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Extracellular vesicle microRNAs in celiac disease patients under a gluten-free diet, and in lactose intolerant individuals

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A R T I C L E I N F O

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A B S T R A C T

Background: Celiac disease (CD) is an autoimmune disorder triggered by an abnormal immunological response to gluten ingestion and is associated with deregulated expression of cellular microRNAs (miRNAs) of the gut mucosa. It is frequently misdiagnosed as lactose intolerance (LI) due to symptom resemblance. Microvilli loss may be counteracted by a rigorous gluten-free diet (GFD). Aims: To identify altered extracellular vesicle miRNAs from plasma among CD patients on GFD (n=34), lactose intolerant individuals on restrictive diet (n=14) and controls (n=23), and to predict biological pathways in which these altered miRNAs may play a part. Methods: Five different small RNA samples of each group were pooled twice and then screened by new-generation sequencing. Four miRNAs were selected to be quantified by RT-qPCR in the entire sample. Results: The levels of four miRNAs – miR-99b-3p, miR-197-3p, miR-223-3p, and miR-374b-5p – differed between CD patients and controls ($P<0.05$). Apart from miR-223-3p, all these miRNAs tended to have altered levels also between LI and controls ($P<0.10$). The results for miR-99b-3p and miR-197-3p between CD and controls were confirmed by RT-qPCR, which also indicated different levels of miR-99b-3p and miR-374b-5p between CD-associated LI and LI ($P<0.05$). Conclusions: These miRNAs may have targets that affect cell death, cell communication, adhesion, and inflammation modulation pathways. Hence, altered miRNA levels could be associated with CD-related aspects and gut mucosa recovery.

Introduction

Celiac disease (CD) affects around 1% of the world's population and is caused by the presentation of gluten peptides by major histocompatibility complex molecules to the effector immunological response, injuring intestinal mucosa in genetically predisposed individuals [2,27]. The proliferation and overstimulation of autoreactive lymphocytes lead to diarrhea, abdominal pain, and decreased absorption of nutrients, e.g., calcium and iron, due to the loss of intestinal microvilli. This mucosa damage is the main factor for impaired lactase production, leading to shared symptoms with individuals presenting lactose intolerance (LI, also used to refer to individuals as lactose intolerant). As a result, CD is often misdiagnosed and considerable time elapses until correct diagnosis and treatment initiation. After the correct treatment with a rigorous gluten-free diet (GFD), LI- and CD-associated symptoms usually improve. However, if CD is not controlled, the function of other organs may be affected, resulting in symptoms characteristic of other diseases, involving salivary glands (in CD-associated Sjögren syndrome),

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pancreas (insulin-dependent diabetes mellitus comorbidity), irritating dermatological blisters [known as dermatitis herpetiformis (DH)], severe anemia, migraine, thyroid impairment (TI), bone mass loss and cancer (thyroid cancer, small intestine cancer, and lymphoma) [7,15,25,45,50].

In fact, alterations in the integrity of the intestinal mucosa associated with changes in the intestinal microbiota may also correlate with CD and/or LI. It is still unclear if pathological conditions primarily lead to intestinal mucosa damage, which in turn leads to changes in the microbiome, or if it is the other way around (changes in the microbiome ultimately lead to intestinal damage culminating in the establishment of various pathological conditions). For instance, the occurrence of dysbiosis – defined as an impairment of the gut microbiota – seems to play a role in several clinical conditions, including LI [13]. Furthermore, growing evidence seems to support the hypothesis that changes in both the composition and function of the intestinal microbiome are associated with a number of chronic inflammatory diseases including CD [48]. On the other hand, dietary gluten-induced gut dysbiosis has already been reported [36].

Nevertheless, it is well established that the human microbiome affects the host's epigenome, as microbiota-derived metabolites (MDM), microbiota-derived components (MDC) and microbiota-secreted proteins (MSP) seem to regulate the host's physiology by modifying its gene expression, including changes in miRNA expression [11,51]. Thus, a better understanding of the pathophysiology of CD and LI, the establishment of miRNA expression profiles, as wells as the development of differential diagnosis and non-invasive biomarkers are still required for both, CD and LI. For all of these purposes, extracellular vesicles (EVs) have proven to be promising. They are released into the peripheral circulation by normal and altered cells and can be collected to investigate and monitor pathological conditions [9,43,49]. EV-associated miRNAs (EV-miRNAs) may be potential disease biomarkers, but can also reveal about and contribute to a better understanding of pathological processes through the analysis of potential miRNA targets and related pathways [9,23].

To the best of our knowledge, the expression profiles of EV-miRNAs have never been studied in biofluids from CD patients, neither among individuals with LI. In fact, the research on circulating miRNAs – e.g., EV-miRNAs as well as cell-free miRNAs – in CD and/or LI is still in its beginning. Even though some studies have reported deregulated expression profiles of several miRNAs in CD patients' intestinal mucosa before and after a GFD [5,6,34,47], only two studies have determined the profile of miRNAs in serum of CD patients, both at diagnosis and after a strict diet [17,44]. Deregulated expression profiles in biofluids from individuals with LI have never been reported in the literature.

Therefore, we investigated whether the miRNA content of EVs is associated with CD or LI, specifically in individuals under restrictive diets. This may be instrumental for the development of minimally invasive approaches to verify the adherence of patients to restrictive diets, especially to a GFD. Nonetheless, the knowledge on EV-miRNAs in CD and LI may be useful to better elucidate the pathological processes of both conditions and to help define common pathological mechanisms affecting the gut mucosal homeostasis and integrity.

Materials and methods

Study population

The present study was approved by The Ethics Committee of the Health Sciences Sector of the Federal University of Paraná (protocol 2.204.113, CAAE 54385616.2.0000.0102). All participants signed a term of informed consent and donated a blood sample. Participants diagnosed with CD and/or LI were recruited during the meetings of the Celiac Disease Patient Association of Paraná (ACELPAR), and samples of controls without restrictive diets were obtained from the panel of the Laboratory of Human Molecular Genetics (LGMH).

Seventy-one individuals (34 CD patients, 14 LI individuals, and 23 controls without restrictive diets) were included. Information on clinical and demographic data as age, gender, ancestry, body mass index (BMI), CD and/or LI diagnosis were collected, as well as on associated comorbidities as TI and DH (Table 1). Most participants were women (54 women and 17 men), of European ancestry, ranging from 12 to 73 years old. No significant differences in age, ancestry, BMI, and sex were found among the evaluated groups (Table 1).

CD diagnosis was based, at least, on a positive result of transglutaminase or anti-endomysium serology, or on duodenum biopsy with marks of inflammation (Marsh II-IV). Data on HLA-DQ haplotype was also available for some patients (presented only as an additional information since it cannot be considered for establishing CD diagnosis). Among CD patients, most (81.5%) of those tested were diagnosed based on positive anti-endomysium serology results, while only 12/34 (35.3%) had jejunum or duodenum biopsy results. All 11 CD patients genotyped for *HLA-DQ* (32.4%) were carriers of the CD-associated *HLA-DQ* haplotype. Five (18.5%) CD patients initiated the GFD before the diagnosis and had negative serology results, therefore requiring confirmation based on gut biopsy or *HLA-DQ* haplotype genotyping. CD patients that informed to have secondary LI had their diagnosis based on altered blood glucose levels after lactose ingestion (lactose tolerance blood test) and were undergoing a lactose-free diet. All individuals presenting only LI took the lactose tolerance blood test, had negative anti-endomysium serology values, and rigorously followed a lactose-free diet, except for three individuals who were on a low lactose diet.

Among CD patients, 20 (58.8%) had LI, 12 (35.3%) had TI, and five (14.7%) had DH diagnosis. Three (21.4%) LI individuals concomitantly presented TI diagnosis. As most CD patients were at the same time LI, we also analyzed the CD and LI groups together. Even though all participants with food-related issues were on restrictive diets for at least three months and, in some cases, up to twenty-two years, they informed having gastrointestinal symptoms or headaches and the need for treatment for comorbidities even after minor contact with gluten. The time elapsed from diet adherence to anti-endomysium negative serology was not precisely known by all participants at the time of enrollment. Despite our intention to do so, additional biopsies and/or blood sampling in the follow-up period were not possible due to COVID-19 restrictions. Consequently, information such as microvilli recovery on GFD was not assessed.

Sample processing

Blood samples were collected into EDTA-coated tubes by

Table 1
Clinical characteristics of patients with celiac disease (CD), individuals with lactose intolerance (LI) and control subjects without food restrictions.

	CD (N=34)	LI (N=14)	Control (N=23)	P-value
Gender (Male / Female)	6 / 28	4 / 10	7 / 16	0.49
Mean age ± SD (year)	41.6 ± 12.8	45 ± 11.2	42.3 ± 12.5	0.80
European /other ancestry	26 / 8	9 / 5	14 / 9	0.42
Mean BMI ± SD	25.7 ± 2.9	25.8 ± 5.2	25.4 ± 5.6	0.84
CD diagnosis				
Endomysium serology + / -	22 / 5	0 / 14	0 / 23	<0.01
Jejunum/duodenum biopsy (Marsh >2) + / -	12 / 0	-	-	-
HLA-DQ2 or HLA-DQ8 + / -	11 / 0	-	4 /11	<0.01
LI diagnosis + / -	20 / 14	14 / 0	0 / 23	<0.01
Comorbidities				
Thyroid impairment + / -	12 / 22	3 /11	1 /22	0.02
Dermatitis herpetiformis + / -	5 / 29	0 / 14	0 / 23	<0.01

N – number of participants, CD – celiac disease, LI – lactose intolerance, SD – standard deviation, BMI – body mass index, + – positive value or presence, and - – negative value or absence. In bold – *P*-values lower than 0.05.

venipuncture, and the plasma was obtained through centrifugation (2,000 x *g* for 15 minutes at room temperature). The obtained plasma was further centrifuged (10,000 x *g* for 20 minutes at room temperature) to remove potential debris, resulting in a clarified plasma.

EV isolation

Proteinase K (20 mg/mL, 0.05 volumes, 10 minutes at 37°C) (Invitrogen, Carlsbad, CA, USA) was mixed with the clarified plasma (1,000 µL of plasma for samples used in RNA sequencing or 300 µL for samples used in RT-qPCR) and incubated with Exosome Isolation Reagent from plasma (Invitrogen, Carlsbad, CA, USA) for an hour at 4°C, according to the manufacturer's instructions. EVs were pelleted after centrifugation (10,000 × *g* for 10 minutes at room temperature), resuspended in PBS buffer 1x, and stored at -20°C or in RIPA cell lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.2% sodium deoxycholate, 0.5% Triton X-100, pH 7.4 plus complete protease inhibitors cocktail from Roche) for protein purification.

EV characterization

EV characterization was performed based on the guidelines of the International Society of Extracellular Vesicles (ISEV) [46]. At first, mean size and quantification of EVs were obtained by light scattering properties through Nanoparticle Tracking Analysis (NTA), performed with the NanoSight LM10 (Malvern, Worcestershire, UK). EV samples were diluted 1:1,000 and evaluated on an equipment with controlled temperature and slow infusion for five recordings of 30 seconds. One sample of EV-depleted plasma was also evaluated by NTA to confirm EV isolation.

Further on, EV size and morphology were evaluated through transmission electron microscopy (TEM). Formvar film-coated grids 300 Mesh (Electron Microscopy Sciences, Hatfield, PA, USA) were immersed in 30 µL of a diluted (1:1,000) sample of EVs for one hour. Grids were rinsed three times with filtered PBS 1x for five minutes and fixed with Karnovsky buffer for 10 minutes. After new rinses with PBS 1x, the EV grid content was contrasted with 5% uranyl acetate for 10 minutes and rinsed again with PBS 1x. Samples were air-dried, covered with carbon and then visualized by JEOL JEM-1400 Plus electron microscope (JEOL Ltd., Tokyo, Japan) (Microscopy Facility, Carlos Chagas Institute, Fiocruz-PR) operating at an acceleration voltage of 80 kV.

Additionally, the presence or absence of classical EV markers (CD9, CD63, and TSG101) was investigated by western blotting (WB), using anti-CD9 (Biolegend, San Diego, EUA), anti-CD63 (Invitrogen, Carlsbad, CA, EUA), and anti-TSG101 (Thermo Fisher Scientific, Waltham, MA, USA) primary antibodies (all at a 1:4,000 dilution). SDS-PAGE with two samples – one of EV proteins and another with proteins purified from an EV-depleted plasma sample – was performed. Proteins were transferred to nitrocellulose membrane and stained with Ponceau 0.2% to verify the immobilization efficiency. Membranes were blocked with 5% non-fat milk in Tris-buffered saline-Tween (TBST) buffer for one hour and then incubated for 16 hours at 4°C with the aforementioned primary antibodies. After three washing steps with TBST, membranes were incubated with secondary anti-mouse IgG coupled to HRP (Sigma Aldrich, Darmstadt, Germany) (at 1:3,000) at room temperature for an hour, followed by an additional washing step. The chemiluminescent reaction was developed with the West Pico Kit (Thermo Fisher Scientific, Waltham, MA, USA) and detection carried out by autoradiography chemistry (Carestream Health, Rochester, NY, USA).

RNA isolation and miRNA enrichment

EV RNA was extracted using the mirVana^{TM} miRNA Isolation kit (Ambion, Austin, TX, USA), following the manufacturer's instructions for small RNA enrichment. We added 0.025 pmol of spike-in cel-miR-39-3p (Qiagen, Hilden, Germany) during the first steps of RNA isolation for technical control and qPCR data normalization. The yield and quality were evaluated by absorbance with the NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

Screening phase – profiling of small RNA content of EVs

EV small RNA profiling was performed by massive parallel cDNA sequencing (RNA-Seq) in pooled samples. Six pools with small RNAs of five samples of each group (CD, LI, and controls) were used to construct a cDNA library with Ion Total RNA-Seq Kit v2 for small RNA libraries (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA libraries were verified with the Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) for quality check, to quantify yield and to detect post-PCR amplification artifacts, and then sequenced in the Ion Proton System (Thermo Fisher Scientific, Waltham, MA, USA). The data were mapped and aligned to the human genome through the miRMaster online tool [16], based on the miRBase v.21 miRNA database [26]. Sequence data (reads) were exported and analyzed with DESeq2 R package on R Studio 3.3.2 [32] in order to determine differential levels of miRNAs among groups after data normalization to the total read count. The corresponding results were plotted with gplots package on R Studio based on unsupervised analyses and Venny online tool [39].

Prediction of biological pathways

The miRpath tool from Diana-Tools was used to reveal biological pathways in which the miRNAs could take part, according to Diana TarBase v8 and KEGG and GO pathways [42]. This strategy was used as a complementary tool to select the best candidate miRNAs among those differentially quantified in EVs, as identified by small RNA-Seq, to be validated by RT-qPCR (see below). Importantly, all differentially quantified EV-miRNAs were also listed in the EVmiRNA database [30].

Here, the intent was not to perform a 'robust' bioinformatics approach, but rather to perform the prediction of biological pathways in order to strengthen the selection of potential candidate miRNAs to be further validated.

Validation phase – quantification of selected EV-miRNAs

Four miRNAs whose quantities differed between the pooled samples (based on fold-change and statistical power values) were selected to be quantified individually in the entire sample set. RT-qPCR was used to quantify four miRNAs as targets and two as references for data normalization (hsa-miR-150-5p and cel-miR-39-3p) from RNA samples of 71 participants. Although miR-150-5p has been described as deregulated in numerous diseases [3,20,24,35], in our miRNA profiling there were no differences among the three groups regarding the levels of miR-150-5p, which was listed among the five-top miRNAs with massive quantities. After quantification by RT-qPCR, we evaluated the quantification cycle (Cq) values for cel-miR-39-3p and miR-150-5p as reference miRNAs and found a standard deviation of 1.35 and 1.36, respectively, and a variation coefficient equal to 6.96 and 4.58, respectively. When analyzed together, these miRNAs presented the best standard deviation of 1.17 and a reasonable variation coefficient of 4.76. Then, we decided to combine the threshold cycle values of miR-150-5p and cel-miR-39-3p as reference miRNAs. Next, cDNA was produced with pre-established TaqMan MicroRNA Assays and TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA), according to the manufacturer's instructions. Reactions were carried out in triplicates for each miRNA in 384-well plates in a QuantStudio Flex 7 machine. Cq values were calculated using the QuantStudio Software v7 (Applied Biosystems, Waltham, MA, USA), and miRNA levels were established by the $2^{-\Delta\Delta Cq}$ comparative method [31]. Results were presented as log2 fold change ($\log2_{FC}$) after being calculated as follows:

$$\Delta Cq = \text{mean } Cq_{\text{target miRNA}} - \left[\left(\text{mean } Cq_{\text{cel-miR-39-3p}} + \text{mean } Cq_{\text{miR-150-5p}}\right)/2\right]$$
$$\Delta\Delta Cq = \Delta Cq_{\text{target group}} - \text{median } \Delta Cq_{\text{reference group}}$$

Statistical analysis

Clinical and demographic data of the three groups were compared using the Chi-square test. Differences concerning EV mean, mode and concentration were assessed by the non-parametric Mann-Whitney test. EV-miRNA levels were compared through the Mann-Whitney test as well. All analyses were performed in R, and graphs were plotted in R or GraphPad Prism 6. *P*-values lower than 0.05 were considered significant. In addition, predicted KEGG and GO pathways associated with differential miRNA levels were predicted using Diana TarBase v8. Statistical significance was determined by the Fisher's exact test, with a *P*-value threshold of 0.05.

Results

EV characterization

EVs were isolated and characterized by NTA, TEM, and WB. Isolated EVs presented mean size of approximately 100 nm (Fig. 1A), exhibiting a cup shape (Fig. 1C) and the presence of CD9, CD63, and TSG101 markers (Fig. 1D), as expected. No differences were found concerning to mean, mode, and concentration between plasmatic EVs isolated from patients and controls (Table S1). In addition, one sample of EV-depleted plasma was also evaluated by NTA and WB, showing particles with mean size around four-fold larger and with around half the quantity, compared to EV samples (Fig. 1B); WB profiles of CD9 and CD63 markers also corroborated such results (Fig. 1D). Together, these results revealed that the purified plasma EVs are enriched in exosomes. Nevertheless, we adopted the EV general term for the isolated fraction, as recommended by the ISEV.

Screening phase – EV miRNA profiling

For 30 individuals, small RNA of EVs from 1mL of plasma was extracted, purified, and concentrated in vacuum to be used to build six cDNA libraries. After aligning and mapping of RNA-Seq data, we obtained 129,416 to 10,751,143 raw reads per sample. The mean of reads mapping to the human genome was of 8.94% (4.25%-18.45%). A mean of 7.09% (2.8-17.6%) of reads corresponded to miRNA sequences after comparison with miRbase v.21. The sequence lengths varied from 17 to 219 nucleotides, with a high quantity (> 700,000) of sequences of 22 nucleotides. LncRNAs (0.77-5.75%), tRNAs (0.22-0.97%), rRNAs (0.03-0.6%), piRNAs (0.3-1.17%), snoRNAs (0.01-0.05%), snRNAs (0-0.01%), and bacterial and viral RNAs were detected as well.

Four hundred and eighty-one miRNAs were detected in the pool of

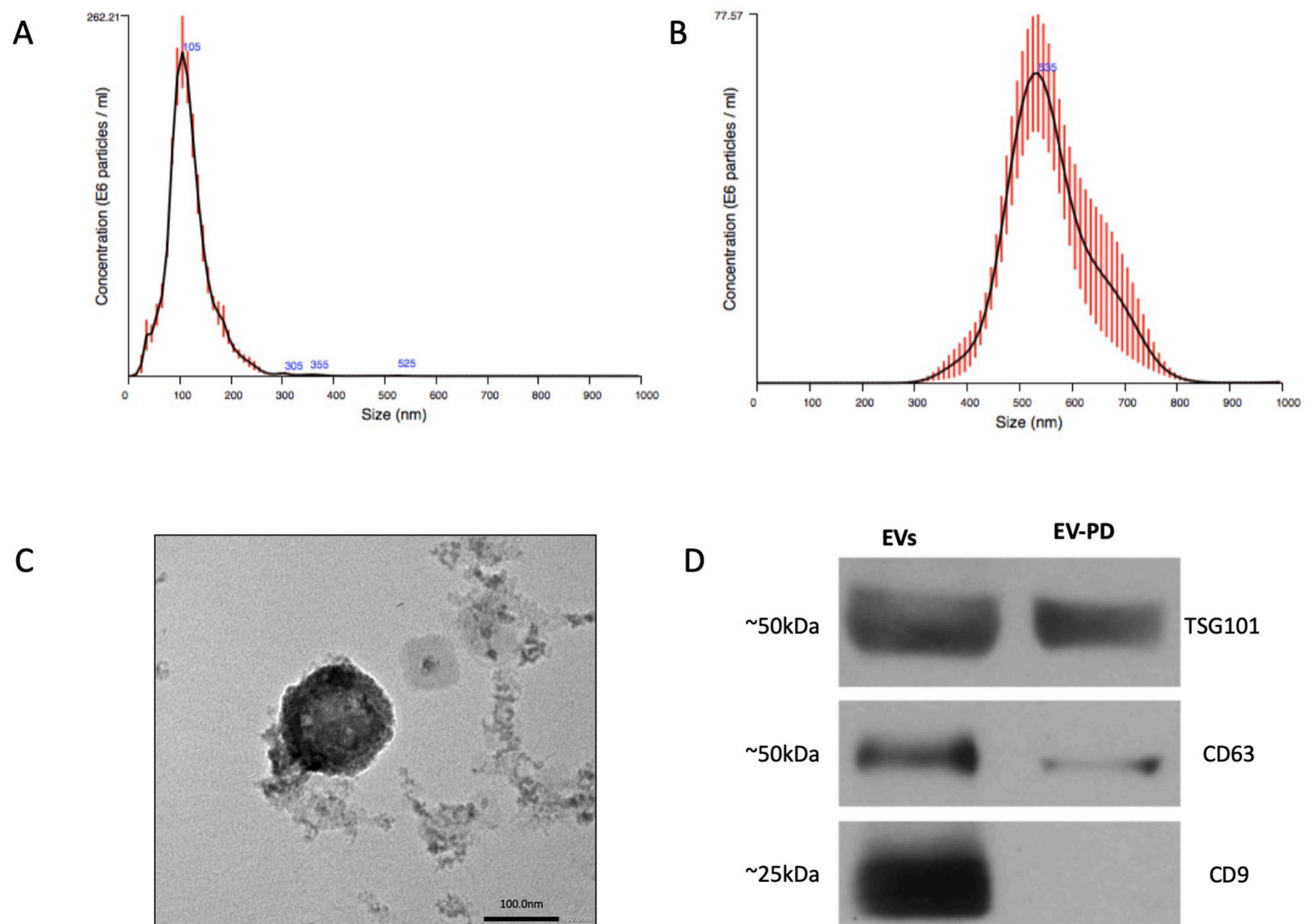

Fig. 1. Purified plasma extracellular vesicles are exosome enriched. A) Diagram of particle distribution based on finite track length adjustment (FTLA) size, showing EVs with approximately 100 nm, compatible with the exosome size, and (B) particles from the EV-depleted plasma fraction larger than isolated EVs. C) The ultrastructural aspect of a vesicle, by transmission electron microscopy (TEM). D) Presence of exosomal proteins in an EV sample (EVs) and in an EV-depleted plasma (EV-PD) sample.

CD patients; 250 of them had less than ten reads, 156 were found in the three groups (CD, LI, and controls) (Fig. 2A), and 94 were shared by all six pools. The quantities of four miRNAs differed between CD patients and controls: miR-99b-3p ($log2_{FC}$=-4.30; P=0.017); miR-197-3p ($log2_{FC}$=3.33; P=0.042); miR-223-3p ($log2_{FC}$=2.41; P=0.008); and miR-374b-5p ($log2_{FC}$=4.83; P=0.008) (Fig. 2B, Table 2). Interestingly, all these miRNAs, except miR-223-3p, presented the same trend patterns between LI and controls as well (P=0.078 for all three miRNAs) (Fig. 2B, Table 2). Furthermore, considering CD and LI individuals together (patients) compared to controls we observed several deregulated miRNAs, including miR-99b-3p ($log2_{FC}$=-6.53; P<0.001), miR-197-3p ($log2_{FC}$=4.14; P=0.003), miR-223-3p ($log2_{FC}$=2.09; P=0.009), and miR-374b-5p ($log2_{FC}$=4.83; P<0.001) (Fig. 2B, Table S2). No differences were found comparing CD *versus* LI individuals (data not shown).

Therefore, miR-99b-3p was found significantly less abundant in CD, as well as in patients in general compared to controls. On the other hand, miR-197-3p, miR-223-3p, and miR-374b-5p were found significantly more abundant in CD and patients in general compared to controls, with miR-374b-5p being five times more abundant in CD and patients in general. Overall, based on the aforementioned profiles for CD, LI (as a trend), and CD and LI together, we selected miR-99b-3p, miR-197-3p, miR-223-3p, and miR-374b-5p as candidate plasmatic EV-miRNAs for further analysis, including quantification in the entire cohort by RT-qPCR.

Predicting biological pathways

Using the miRpath Diana online tool, pathways regulated by miR-99b-3p, miR-197-3p, miR-223-3p, and miR-374b-5p were predicted. KEGG annotation analysis resulted in 10 pathways related to cell cycle, proliferation and communication, adherens junction, carcinogenesis and extracellular matrix (ECM) organization (Fig. S1). Remarkably, mir-197-3p was found significantly associated with eight of ten predicted pathways (P<0.05), including regulation of ECM-receptor interaction, protein processing in endoplasmic reticulum, and adherens junction. The other studied miRNAs, as miR-374b-5p and miR-99b-3p, were also found significantly associated with different predicted pathways (P<0.05), while miR-223-3p was only associated with the 'proteoglycans in cancer' pathway. Curiously, all miRNAs except miR-99b-

Table 2
List of top 10 microRNAs from small RNA sequencing based on the smallest adjusted *P*-value for each analysis (CD *versus* control and LI *versus* control).

Analysis	microRNA	log2 Fold Change	Adjusted *P*-value
CD *vs* control	hsa-miR-223-3p*	2.41	**0.008**
	hsa-miR-374b-5p*	4.83	**0.008**
	hsa-miR-99b-3p*	-4.30	**0.017**
	hsa-miR-197-3p*	3.33	**0.042**
	hsa-miR-28-3p	3.81	0.061
	hsa-miR-375	-3.59	0.067
	hsa-miR-4732-5p	-3.16	0.067
	hsa-let-7f-1-5p	2.19	0.071
	hsa-let-7f-2-5p	2.18	0.071
	hsa-miR-221-3p	2.18	0.071
LI *vs* control	hsa-miR-197-3p*	3.57	0.078
	hsa-miR-374b-5p*	3.78	0.078
	hsa-miR-99b-3p*	-4.18	0.078
	hsa-let-7f-1-5p	2.34	0.186
	hsa-let-7f-2-5p	2.33	0.186
	hsa-miR-181a-1-5p	-2.22	0.186
	hsa-miR-181a-2-5p	-2.22	0.186
	hsa-miR-125a-5p	2.69	0.356
	hsa-miR-181b-2-5p	-2.22	0.356
	hsa-miR-181b-1-5p	-2.156	0.411

* The microRNAs quantified in the entire cohort. *vs* – *versus*. In bold – *P*-values lower than 0.05.

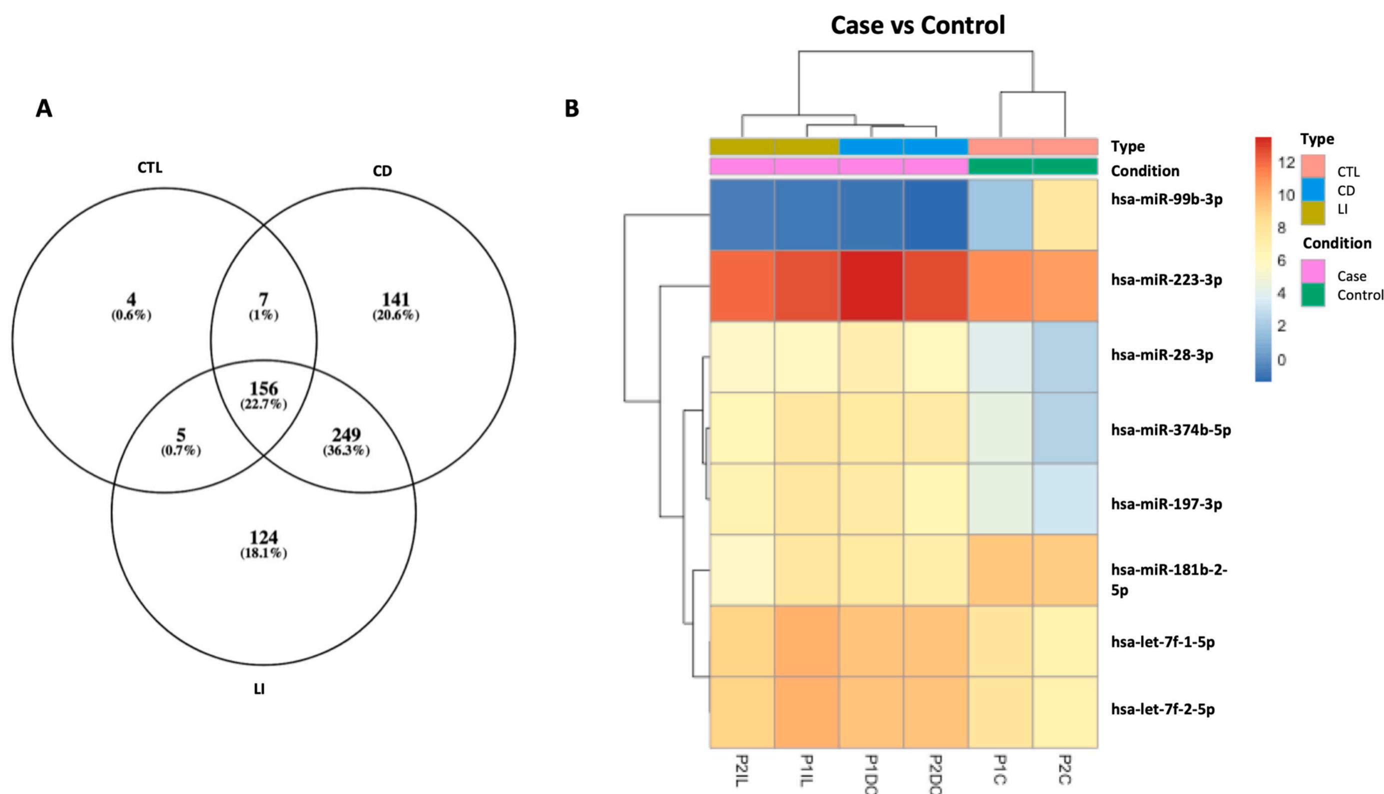

Fig. 2. The celiac disease (CD) and lactose intolerance (LI) miRNA profiles differed from the control profile for the number and quantity of detected miRNAs. A) MiRNAs detected in each studied group (CD, LI and control). B) MiRNAs with differential quantities between patient and control pools. Abbreviations: CTL – control, CD – celiac disease, LI – lactose intolerance, P1C – pool 1 of controls, P2C – pool 2 of controls, P1CD – pool 1 of celiac disease, P2CD – pool 2 of celiac disease, P1LI – pool 1 of lactose intolerance, and P2LI – pool 2 of lactose intolerance.

3p were significantly associated with this pathway.

Additionally, GO analysis showed 90 pathways associated with several biological, metabolic and catabolic processes, including response to stress, innate immunity, ECM modulation and intestinal epithelial cell development (Fig. S2). As for KEGG analysis, miR-197-3p and miR-374b-5p were found significantly associated with the great majority of predicted pathways.

Validation phase – quantification of EV-miRNAs

MiR-99b-3p, miR-197-3p, miR-223-3p, and miR-374b-5p levels were quantified by RT-qPCR in 71 plasmatic EV samples, including CD patients with LI (CD-associated LI, CD+LI) or without LI (CD not associated with LI, CD-LI), LI individuals, and controls. Altered levels were observed for miR-99b-3p and miR-197-3p among different groups. MiR-99b-3p levels significantly differed between CD patients and controls ($log2_{FC}$=-0.884; P=0.0343), and between patients in general and controls ($log2_{FC}$=-0.808; P=0.0471) (Fig. 3, Table 3). Curiously, reduced levels of miR-99b-3p were noted between CD-associated LI (CD+LI) and controls ($log2_{FC}$=-0.932; P=0.0264) (Fig. 3, Table 3), and between CD-associated LI (CD+LI) and LI individuals ($log2_{FC}$=-0.333; P=0.0487) (Fig. S4, Table 3). No significant difference was found comparing LI individuals to controls ($log2_{FC}$=-0.175; P=0.2993) (Table 3), or CD-LI with controls ($log2_{FC}$=-0.842; P=0.1555) (Fig. 3, Table 3). Taken together, this may suggest that a significant reduction on miR-99a-3p levels might be observed when CD and LI are associated.

In turn, miR-197-3p levels were found decreased in CD patients in comparison to controls ($log2_{FC}$=-0.881; P=0.0132), between patients in general (CD and LI groups together) and controls ($log2_{FC}$=-0.819; P=0.0326), between CD-associated LI (CD+LI) and controls ($log2_{FC}$=-0.849; P=0.0427), as well as in CD not associated with LI (CD-LI) against controls ($log2_{FC}$=-1.198; P=0.0196) (Fig. 3, Table 3). No significant difference was found comparing LI to controls ($log2_{FC}$=-0.510; P=0.3089) (Table 3), nor among other comparisons (Fig. 3, Fig. S4, Table 3). Together, these results may indicate that miR-197-3p decreased levels in EVs are specific of CD. Moreover, miR-374b-5p levels differed only between CD-associated LI (CD+LI) and LI individuals ($log2_{FC}$=-1.146; P=0.0384), being significantly decreased in CD-associated LI (CD+LI) in comparison to LI exclusive individuals (Fig. S4, Table 3), which, even though not so clearly, could also be suggestive of a CD-exclusive pattern. Furthermore, miR-223-3p levels did not differ among the proposed comparisons (Table S3).

Additionally, CD patients were also stratified according to CD-associated comorbidities – TI and DH status –, such as CD patients with TI (CD-associated TI, CD+TI) or without TI (CD not associated with TI, CD-TI), and CD patients with DH (CD-associated DH, CD+DH) or without DH (CD not associated with DH, CD-DH). MiR-99b-3p levels differed between CD-associated TI patients and controls ($log2_{FC}$=-1.548; P=0.0363) and between CD patients without DH and controls ($log2_{FC}$=-0.986; P=0.0222). Differences between CD and controls for miR-197-3p remained when stratifying CD patients in CD-associated TI ($log2_{FC}$=-1.311; P=0.0254), CD without TI ($log2_{FC}$=-0.868; P=0.0152), and CD without DH ($log2_{FC}$=0.548; P=0.0152). Different levels of miR-374b-5p were observed between CD patients without DH and LI exclusive individuals ($log2_{FC}$=0.485; P=0.0402). Additional comparisons were not statistically significant (Fig. 3, Fig. S4, Table 3 and Table S3). Even taking into account that all significant associations involved the comparison between groups with more than 10 individuals (except for the comparison between CD-associated LI and controls), we recommend considering these results with caution, especially those on CD-associated TI, LI or DH. With the aforementioned comparisons, we aimed to preliminarily explore the potential influence of TI and DH on EV-miRNA levels, since both conditions affect a considerably higher proportion of individuals with CD than with LI or controls (P<0.05) (Table 1). Therefore, even aware of sample size limitation, we decided to perform and present such analyses given their importance.

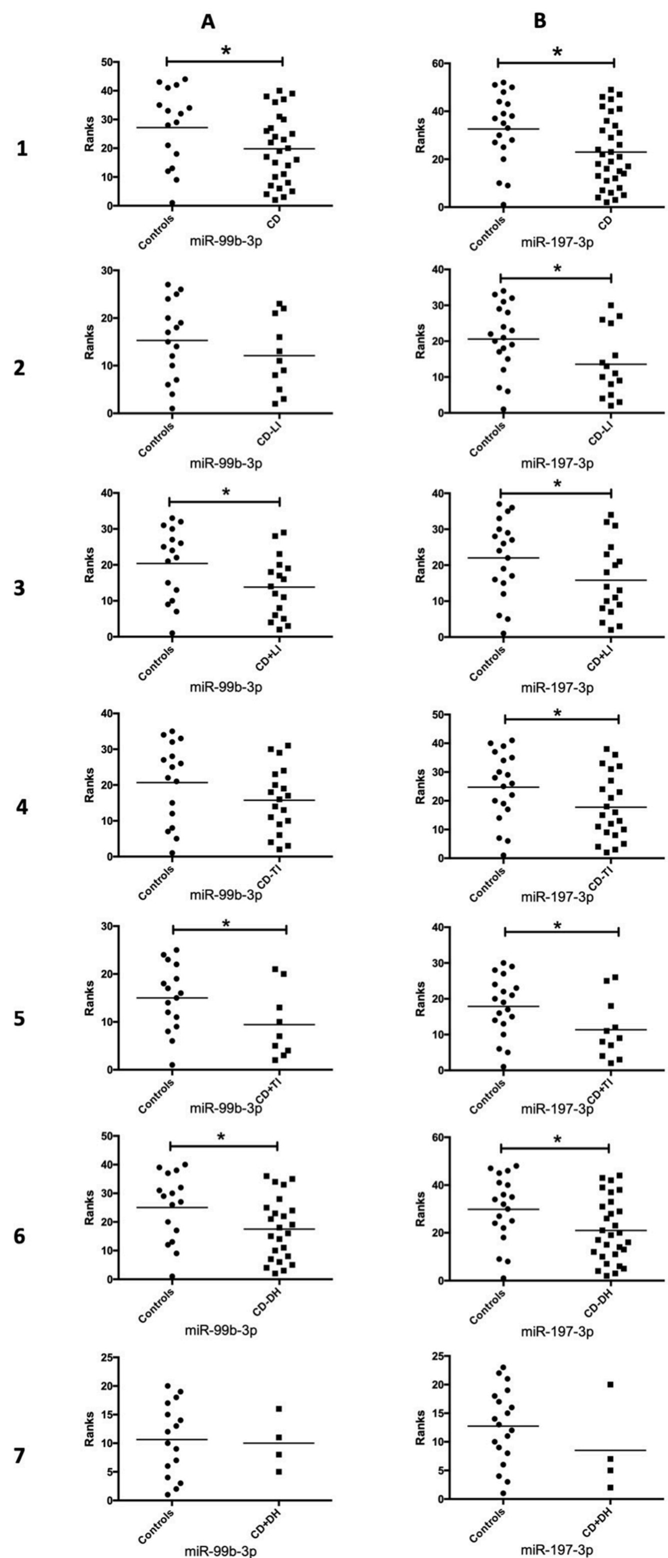

Fig. 3. Comparison of the levels of (A) miR-99b-3p and (B) miR-197-3p between: (1) celiac disease (CD) and controls; (2) CD not associated with lactose intolerance (CD-LI) and controls; (3) CD-associated LI (CD+LI) and controls; (4) CD not associated with thyroid impairment (CD-TI) and controls; (5) CD-associated TI (CD+TI) and controls; (6) CD not associated with dermatitis herpetiformis (CD-DH) and controls; (7) CD-associated DH (CD+DH) and controls. Abbreviations: CD – celiac disease, LI – lactose intolerance, TI – thyroid impairment, DH – dermatitis herpetiformis. * P-values lower than 0.05 (statistical significance was determined by Mann Whitney Test).

Table 3
Results of stratified analysis, reporting log2 Fold Change values and *P*-values.

	miR-99b-3p		miR-197-3p		miR-374b-5p	
	log2 Fold Change	*P*	log2 Fold Change	*P*	log2 Fold Change	*P*
CD *vs* control	-0.884	**0.0343**	-0.881	**0.0132**	0.051	0.3991
LI *vs* control	-0.175	0.2993	-0.510	0.3089	0.463	0.1217
patient *vs* control	-0.808	**0.0471**	-0.819	**0.0326**	0.276	0.3943
CD-LI *vs* control	-0.842	0.1555	-1.198	**0.0196**	0.438	0.3761
CD+LI *vs* control	-0.932	**0.0264**	-0.849	**0.0427**	-0.683	0.2380
CD-TI *vs* control	-0.811	0.0801	-0.842	**0.0323**	-0.056	0.3722
CD+TI *vs* control	-1.548	**0.0363**	-1.311	**0.0254**	-1.015	0.1610
CD-DH *vs* control	-0.986	**0.0222**	-0.868	**0.0152**	-0.022	0.2690
CD+DH *vs* control	-0.311	0.4169	-1.123	0.1309	1.523	0.1828
CD *vs* LI	0.710	0.0790	0.364	0.0954	0.411	0.0743
CD-LI *vs* LI	-0.664	0.2695	-0.680	0.0852	-0.025	0.2494
CD+LI *vs* LI	-0.333	**0.0487**	-0.561	0.1773	-1.146	**0.0384**
CD-TI *vs* LI	0.633	0.1270	0.332	0.1627	0.194	0.1459
CD+TI *vs* LI	1.373	0.0706	0.800	0.0815	1.153	0.0529
CD-DH *vs* LI	1.751	0.0521	0.356	0.1009	0.485	**0.0402**
CD+DH *vs* LI	0.808	0.500	0.611	0.2304	-1.062	0.3605
CD+LI *vs* CD-LI	-0.092	0.3195	0.347	0.2509	0.463	0.1217
CD+TI *vs* CD-TI	-0.737	0.0994	-0.468	0.2612	-0.960	0.2232
CD+DH *vs* CD-DH	0.674	0.1047	-0.255	0.4283	1.545	0.0940

vs – *versus*, CD – celiac disease, LI – lactose intolerance, TI – thyroid impairment, DH – dermatitis herpetiformis. In bold – *P*-values lower than 0.05.

Discussion

This pioneering study investigated the profile of circulating EV-miRNAs of CD patients on GFD and LI individuals on lactose restrictive diet, compared to controls with no report of food-related issues. Since CD patients were on a GFD, most of the symptoms were not present at the time of sample assessment; however, all complained about symptoms following minor contact with gluten, and some developed other associated conditions, like secondary LI, TI, and DH, as already reported [7,45]. Adherence to a GFD is a challenge involving economic, financial, social and individual factors, despite delivering numerous benefits [37]. Unfortunately, predisposition to some diseases as thyroid cancer continues even on a GFD [25,50]. There are no good diagnostic tests available to evaluate the adhesion to a GFD [10]. Thus, this study aimed to identify EV-miRNAs that in the future could be used to evaluate GFD adherence, based on a blood test, potentially avoiding the realization of invasive tests, and to understand the response of CD patients on GFD, compared with LI individuals and controls without food restrictions.

We would also like to highlight that this is an essentially exploratory study, aiming to generate preliminary data on EV-miRNAs in CD patients and/or LI individuals on restrictive diets. Based on the study design and the obtained results, it is not possible to propose candidate miRNAs, neither individually nor in a composed panel, as potential molecular biomarkers for CD, for which a comparison with celiac patients at the time of diagnosis would have been necessary.

Based on the obtained results, we found a similar profile between CD and LI that could be related to shared gut microbiota modifications since one-third of the CD participants reported symptoms resulting from lactose ingestion. Interestingly, the abundance of two EV-miRNAs (miR-99b-3p and miR-374b-5p) differed between CD-associated LI and LI exclusive individuals. They may be associated with intestinal damage and with extraintestinal symptoms and shall be further investigated.

The role of miRNAs in CD has been investigated in different settings, but none of them have evaluated the EV miRNA cargo [5,6,34,47]. We detected three miRNAs whose quantity in EVs differed (significantly or as a trend) between CD patients and controls or LI – miR-99b-3p, miR-197-3p, and miR-374b-5p. Only miR-197-3p had been formerly described as deregulated in the mucosa of CD patients with active disease [34]. MiR-197-3p levels were reduced in the CD-injured duodenal mucosa, compared with the controls' healthy mucosa, with its expression varying among patients with different Marsh levels [34]. As expected, its abundance was low in plasmatic EVs isolated from CD patients, varying among CD patients with CD-related conditions. However, in the pooled small RNA-Seq data, the result was the opposite. This discrepancy may be due to a differential distribution of miRNA content among CD-related diseases and to distinct GFD duration.

MiR-197-3p has been widely investigated in the context of cancer, as well as in CD. The down-regulation of the mRNA of metadherin (*MTDH*) – a component of the adherent junctions – by miR-197-3p is associated with the reduction of the progression to gastric cancer when this miRNA is under-expressed [28]. Also, the mRNAs of laminin subunit gamma 2 (*LAMC2*) – a signaling element of extracellular matrix receptors – and of metastasis suppressor kangai-1 (*CD82*) – a p53 signaling suppressor – are negatively regulated by miR-197-3p and the increase in the levels of these mRNAs by down-regulation of miR-197-3p may contribute to the development of gastric cancer [53]. The decrease in the expression of miR-197-3p in some types of cancer may be associated with an increase in the expression of HOTAIR long non-coding (lncRNA). HOTAIR may act as a "sponge" for miR-197-3p, impairing its ability to regulate its particular mRNA targets, ultimately contributing to disease progression [33].

Moreover, we highlight that two – miR-99b-3p and miR-374b-5p – out of three miRNAs whose level alterations were observed in the present study have not yet been described in CD, although their influence on CD-associated diseases has already been suggested. Differential expression of miR-374b-5p was described in gastric and intestinal cancers [41,52]. Increased levels of miR-374b-5p in gastric tumor tissues might contribute to tumor invasion and metastasis, regulating the mRNA levels of reversion inducing cysteine-rich protein with kazal motifs (*RECK*) [52]. Contrarily, reduced levels of miR-374b-5p were found in colon cancer cells in comparison with non-tumor cells. MiR-374b-5p seems to be involved in the AKT serine/threonine kinase 1 (AKT1) pathway by negatively regulating, at the mRNA and protein levels, the expression of liver receptor homolog-1 (*LRH1*), a protein involved in tumorigenesis of colon cancer [41].

In addition, reduced expression of miR-374b-5p was detected in individuals with lymphoma. Furthermore, the overexpression of two crucial proteins – AKT1 and Wnt16 – that also participate in the AKT signaling pathway was also observed within the tumor. The increase of miR-374b-5p expression reflected an increase in apoptosis and suppression of tumor cell proliferation [40]. Moreover, the pronounced down-regulation of Claudin 14 (*CLDN14*) mRNA in renal tissue by miR-374b-5p overexpression [18] could contribute to the dysfunctions of the cell barrier and be associated with intestinal diseases, such as CD [54]. Here, we found a higher abundance of miR-374b-5p in plasmatic EVs from patients with CD-associated LI, compared with LI only. The regulation of its putative targets remains to be investigated in both conditions.

MiR-99b-3p was found down-regulated in thyroid cancer patients [14] and could be involved in increased risk of thyroid cancer in CD patients [25]. The involvement of miR-99b-3p was also described in intestinal epithelial cell transformation [12], immune modulation [21],

and promotion of migration, invasion and proliferation of hepatocellular carcinoma cells (HCC) by targeting the protocadherin 19 (*PCDH19*) mRNA [55]. Curiously, differences in miR-99b-3p expression levels were observed in gastric cancer samples depending on *Helicobacter pylori* infection status, reinforcing the idea that differences in the intestinal microbiome may influence miRNA profiles [8]. In CD pathogenesis, however, miR-99b-3p function is still unclear. In our setting, the levels of this miRNA in plasmatic EVs decreased upon GFD compliance, which may indicate a pathogenic role in microvilli aplasia.

Moreover, the *in silico* analysis of targets and pathways of miRNAs with differential quantities between CD patients and controls allowed to point out possible targets of miRNAs involved in the pathogenesis of CD. The modulation of the levels of these miRNAs has already been described as favoring carcinogenic processes. Similar molecular processes are involved in CD, such as changes in adhesion molecules [28]. They may increase permeability of the intestinal epithelium, aggravating the toxic effects of gluten and affecting the modulation of immune responses, via alteration of the levels of cytokines and other regulators of immunity [22]. Regulatory pathways of cell metabolism and ECM receptors were also associated with the differential quantities of EV-miRNAs, which could alter cell remodeling and food absorption, therefore contributing to the development of lactose intolerance and predisposition to cancer.

Among the limitations of our study, we highlight the lack of a group composed of CD patients before adherence to a GFD. In general, patients immediately adhere to a GFD as CD diagnosis is suspected or established, turning it extremely difficult to recruit recently diagnosed CD adult patients that are not yet on a GFD, especially in the case of a transversal study. In contrast, the authors of two other studies investigated circulating miRNAs (cell-free miRNAs) associated with CD development in infancy [17,44]. Among 53 infant participants of the longitudinal PreventCD study, only 33 developed CD during follow-up [44], whereas 40 untreated patients were enrolled in the second study [17]. This exemplifies that even in specific pediatric cohorts designed to follow-up CD patients the number of CD patients remain modest. Even so, we aim to validate our results in the near future by (i) conducting a follow-up study in adults starting from CD diagnosis, (ii) increasing sample size by an extended period of recruitment, and (iii) characterizing miRNA profiles in biopsies taken from patients with defined lymphocyte infiltration, anti-transglutaminase IgA titers and tissue deposition. This approach would also enable correlations between circulating EV-miRNA profiles, biopsy tissue composition and CD disease status.

In general, we expect that cellular and circulating miRNA expression profiles reflect one another to some extent. In fact, liquid biopsies support the concept that punctual changes in EV-miRNA profiles systemically reflect a subjacent physiological (pathological) condition [29,56]. However, circulating miRNA levels arise from several cellular types. For instance, plasma EV-miRNAs represent a pool of miRNAs originated from EVs released by immune cells, cells from the intestinal mucosa, among others. Moreover, clinical and sociodemographic characteristics such as age, gender, ancestry, and BMI [4,19,38], as well as parameters related to individual miRNA stability, as transcription or decay rates [1], may also impact miRNA levels. Thus, follow-up studies shall compare intracellular and (free or EV-associated) extracellular miRNA profiles between untreated (CD at diagnosis) and treated (CD on GFD) patients, as well as correlate these profiles with autoantibody production and microvilli recovery.

Considering the aforementioned limitations, we would like to list some potential extra experiments that would expand the knowledge on miRNAs in CD: (i) the analysis of miRNA levels in CD at diagnosis; (ii) the correlation of plasma miRNA levels with autoantibodies in untreated (CD at diagnosis) and treated (CD on GFD) patients; and (iii) the correlation of intracellular and extracellular miRNA profiles taking into account disease status.

As positive aspects, we highlight that, to the best of our knowledge, this is the first study evaluating the levels of EV-miRNAs in biofluids from CD patients and LI individuals. Our preliminary data may pave the way to the understanding of key miRNAs involved in the response to GFD and lactose restrictive diets, which, in turn, may lead to potential molecular biomarkers for diet adherence and disease recovery. Additionally, another positive aspect of our study was the discrimination of CD-associated LI patients from CD exclusive patients (an aspect that can be misreported in a CD cohort, since LI is frequently associated with CD). Taking into account this frequent association [in our cohort, approximately 60% (20/34) of CD patients were diagnosed with both conditions], we considered discriminating CD-associated LI patients as a positive aspect of our study. In addition, when reporting a 'CD not associated with LI' group we may be addressing CD exclusive patients and, therefore, be able to discriminate specific miRNAs potentially associated with CD only. Stratifying and reallocating samples in different groups allowed us to make different comparisons. Therefore, we could infer which miRNAs are indeed potentially associated with CD, or with LI, as well as with both conditions.

In summary, we found altered EV-miRNAs for CD and LI, including miR-99b-3p decreased levels for CD-associated LI, miR-197-3p decreased levels specifically for CD not associated with LI, and miR-374b-5p decreased levels in CD-associated LI compared to LI. The participation of these miRNAs in cell adhesion and immune modulation pathways has been predicted, and their altered levels could be involved in the hypersensitivity to gluten and possibly contribute to CD-associated diseases, such as LI, TI, and DH. Altered levels of EV-miRNAs might also be associated with restrictive diets, as gluten-free diet and lactose-free diet, and affect gut recovery.

Author contributions

DSL, DM, GAC and ABWB designed the study. DM, RCA, GAC and ABWB supervised the study. DM, MLPE, ESM and SMZ obtained funding. DSL, ABWB, HCB and LCO interviewed and selected cases and collected blood samples. PMMO, ILMS, VCSP, TRT, ESM, RVS and PFW performed technical analysis. DSL, RCA and FCBB performed bioinformatics and statistical analysis and interpreted data. DSL and FCBB drafted the manuscript. DM, MLPE, ABWB, SMZ, PFW and GAC revised and approved the manuscript.

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Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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