas Bmpr2 mRNA expression did not show any variation in mice undergoing hypoxia compared to normoxia. In addition, Hfe2 mRNA resulted significantly decreased only up to 24 hours of exposure. Analyses in silico revealed that some miRNAs, identified as up-regulated in the microarray, had possible binding sites on these molecules. In particular, miR-101a-3p, which had a binding site on BmpR1a, showed in our samples an increased expression, and inversely correlated with its target. These data, which suggest its role in hypoxia-dependent regulation, will however require in vitro confirmation, either by transfection studies with reporter constructs or with miRNA mimic/antimiRNA oligos. On the contrary, miR-192-5p, acting on Bmpr2, showed a statistically significant up-regulation at 24 and 48 hours of exposure, but that wasn't a correlation with its target, suggesting that in hypoxic conditions miR-192-5p is probably directed on other genes. The data obtained up to now will also need to be validated at the protein level; it must be remembered, however, that signal transduction through these receptors involves the phosphorylation of their intracytoplasmic portion, and the only available antibody recognizes only one phosphorylation on BmpR2.

Among the miRNAs identified as differentially expressed in the microarray we were not able to detect, by in silico analysis, any of them as interacting with hemojuvelin. However, previous data in the literature prompted us to assess the expression of miR-122-5p, which is one of the most expressed miRNA in the liver tissue *(Hsu SD et al., 2015),* and has been demonstrated having a binding sites on Hfe2 3'UTR *(Castoldi M et al., 2011)*. Interestingly, we observed a significant up-regulation in mice exposed to hypoxia only at 12 and 24 hours of exposure, and a significant hemojuvelin down regulation. If only these time-points were included in the analysis, miR-122-5p inversely correlated with Hfe2 mRNA expression. These data thus suggest that a different mechanism intervenes after 24 hours of exposure to level out their expression, but further investigations are necessary.

Considering these data we then evaluated the influences of hypoxia on the other actors of BMP/SMAD pathway.

We focused on the variations taking place into the nucleus, in particular evaluating SMAD1/5/8 and its effects on the transcription of the downstream genes. The phosphorylation of this complex resulted decreased at all the time points of hypoxia considered, confirming data previously reported in in vitro experiments *(Chaston TB et al., 2011).* One of the downstream target of this process is Hepcidin (Hamp), an hepatic hormone which, at systemic level, controls iron storage into the cells through the regulation of Ferroportin (Fpn1), the only known iron exporter *(Hentze MW et al., 2010).* In our mice liver samples, Hamp mRNA resulted significantly reduced by 50% immediately after 12 hours of exposure to hypoxia, and this down-regulation was maintained for the other time points; moreover our analyses showed an increase in Fpn1 expression only at protein level, confirming data reported in the literature indicating that its regulation is not at transcriptional level *(Frazer DM et al., 2014).* The data here reported further demonstrate that, under hypoxic conditions, iron needs to be released from the cells, event that takes place through the reduction of Hamp and the consequent increase in iron exporter expression *(Ramey G. et al., 2010).* Taken together these data suggest that all the entire pathway of Bmp/Smad is hypoxia-dependent, fact important to clarify the mechanism to decrease Hamp expression in hypoxic conditions. This observation, however, cannot exclude that other regulatory pathways could intervene. For example, one of the candidates is Atoh8, which regulates Hamp expression in two different ways: firstly binding directly to its promoter and secondly acting on the BMP/SMAD pathway, in particular on Smad phosphorylation *(Patel N et al., 2014).* Among the miRNAs identified by microarray, miR-351-3p showed a sequence complementary to that of Atoh8 3'UTR. However, when we analyzed the liver samples of hypoxic mice, we did not detect any variation in Atoh8 mRNA expression. On the other hand, Atoh8 protein resulted significantly down-regulated after hypoxia exposure, showing a trend inversely correlated with miR-351-5p, data consistent with Atoh8 translational regulation. Taken together these data suggested that in hypoxic conditions miR-22-3p, miR-101a, miR-122-5p and miR-351-5p had a pivotal role, in the hypoxia-dependent regulation of genes involve in iron metabolism, and in particular in BMP/SMAD pathway that

entailed the down-regulation of Hepcidin, the main regulator of iron homeostasis in hypoxic conditions.

The down-regulation of HAMP is thus a crucial point in organism hypoxia response, due to the need to mobilize iron stores, as well as to increase iron absorption in order to fulfill the request of the increased erythropoiesis. However the molecular mechanisms involved could be different according to the time of hypoxia exposure and may occur consequently. This idea is supported also by studies performed at high altitude, where mobilization of iron stores (assessed by serum ferritin level) was observed prior to the erythopoietic drive *(Piperno A et al., 2011).* It is thus possible that the hypoxia-driven mechanisms here described represent the first line of response, and longer time points as well as the analysis of the erythroid regulators will be necessary to provide a full outline of the involved mechanisms.

5.3. CONCLUSION

The data reported in the thesis, although obtained in two different conditions, in humans and in a mouse model, again support the essential role of miRNAs as expression regulators. However the obtained results confirm also the idea that miRNAs work as a network, either concurring at the same results (as hepcidin downregulation in hypoxia) or selecting the most appropriate targets according to the moment's need (CXCL2 and NOD2 vs MAD2L1). Lastly, as also observed by other researchers, miRNA regulation seems to work varying miRNAs concentrations according to the cell condition, not as an on/off mechanism but rather providing a fine tuning of gene expression.

VI.

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