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RESEARCH PAPER

miRNA-regulated gene expression differs in celiac disease patients according to the age of presentation

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Abstract Celiac disease is an intestinal disease which shows different symptoms and clinical manifestations among pediatric and adult patients. These variations could be imputable to age-related changes in gut architecture and intestinal immune system, which could be characterized by gene expression differences possibly regulated by miRNAs. We analyzed a panel of miRNAs and their target genes in duodenal biopsies of Marsh 3AB and 3C pediatric celiac patients, compared to controls. Moreover, to assess variation of expression in plasma samples, we evaluated circulating miRNA levels in controls and patients at diagnosis or on gluten-free diet. We detected a decreased miR-192-5p expression in celiac patients, but no variations in NOD2 and CXCL2, targets previously identified in adults. Conversely, we detected a significant increase in mRNA and protein levels of another target, MAD2L1, protein related to cell cycle control. miR-31-5p and miR-338-3p were down-regulated and their respective targets, FOXP3 and RUNX1, involved in Treg function, resulted up-regulated in celiac patients. Finally, we detected, in celiac patients, an increased expression of miR-21-5p, possibly caused by a regulatory loop with its putative target STAT3, which showed an increased activation in Marsh 3C patients. The analysis of plasma revealed a trend similar to that observed in biopsies, but in presence of gluten-free diet we could not detect circulating miRNAs values comparable to controls. miRNAs and their gene targets showed an altered expression in duodenal mucosa and plasma of celiac disease pediatric patients, and these alterations could be different from adult ones.

Keywords Celiac disease · Pediatrics · MicroRNA · Immunity

Introduction

Celiac disease (CD) is an inflammatory disease of the small intestine affecting at least 1 % of world population, with an increase in incidence, in particular considering children (Kenrick and Day 2014). The clinical symptoms of CD are variable and differ according to the age of presentation (Diamanti et al. 2014). Adults patients can have intestinal and extra-intestinal clinical manifestations, whereas children often present CD with "classic" symptoms including abdominal pain, malabsorption and growth retardation (Telega et al. 2008). CD is triggered by the ingestion of gluten peptides in genetically predisposed individuals; this fact starts a cascade of events involving immune response, which results in intestinal damage and increase in intestinal permeability (Schuppan et al. 2009).

The major genetic risk factor identified up to now is the presence of a specific HLA heterodimer, namely DQ2.5 [DQA1*0501–DQB1*0201] (either in *cis* or *trans* position), or DQ8 [DQA1*03-DQB1*0302/0305] haplotypes; the presence of the various combination of different

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haplotypes allows also to categorize subjects with a moderate (single DQ2.5 or DQ8) or a high risk of celiac disease (homozygosity for DQA1*0501-DQB1*0201 or DQA1*0501-DQB1*0201 and DQA1*0201-DQB1*02) (Romanos et al. 2014). Genome-wide association studies have identified several additional loci (mostly including genes related to the immune response), but in total only 50 % of the genetic predisposition can be accounted for (Dubois et al. 2010; Gutierrez-Achury et al. 2015). The presence of a genetic risk is, however, not sufficient for the development of the disease, since interaction with gluten is essential. The difference in the time of development had been hypothesized to depend upon the age of gluten introduction in the diet, but recent multicentric studies in high-risk children have demonstrated that the delayed introduction of gluten in the diet can have only a slight effect, since at 5 years of age the prevalence of autoantibody positivity or of CD was similar in the early versus late gluten introduction group (Lionetti et al. 2014; Vriezinga et al. 2014).

The development of CD either in children or adults could thus be associated with a different immune response to gluten (rather than the age of introduction), possibly due to the maturation of the intestinal immune system or its abnormal regulation. Thus processes regulating immunity, at transcriptional and post-transcriptional level, could affect the clinical manifestation and the severity of the disease. A pivotal role could be played by microRNAs (miRNAs), a class of small non-coding RNAs, which bind the complementary sequences in the 3′UTR of target genes causing their degradation or translational inhibition (Runtsch et al. 2014). We previously focused our attention on the role of miRNAs in the regulation of gene expression in CD, reporting a microarray analysis performed on adult CD patients with different phenotypes (Vaira et al. 2014) and we also described the different expression of some miRNAs and their target genes, involved in the immune response, according to the severity of the intestinal lesion (Magni et al. 2014). However, we obtained these data in adult patients, and the same miRNAs identified by us as down-regulated in adult CD had not been previously detected as altered in biopsies obtained from CD children (Sapone et al. 2011). There are very few data on the morphological and histological differences in small intestinal architecture at different ages (Ren et al. 2014), and on the development of the immune response in intestine (Mabbott et al. 2015), and no data on miRNA profile that could be different according to age.

Moreover, although diagnostic criteria for pediatric CD have been recently revised (Husby et al. 2012; Giersiepen et al. 2012), additional markers could be useful in CD diagnosis in children with various clinical manifestations or in follow-up. A panel of miRNAs differently expressed in the serum of CD patients could thus be used as molecular biomarkers. To assess whether the miRNAs and targets identified in adults as differentially expressed, presented the same pattern in children, we evaluated a cohort of pediatric CD patients and analyzed miRNA levels in the serum at diagnosis and in patients on gluten-free diet (GFD) to verify whether they could also represent a good indicator of compliance and mucosal recovery.

Materials and methods

Patients' cohorts

Duodenal biopsies were obtained from a total of 28 children, who underwent upper gastrointestinal endoscopy for diagnostic purposes, after having obtained the informed consent from their parents. Analyses were performed on 8 controls (subjects with normal endoscopic and histological duodenal findings, 4 girls and 4 boys, mean age 11 ± 3.8 years) and 20 patients with untreated CD (11 girls and 9 boys, mean age 8 ± 3.6 years), divided into Marsh 3AB (either with Marsh 3A or Marsh 3B) and Marsh 3C group (8 and 12 patients, respectively) (Oberhuber 2000) in whom diagnosis was based on positive anti tTG and/or anti-endomysium antibody testing and duodenal histology. Duodenal biopsies were frozen for RNA isolation and protein extraction and maintained at −80 °C until use. For experiments on plasma, we enrolled a total of 36 subjects: 12 controls (6 girls and 6 boys, mean age 10 ± 3.6 years) and 17 CD patients (7 Marsh 3AB and 10 Marsh 3C, 9 girls and 8 boys, mean age 7 ± 3.4 years) at the first diagnosis (13 of them were the same subjects undergoing biopsy) and additional 7 CD patients (4 girls and 3 boys, mean age 9 ± 4.3 years) on GFD for at least 1 year. Blood samples were immediately centrifuged at 3000 g to obtain plasma fractions, which were aliquoted and stored at −80 °C until use. No statistically significant difference in demographic parameters was observed between the groups (Table 1S).

As assessed by the diagnostic guidelines of ESPGHAN, HLA typing is not required for CD diagnosis. However, HLA typing was performed in 20 CD patients; 19 subjects resulted DQ2 positive and one DQ8 (15 first diagnosis patients and 5 on GFD).

For miR-486-5p and MAD2L1, the data obtained in the children cohorts were compared to those observed in the previously analyzed adult cohorts (Magni et al. 2014).

The study was approved by the pertinent ethics committee of San Gerardo Hospital—Monza (MB) and it is conformed to the standards set by the Declaration of Helsinki.

RNA extraction

Total RNA was extracted from biopsies using a MiRcury RNA Isolation Kit (Exiqon, Vedbaek, Denmark) following the manufacturer's instructions. Total RNA extraction from plasma was performed using miRNeasy Serum/Plasma Kit (Qiagen, Valencia, CA) and following the manufacturer's instructions. Quality and quantification of extracted RNA was performed by NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

RT-qPCR

qPCR was performed using 7900HT Fast Real-Time PCR System (Applied Biosystems-Life Technologies, Carlsbad, CA, USA). For miRNAs analyses, RNA was reverse transcribed using TaqMan MicroRNA Reverse Transcription kit (Applied Biosystem), and the commercial available kit TaqMan MicroRNA Assays (Applied Biosystem) were used for quantitative PCR. Identification of miRNAs' possible targets was performed using the following softwares and databases: 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/), considering their miR SVR score, focusing in particular on those with an in vitro validation.

For gene expression, instead, TaqMan Reverse Transcription Reagents kit (Applied Biosystems) was used with random primers to obtain cDNAs, and Sybr green PCR Master Mix (Applied Biosystems) was employed for the detection of target mRNAs with the exception of FOXP3 and MAD2L1 (assessed by TaqMan Gene Expression Assay, Applied Biosystem). Primers were designed to be 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′)

For all analyses, each sample was examined in triplicate. All data were normalized to the Let-7b for miRNA analysis and to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) for genes. The relative quantification with $2^{(-\Delta\Delta Ct)}$ method was employed to calculate relative changes in gene expression using an external reference sample (RNA obtained from duodenal biopsies of a control subject, not included in the analysis).

Western blot analysis

Protein extraction was performed with RIPA buffer containing protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA), after having disrupted manually duodenal tissues. Proteins were separated on NuPAGE Novel Bis–Tris (4–12 %) gels (Invitrogen-Life Technologies, Carslbad, CA, USA). They were transferred on nitrocellulose membrane (Amersham, Buckinghamshire, UK). Membranes were blocked with TTBS and 5 % non-fat milk and hybridizations performed at 4 °C overnight with the following antibodies: mouse anti-human NOD2 (Anti-CARD15 antibody [2D9]—ab31488, Abcam, 1:1000) (Abcam, Cambridge, UK), goat anti-human CXCL2 (Anti-GRO beta antibody—ab91511, Abcam, 1:2000), mouse anti-human FOXP3 (Anti-FOXP3 antibody—ab22510, Abcam, 1:500), mouse anti-human MAD2L1 (WH0004085M1, Sigma-Aldrich, 1:1000 + 2 % milk), rabbit anti-human phospho-STAT3 (ser727) (#9134, Cell Signalling, 1:1000) (Cell Signalling, Danvers, MA, USA), and rabbit Anti-human actin (A2066, Sigma, 1:1500). SuperSignal West Pico Chemiluminescent 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).

Statistical analysis

ANOVA and Student's *t* test was performed for the comparison of the data. ANOVA on ranks followed by Dunn post hoc test was used when data failed the equal variance test. The significance level was set at p value <0.05. Statistical evaluation was performed with the SYSTAT software package (SPSS, Chicago, IL).

Results

We initially analyzed some miRNAs previously detected as differentially expressed in the duodenum of CD adult patients compared to controls (Magni et al. 2014).

miR-192-5p expression was significantly reduced in biopsies obtained from 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) (Fig. 1). We also studied the expression of chemokine (C-X-C motif) ligand 2 (CXCL2) and nucleotide-binding oligomerization domain containing 2 (NOD2), validated targets of miR-192-5p in adults. Differently from what expected, the expression of these molecules did not inversely correlate with miR-192-5p levels: CXCL2 mRNA expression was significantly reduced in biopsies obtained from 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), whereas protein expression in controls and marsh 3C, did not show any variations (0.43 ± 0.11 vs

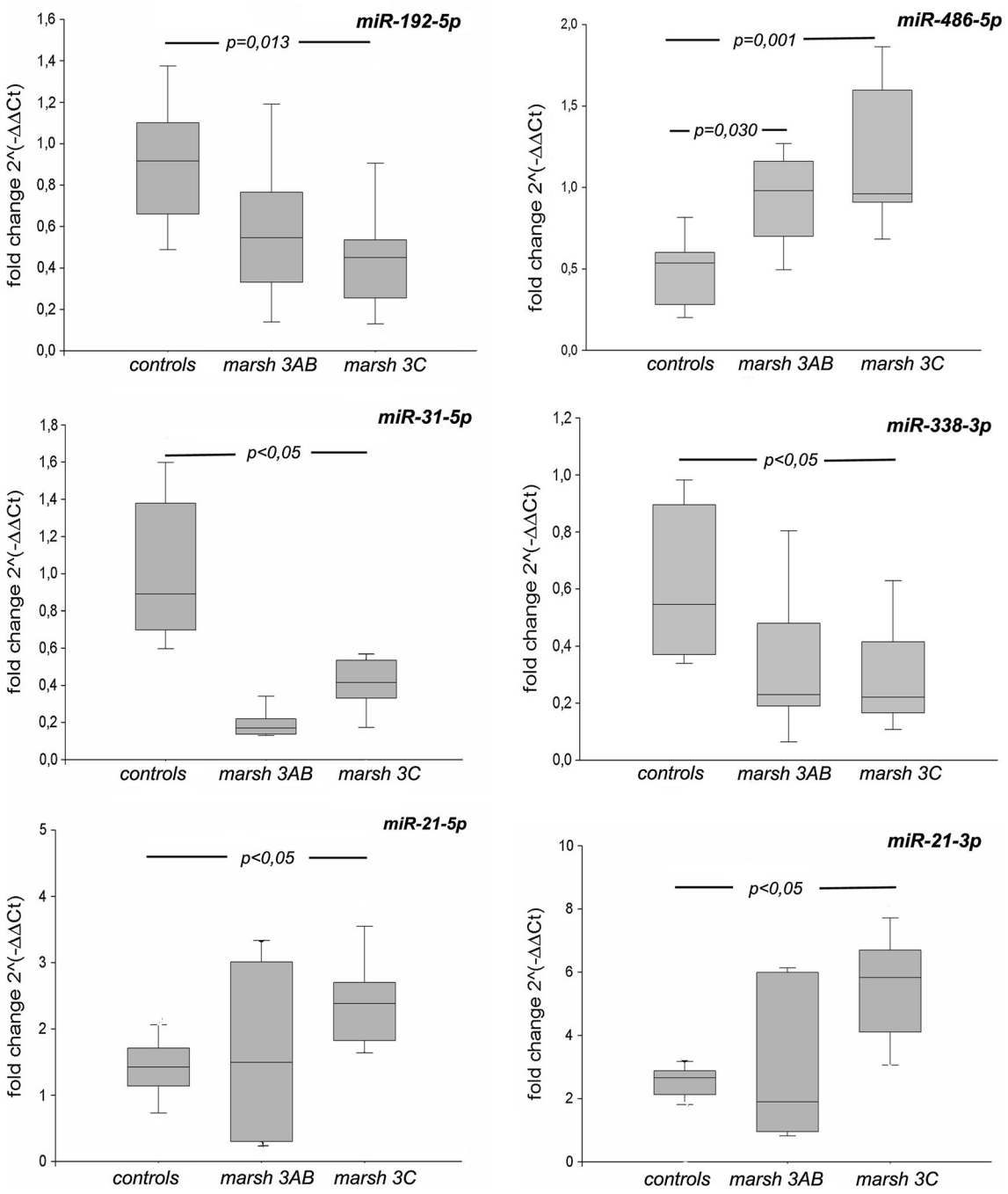

Fig. 1 miRNAs expression. Expression level of miR-192-5p, miR-31-5p, miR-338-3p, miR-21-5p, miR-21-3p and miR-486-5p in duodenal biopsies of controls ($n = 8$), Marsh 3AB ($n = 8$) and Marsh 3C ($n = 12$) celiac pediatric patients. Data obtained by quantitative PCR are represented as fold change compared to an external sample. The *box plots* show median value and the 25 and 75 percentiles, whiskers the maximum and minimum value. Statistical analysis was performed by one-way ANOVA

0.52 ± 0.30, controls and Marsh 3C, respectively, p = ns by t test). Moreover, NOD2 mRNA levels in children 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) as well as NOD2 protein expression (0.44 ± 0.20 vs 0.60 ± 0.59 in controls and Marsh 3C, respectively).

Several miRNAs can bind the same mRNA and the final effect is due to the balance among them. To assess whether other miRNAs could bind NOD2 and CXCL2, we performed in silico analyses that identified miR-486-5p seeding sequence within their 3′ UTR. This miRNA was significantly up-regulated in pediatric CD 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) (Fig. 1) but not in adult ones (1.13 ± 0.82 and 1.05 ± 0.78 in controls and Marsh 3C patients, respectively, data not shown), thus supporting the presence of a different miRNA-regulatory pattern.

In silico analysis identified 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) (Fig. 2, panel a). MAD2L1 mRNA expression was up-regulated in children with CD, with a threefold 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. 2, panel b). 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; data not shown). MAD2L1 protein expression paralleled mRNA expression: its level was up-regulated in CD 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. 2, panel c, d).

In our previous study, we found that other miRNAs (miR-31-5p and miR-338-3p) were down-regulated in the duodenum of adults CD patients. miR-31-5p was significantly down-regulated in CD (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) (Fig. 1). Furthermore, increased mRNA expression of its target forkhead box P3 (FOXP3) was detected in Marsh 3C, with 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. 3, panel a). FOXP3 protein expression was similar to mRNA expression, being up-regulated in marsh 3C patients compared to controls (1.88 ± 0.49 and 3.94 ± 0.89, controls and Marsh 3C respectively, $p = 0.012$ by ANOVA) (Fig. 3, panel b, c).

We then analyzed miR-338-3p expression; also in this case, 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) (Fig. 1). We also evaluated its putative target, runt-related transcription factor 1 (RUNX1); similarly to FOXP3, it was significantly up-regulated in Marsh 3C patients compared to controls, with a fourfold increase (0.51 ± 0.42 in controls and 2.01 ± 1.42 in Marsh 3C, $p < 0.05$) (Fig. 3 panel d).

Other miRNAs can be involved in the regulation of the immune response in celiac duodenum; another possible candidate is miR-21-5p, which has been previously reported as involved in inflammatory and immune response, in particular being able to stimulate cytokine expression (Schetter et al. 2009; Zhang et al. 2015).

We detected a significant up-regulation of miR-21-5p expression in Marsh 3C 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). Since the processing of miRNAs leads to the generation of two mature forms, we also wanted to assess whether miR-21-3p had the same trend and we observed an up-regulation in Marsh 3C patients, with a twofold 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) (Fig. 1). In silico analysis of possible targets of miR-21-5p revealed a binding site for this miRNA on 3′UTR of programmed cell death 4 (PDCD4) (Shen et al. 2014); nevertheless, we did not detect any correlation, since PDCD4 mRNA showed an increase in CD patients compared to controls, although not significant (1.48 ± 0.69, 2.10 ± 1.26 and 2.84 ± 2.26 in controls, Marsh 3AB and Marsh 3C, respectively) (data not shown).

Among other possible targets of miR-21-5p, we analyzed signal transducer and activator of transcription 3 (STAT3) (Kim et al. 2012). In this case, we observed a significant increase in STAT3 mRNA 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. 4, panel a). However, the relationship between miR-21 and STAT3 could be complex, since STAT3 can cause the transcriptional activation of miR-21 (Kohanbash and Okada 2012). We thus evaluated STAT3 phosphorylation, detecting an increase in serine 727 phosphorylation in CD patients, although not significant (0.86 ± 0.36 vs 1.42 ± 1.08 in controls and Marsh 3C, respectively) (Fig. 4 panel b, c).

We then assessed the circulating levels of the miRNAs described above in the plasma of CD patients at diagnosis or on gluten-free diet (GFD) (Fig. 5). miR-192-5p

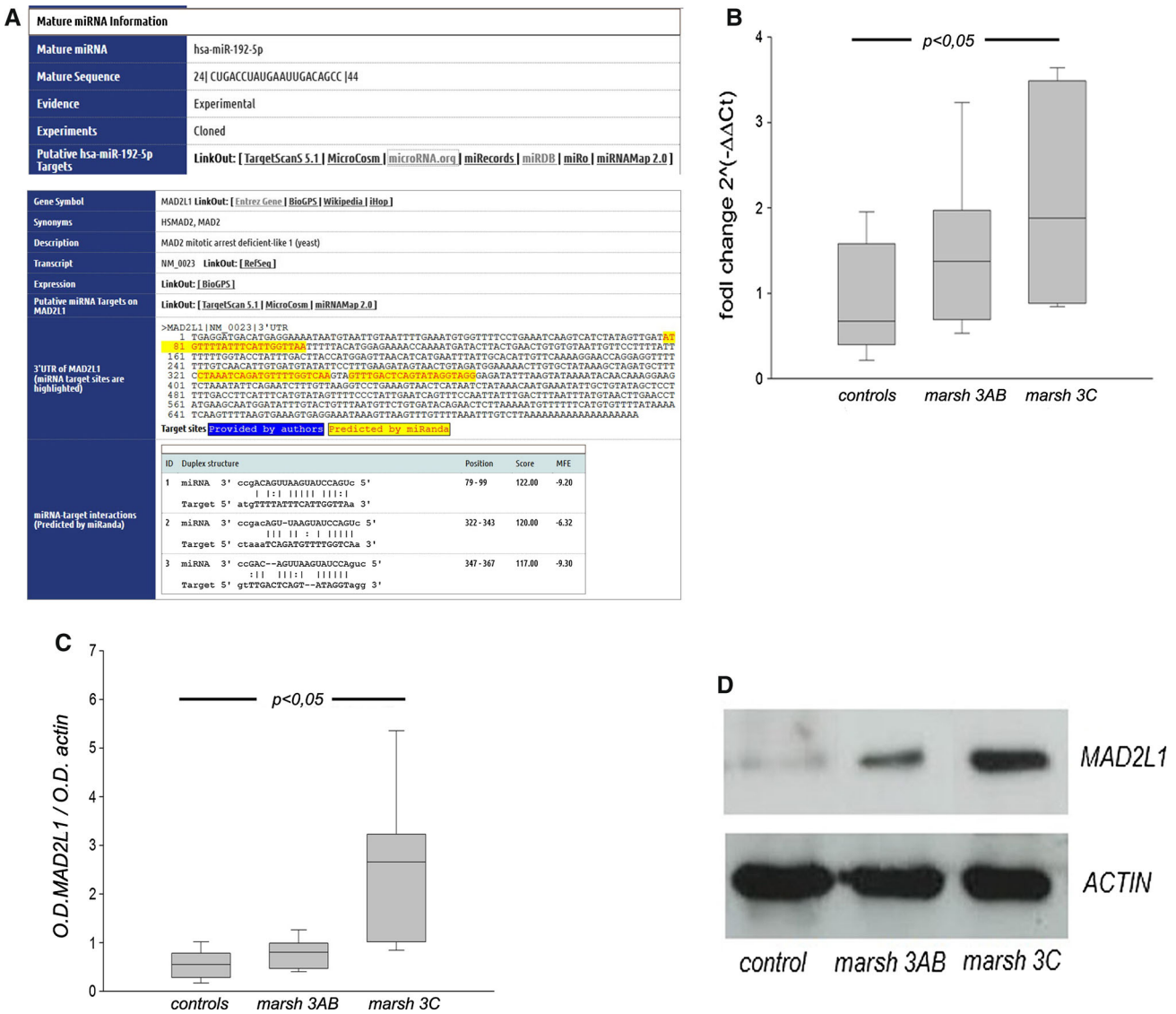

Fig. 2 MAD2L1 mRNA and protein expression. **a** Alignment between miR-192-5p and predicted target MAD2L1 using miRTarBase software. **b** Expression level of MAD2L1 mRNA in duodenal biopsies of controls ($n = 8$), Marsh 3AB ($n = 8$) and Marsh 3C ($n = 12$) celiac pediatric patients. Data obtained by quantitative PCR are represented as fold chance compared to an external sample. The *box plots* show median value and the 25 and 75 percentiles, *whiskers* the maximum and minimum value. Statistical analysis was performed by ANOVA. **c** Densitometric analysis of MAD2L1 protein in duodenal biopsies of celiac patients and controls normalized on actin expression ($n = 7$ controls, n = 8 Marsh 3AB and $n = 8$ Marsh 3C) and **d** western blot image of MAD2L1 and actin levels in controls and CD patients

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 reach a level comparable to controls (0.24 ± 0.14, $p = 0.001$) (Fig. 5, panel a). miR-486-5p showed an up-regulation trend, as detected in biopsies, but it was not significant. Furthermore, the analysis of GFD plasma revealed an expression level still higher than controls (2.04 ± 1.16, 3.79 ± 3.29, 3.66 ± 2.66 and 4.18 ± 1.57 in controls, Marsh 3AB, Marsh 3C and GFD, respectively) (Fig. 5, panel b). 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). Moreover, the difference between controls and GFD levels (0.59 ± 0.35) resulted not statistically significant (Fig. 5, panel c). 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 the difference in expression levels of GFD compared to

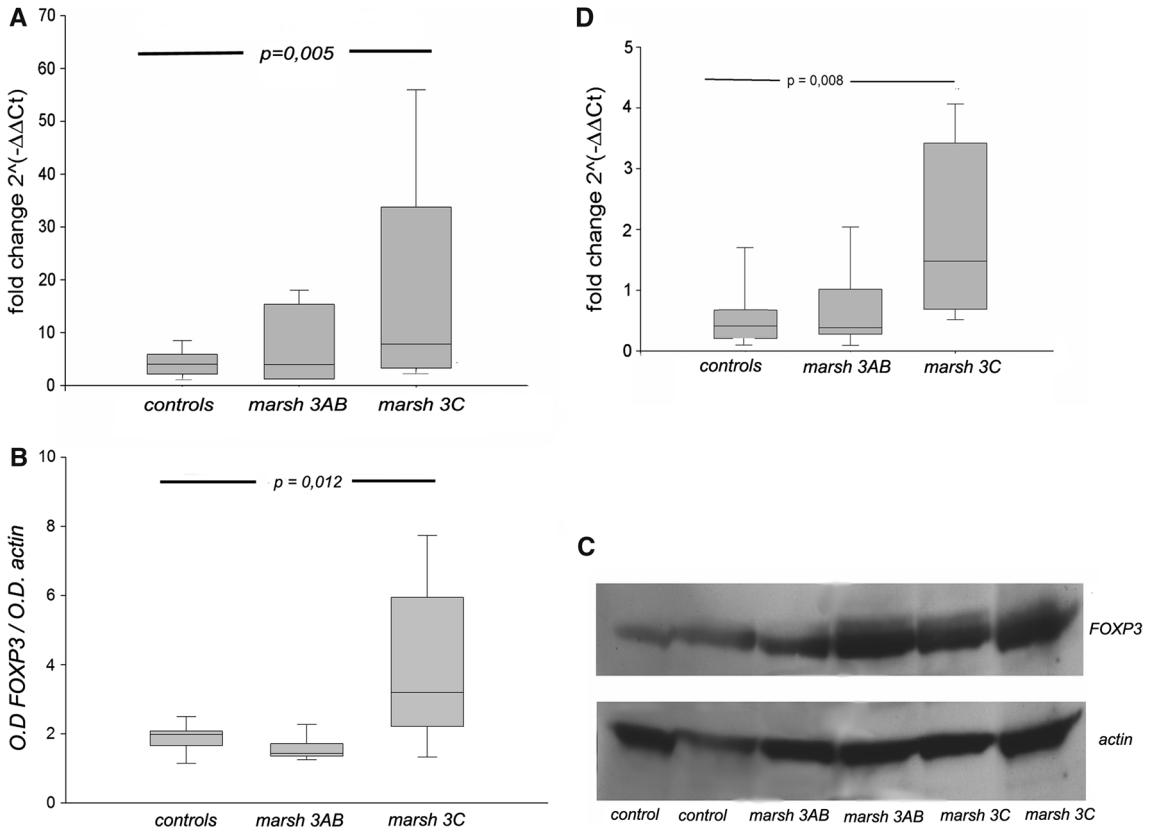

Fig. 3 FOXP3 mRNA and protein, and RUNX1 mRNA expression. **a** Expression level of FOXP3 mRNA in duodenal biopsies of controls ($n = 8$), Marsh 3AB ($n = 8$) and Marsh 3C ($n = 12$) celiac pediatric patients. Data obtained by quantitative PCR are represented as fold change compared to an external sample. The *box plots* show median value and the 25 and 75 percentiles, *whiskers* the maximum and minimum value. Statistical analysis was performed by ANOVA. **b** western blot image of FOXP3 and actin levels in controls and CD patients and **c** densitometric analysis of FOXP3 protein in duodenal biopsies of celiac patients and controls normalized on actin expression ($n = 7$ controls, $n = 7$ Marsh 3AB and $n = 8$ Marsh 3C). **d** Expression level of RUNX1 mRNA in duodenal biopsies of controls ($n = 8$), Marsh 3AB ($n = 8$) and Marsh 3C ($n = 12$) celiac pediatric patients. Data obtained by quantitative PCR are represented as fold chance compared to an external sample and statistical analysis performed by ANOVA

controls, resulted not statistically significant (Fig. 5, panel d). Finally, 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), but the results were not statistically significant; however, GFD group showed expression levels similar to controls (0.52 ± 0.12) (Fig. 5, panel e). Results obtained in plasma in GFD patients are also summarized in Table 2S).

Discussion

The present study shows that the pattern of expression of some miRNAs and related gene targets in the duodenal mucosa of pediatric patients with CD differs from that observed in controls or adults celiac patients.

In a previous study (Magni et al. 2014), we detected, in adult CD patients, down-regulated miRNAs associated with the up-regulation of molecules involved in innate or adaptive immunity.

We demonstrate that also in pediatric CD patients, miR-192-5p was down-regulated with a major decrease in Marsh 3C patients; however, when we analyzed the hypothetical targets of this miRNA, NOD2 and CXCL2, we could not detect an inverse correlation either with mRNA or protein expression levels.

NOD2 is a member of pattern recognition receptors, which plays an important role in immune response at the mucosal level, recognizing peptidoglycan of the bacterial membrane and activating the NF-kB pathway (Carneiro et al. 2008). CXCL2 is a chemokine produced by epithelial intestinal cells (Ohtsuka and Sanderson 2003) and secreted by monocytes and macrophages after activation of Toll-Like Receptors (TLRs) (Areschoug and Gordon 2008). Both proteins could have a role in the pathogenesis of CD, since they are strictly correlated with the immune response and/or tolerance to antigens. Despite these evidences, we did not observe any variation in their protein expression in pediatric CD patients compared to controls. The unchanged levels of both proteins could be explained by the action of

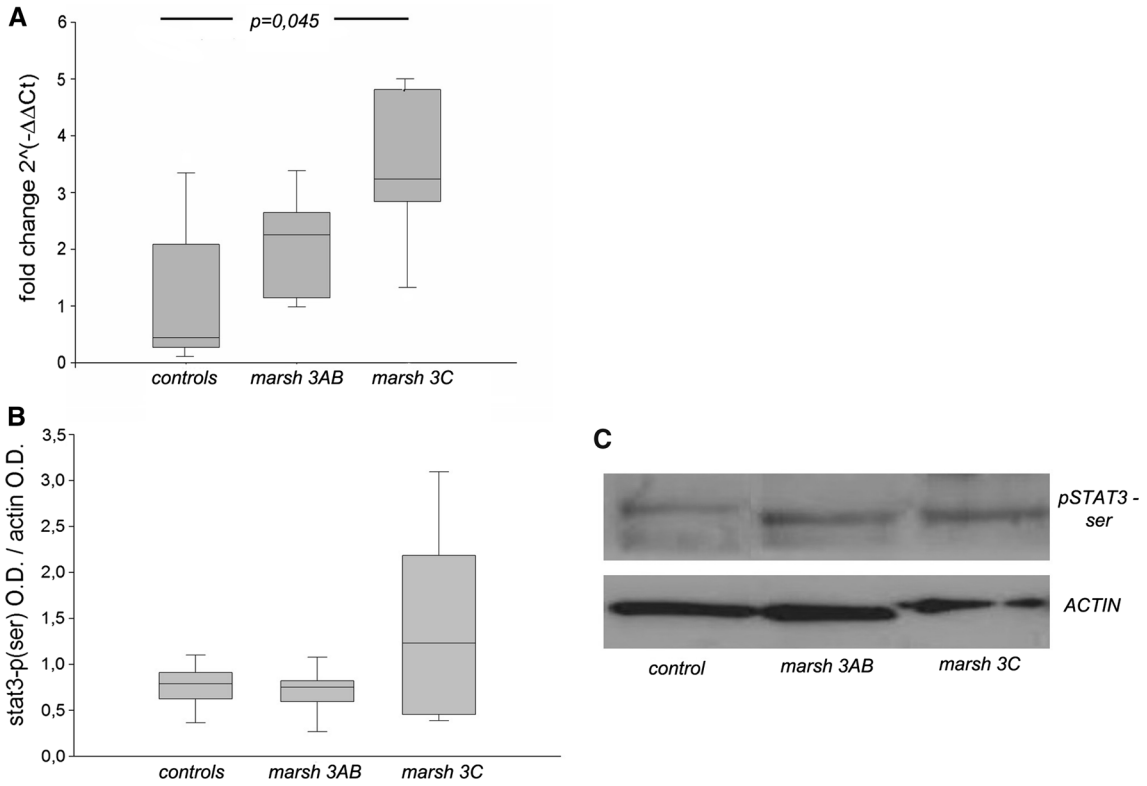

Fig. 4 STAT3 mRNA and phosphorylated protein expression. **a** Expression level of STAT3 mRNA in duodenal biopsies of controls ($n = 8$), Marsh 3AB ($n = 8$) and Marsh 3C ($n = 12$) celiac pediatric patients. Data obtained by quantitative PCR are represented as fold change compared to an external sample. The *box plots* show median value and the 25 and 75 percentiles, whiskers the maximum and minimum value. Statistical analysis was performed by ANOVA. **b** Western blot image of STAT3 phosphorylated on serine 727 and actin levels in controls and CD patients and **c** relative densitometric analysis ($n = 7$ controls, $n = 7$ Marsh 3AB and $n = 8$ Marsh 3C)

other miRNAs, since several binding sites for various miRNAs are present in the 3′UTR of these two mRNAs. In silico analyses revealed NOD2 and CXCL2 as target genes of miR-486-5p, involved in gastrointestinal diseases (Zhu et al. 2014) and in the development of the gut (Liang et al. 2014). In our cohort it was up-regulated in pediatric CD patients; we thus speculate that the expression of miR-486-5p could contrast the action of miR-192-5p on NOD2 and CXCL2 in the pediatric gut, in CD patients. Interestingly, no variation in miR-486-5p expression was observed in adult CD patients, further supporting the hypothesis of a different miRNA regulation in children.

The development of the intestinal immune system starts in early natal life (Steege et al. 1997), and the postnatal period is a critical window for maturation, since the microbiota composition diverges from the maternal one (Donovan et al. 2014). Hornef and Fulde (2014), showed the differential expression of immunity genes in the murine neonate and adult intestine, and age-related transformations have also been reported for adaptive immunity (Howie et al. 1998; He et al. 2013). All these findings support the idea that the gut (and its immune system) shows gene expression changes clearly related to age (Lee et al. 2001; Steegenga et al. 2012), modifications that could be controlled by specific miRNA patterns. In celiac disease a variation in proteins of the innate immune system could be part of the complex relationship among innate immunity, microbiota and food tolerance. NOD2 can mediate the degree of inflammation in the intestinal mucosa caused by an alteration of the microbiota, as demonstrated in NOD2 ko mice (Ramanan et al. 2014). However, the alteration of the microbiota itself could depend on the absence of NOD2 protein (Rehman et al. 2011) and, in turn, variation in the microbiota could affect the processing of peptides (including those of gliadin). In fact the presence of a dysbiosis has been reported in CD (Collado et al. 2009) and has also been related to the presence of symptoms in patients on GFD (Sánchez et al. 2011).

Conversely, we noticed variations in another verified target of miR-192-5p, MAD2L1 (Georges et al. 2008), implicated in the checkpoint that monitors proper chromosome attachment to spindle microtubules during cell division (Skinner et al. 2008). In presence of an abnormal metaphase alignment, it converts its inactive open form in the active close one and links Bub1-related kinase (BubR1) and cell division cycle 20 (Cdc20) proteins, inhibiting cell

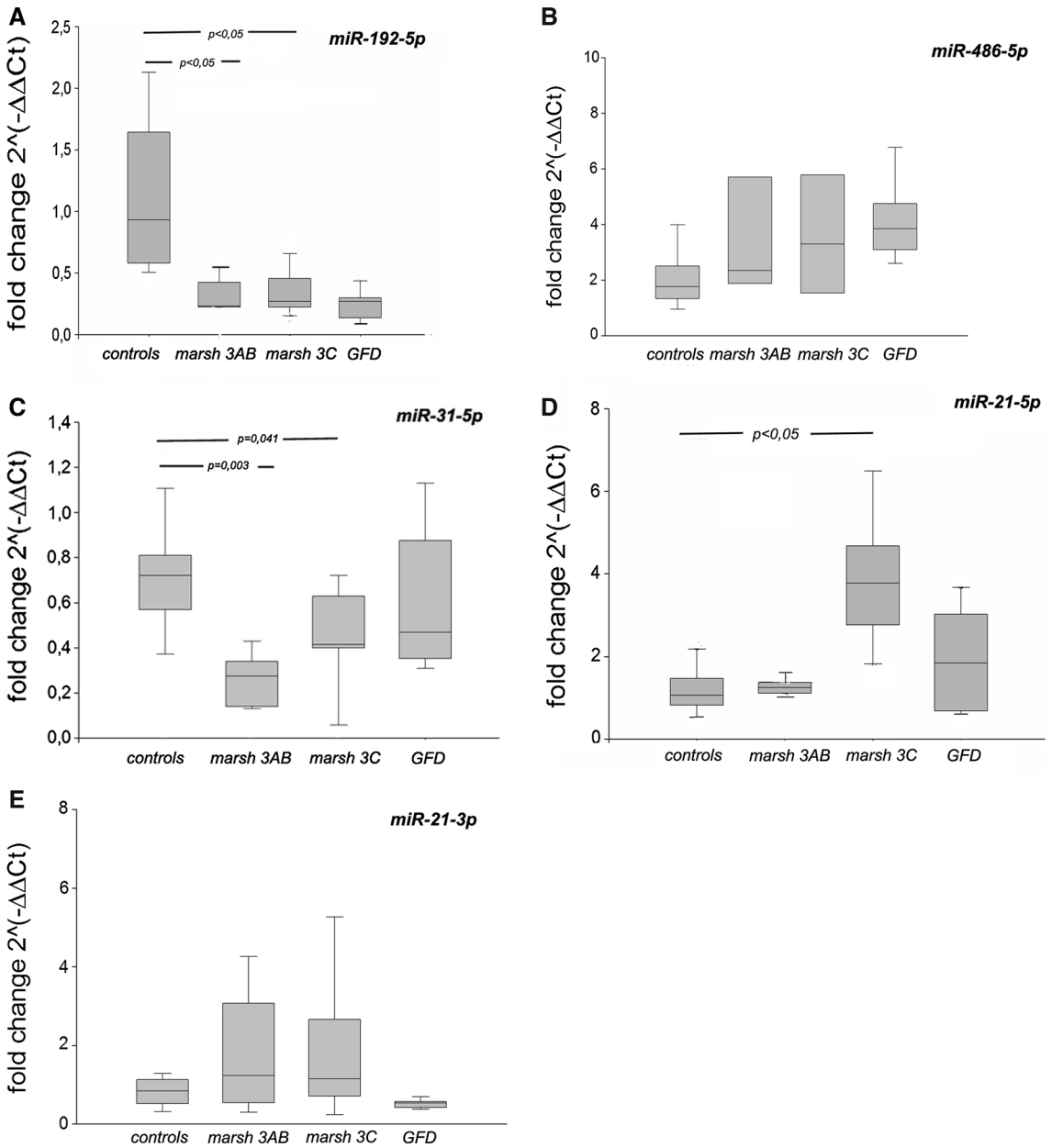

Fig. 5 miRNAs expression in plasma samples. Expression level of miR-192-5p, miR-31-5p, miR-21-5p, miR-21-3p and miR-486-5p in plasma samples of controls ($n = 12$), Marsh 3AB ($n = 7$), Marsh 3C ($n = 10$) and GFD ($n = 7$) celiac pediatric patients. Data obtained by quantitative PCR are represented as fold change compared to an external sample. The *box plots* show median value and the 25 and 75 percentiles, *whiskers* the maximum and minimum value. Statistical analysis was performed by one-way ANOVA

cycle progression (Han et al. 2013). In ulcerative colitis, MAD2L1 protein expression was increased as compared to controls (Burum-Auensen et al. 2007), whereas its mRNA overexpression was detected investigating gene expression profile in mucosa of CD pediatric patients (Bragde et al. 2011). Since this protein causes cell cycle arrest (Wassmann and Benezra 2001), its increased expression could be interpreted as an attempt to control anomalous processes of cell replication that may be present in the intestine of pediatric CD patients.

Also miR-21-5p could have a role in the regulation of innate immunity and/or inflammation; in fact, in silico analysis of possible targets of miR-21-5p, revealed a binding site on the 3′UTR of STAT3 (Kim et al. 2012). In our previous study, miR-21-5p did not show any variations in its expression in duodenal biopsies of adult CD patients compared to controls (Magni et al. 2014), whereas in children we detected a significant increase in miR-21-5p in the duodenal biopsies of Marsh 3C CD patients, which was paralleled by an increase in STAT3 mRNA expression, fact

that does not agree with a direct effect of miR-21-5p on STAT3. However, it has been demonstrated that the connection between them could be complex, since STAT3 is responsible for the transcriptional activation of miR-21-5p (Kohanbash and Okada 2012; Löffler et al. 2007; Han et al. 2012). STAT3 plays a major role in inflammation, and Musso et al. (2005), described an involvement of STAT3 in inflammatory bowel disease and celiac disease, in areas of active inflammation and infiltrating macrophages. STAT3 is targeted by miR-21-5p, but its role as a transcription factor could prevail on the miRNA inhibitory effect, thus enhancing miR-21-5p transcription. In fact, in CD patients, STAT3 phosphorylation on serine 727, which is responsible for STAT3 activity as a transcriptional factor, was increased (Yokogami et al. 2000). Since STAT3 is a gene regulated by interleukin-6 (IL-6) and interleukin-15 (IL-15) pathways (Sarra et al. 2013), we speculate that in pediatric patients with CD, the activation of inflammation/innate immunity induces STAT3 activity.

As regards the other miRNAs, we detected a significant down-regulation of miR-31-5p and an up-regulation of its target, FOXP3, transcription factor essential for Treg development (Rouas et al. 2009), which represents a pivotal event in the regulation of the immune response also in CD (Brazowski et al. 2010). Similarly, we observed a down-regulation of miR-338-3p in pediatric Marsh 3C patients which inversely correlated with its target RUNX1, an activator factor of FOXP3 transcription (Ono et al. 2007). Both these genes have a role in the regulation of intestinal adaptive immunity, and the results are similar to those observed in adult CD patients (Magni et al. 2014), suggesting that their activation could depend on local stimuli rather than on pure age-related changes.

The identification of a panel of miRNAs that could be used as a diagnostic/follow-up tool surely could be extremely useful, in particular in pediatric patients. For this reason we analyzed miRNAs expression in plasma. Results showed the same trend of duodenal biopsies; miR-192-5p and miR-31-5p decreased in CD patients compared to controls, consistently with the severity of mucosal damage; similarly, miR-21-5p and miR-21-3p increased their expression even though miR-21-3p did not reach statistical significance. We were unable to detect miR-338-3p in plasma, probably due to very low expression of this miRNA in the samples. However, when we evaluated in the whole cohort the biopsy—plasma pairs, we did not detect a significant positive correlation, thus suggesting the presence of other factors (or sources) determining the circulating level of each miRNA.

In GFD patients, miR-192-5p and 486-5p expression in plasma did not return to levels comparable to controls, in fact they remained significantly down-regulated. For miR-31-5p, 21-5p and 21-3p in GFD, indeed, expression levels tended to return to controls levels and the difference in expression between the two groups resulted not statistically significant (Table 2S). GFD patients were comparable to untreated ones in terms of HLA typing and had undergone GFD for at least 12 months; although we could not obtain a second biopsy to verify the level of inflammation, all biochemistry tests were negative, and interviews confirmed a good adhesion to the diet. However, negative antibodies titer may not be a perfect predictor of mucosal healing in the first years after diagnosis (Vécsei et al. 2014). Thus, the lack of normalization of miRNA levels will need further investigation, both evaluating biopsy expression levels (if available) as well as possible polymorphisms in CD patients able to affect their expression levels.

Taken together, these data suggest that a wider panel of plasmatic miRNAs should be analyzed to provide reliable information on the intestinal mucosa status and, possibly, reduce the need for invasive procedures.

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Compliance with ethical standards

Conflict of interest Gaia Buoli Comani, Roberto Panceri, Marco Dinelli, Andrea Biondi, Clara Mancuso, Raffaella Meneveri and Donatella Barisani declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants, or their parents if children, included in the study.

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