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Crosstalk between MicroRNAs and Oxidative Stress in Coeliac Disease

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Abstract
MicroRNAs (miRNAs) are small, non-coding RNA molecules involved in regulating gene expression. Many studies, mostly conducted on pediatric patients, suggested that oxidative stress and several miRNAs may play an important role in coeliac disease (CeD) pathogenesis. However, the interplay between oxidative stress and miRNA regulatory functions in CeD remains to be clarified. In this review, we aimed to perform a literature review on the role of miRNAs and oxidative stress in adult CeD patients and to analyze their potential interactions. In this direction, we also reported the preliminary results of a pilot study we recently performed.

Introduction

Celiac disease (CeD) is an immune-mediated disease triggered by the ingestion of gluten in genetically predisposed subjects [1]. Many patients remain undiagnosed for years before being correctly diagnosed and receiving appropriate treatment [2]. Recently published epidemiologic data set the histological and serological prevalence rates of CeD at 1.4% and 0.7%, respectively [3]. In adults, CeD diagnosis is based on the positivity of serum anti-tissue transglutaminase IgA antibodies, confirmed by duodenal damages such as increased intraepithelial lymphocytes, crypt hyperplasia, and villous atrophy [1]. A lifelong gluten-free diet (GFD) is the only proven and widely accepted treatment for CeD. The large majority of patients respond to the GFD and have an excellent disease prognosis, but, in less than 1% of cases, some preneoplastic and malignant conditions may complicate the disease course [4].

In recent years, several researchers have tried to unravel the mechanisms underlying the pathogenesis of CeD and to find novel molecular biomarkers for the diagnosis in the search for alternative methods that could avoid invasive procedures (i.e., duodenal biopsies) or follow-up of intestinal damage (as markers of dietary adherence). In this setting, the role of microRNAs (miRNAs) and oxidative stress in the pathogenesis of CeD has been studied, and their use as potential markers of active disease in clinical practice has been proposed [5, 6]. In this review, we aimed to summarize the available evidence on the role of miRNAs and oxidative stress in

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adult CeD patients and to highlight their potential interaction in the pathogenesis of the disease. Moreover, some preliminary data from our pilot study regarding crosstalk between miRNAs and oxidative stress are also presented.

Role of miRNAs

miRNAs are small non-coding RNA molecules (~20–25 nucleotides long) extensively involved in regulating gene expression. They play a role in almost all cellular pathways, acting at the post-transcriptional level by binding complementary recognition sequences of target mRNAs, thus upregulating or downregulating the expression of specific genes [7]. More than 700 miRNA genes are estimated in the human genome, accounting for 1–4% of expressed genes [8]. A single miRNA might control hundreds of distinct targets, and it is estimated that approximately a third of all animal genes are potentially under miRNA control. Several mechanisms have been proposed to explain how miRNAs exert post-transcriptional control over their targets, most commonly regulating their targets in a 3′UTR-dependent manner, resulting in downregulation of gene expression via translational repression and/or mRNA degradation.

miRNAs contribute to a variety of physiologic and pathologic processes, including some autoimmune diseases and several types of cancer [9–14]. Moreover, miRNAs play a pivotal role in regulating immune response by modulating the functioning and differentiation of immune cells. In diseases characterized by chronic inflammation, such as CeD, they have been discussed as potential biomarkers of mucosal inflammation [15–17]. Indeed, in CeD patients, several genes involved in innate and adaptative immunity are targeted by miRNAs [15], miRNAs being implicated in the pathogenesis of CeD, with several studies investigating differential miRNA expression on duodenal tissue biopsies. Moreover, miRNAs have been evaluated as non-invasive circulating biomarkers in the diagnostic setting and for disease monitoring during follow-up. Table 1 summarizes the studies about miRNAs in CeD patients.

Tissue miRNAs and Celiac Disease

Inflammatory disorders, such as inflammatory bowel diseases or CeD, are characterized by a functional impairment of the intestinal epithelium. Some preclinical data demonstrate that miRNAs are involved in these intestinal epithelium alterations and intestinal barrier function deregulation [18, 19]. In particular, McKenna et al. [18] demonstrated that, among the 453 miRNAs identified in the intestinal mucosa, mmu-miR-192, mmu-miR-215, and mmu-miR-let7 resulted the most highly expressed in both the small and large intestine. The role of miRNAs in regulating intestinal homeostasis is demonstrated by the fact that mice deficient for Dicer (a pivotal protein of the miRNA biogenesis machinery, which is responsible for the synthesis of mature miRNAs) have a disorganized intestinal epithelium, with a decrease in goblet cells, a significant increase of apoptosis in the crypts of both the jejunum and colon, and accelerated jejunal cell migration. Moreover, intestinal barrier function is impaired in this mouse model, resulting in intestinal inflammation, with lymphocyte and neutrophil infiltration [18].

Enteropathies with intestinal inflammation are characterized by an alteration in intestinal barrier permeability, which can be modulated by miRNAs. Indeed, it has been demonstrated both in vitro (in the intestinal epithelial Caco-2 cell model) and in vivo (in a mouse small intestinal model) that mmu-miR-122a, by recognizing the mRNA of occludine at 3′UTR, can induce depletion of occludin in epithelial tight junctions (TJ) [19]. The activation of immune response is stimulated by the release of chemokines and cytokines, such as tumor necrosis factor (TNF)-α, that can upregulate mmu-miR-122a [19].

The regulation of gene expression in intestinal epithelial cells is a complex process, modulated by many different signaling pathways commonly altered in CeD (such as those that regulate proliferation/differentiation balance) [20]. Being miRNAs, fine regulators of gene expression, their altered expression in CeD may be implicated in the pathogenesis of the disease and can be associated with specific clinical features [21]. In the last few years, several studies have evaluated the expression profiles of miRNAs in the human small intestine of patients with CeD in an attempt to understand the pathophysiologic mechanisms implicated [15, 17, 22–24]. In a cohort of 40 children (20 with active CeD, 9 on a GFD, and 11 controls), 9 miRNAs were upregulated, and 21 were downregulated in the duodenal tissue of CeD patients compared to controls (but with similar expression levels in active and GFD CeD). Among the upregulated miRNAs, miR-449a was expressed at very high levels in CeD and GFD children, targeting and reducing NOTCH1 and KLF4 [22], both involved in the control of mouse intestinal homeostasis, guiding cell proliferation and differentiation [25, 26]. These data indicate a miR-449a-mediated NOTCH1 and KLF4 pathway regulation

Table 1. miRNAs in CeD

First author	Year	miRNAs	Number	Tissue/ blood	Pediatric/ adult population	Main findings
Capuano	2011	miR-449a	Active CeD, *n* = 20 GFD, *n* = 9 Controls, *n* = 11	Tissue	Pediatric	miR-449a inhibits the expression of NOTCH1 and KLF4
Vaira	2014	miR-31-5p, miR-192-3p, miR-194-5p, miR-551a, miR-551b-5p, miR-638 and miR-1290	Active CeD, *n* = 33 GFD, *n* = 34 Controls, *n* = 17	Tissue	Adult	Downregulation of miR-194-5p and overexpression of miR-638 in CeD with anemia. Downregulation of miR-31-5p and miR-192-3p, and overexpression of miR-1290 related to CeD regardless of the clinical presentation
Magni	2014	miR-192-5p, miR-31-5p, miR-338-3p, and miR-197	Microarray: CeD, *n* = 6; controls, *n* = 5 Validation: controls, *n* = 10; active CeD, *n* = 21 (Marsh 3A-B, *n* = 9 and Marsh 3C, *n* = 12) In vitro experiments, GDF, *n* = 9; controls, *n* = 5	Tissue	Adult	Decreased expression of miR-192-5p, miR-31-5p, miR-338-3p, and miR-197 Among possible miRNAs targets, increased mRNA and protein of CXCL2 and NOD2 in Marsh 3C, and significant inverse correlation with miR-192-5p Alterations in CXCL2 and NOD2, FOXP3, miR-192-5p, and miR-31-5p expression were triggered by gliadin exposure
Buoli Comani	2015	miR-192-5p, miR-31-5p, miR-338-3p, miR-21-5p	Tissue analysis: Controls, *n* = 8; active CeD, *n* = 20 Plasma analysis: controls, *n* = 12; active CeD, *n* = 17 GFD, *n* = 7	Tissue and plasma	Pediatric	Downregulation of miR-192-5p, miR-31-5p and miR-338-3p expression in CeD. Upregulation of miR-21-5p Plasma analyses demonstrated a similar trend to that observed in biopsies
Comincini	2017	miR-17, miR-30a	Tissue analysis: controls, *n* = 24; active CeD, *n* = 25 Blood analysis: controls, *n* = 33; active CeD, *n* = 23	Tissue and blood	Pediatric	Autophagy-related genes (ATG7 and BECN1) are regulated bymiR-17 and miR-30a. These molecular markers may be useful to increase the accuracy in CeD diagnosis
Bascunan	2020	miRNA-146a, miRNA-155, miRNA-21, miRNA-125b	Active CeD, *n* = 10 GFD, *n* = 10 Controls, *n* = 10	Tissue and blood	Adult	CeD versus controls in peripheral blood mononuclear cells: miRNA-146a (AUC = 0.91) and miRNA-155 (AUC 0.92) In plasma, high accuracy for miR-155 (AUC 0.98)
Tan	2021	>200 miRNAs	*n* = 53 (*n* = 33 developed CeD during follow-up; controls, *n* = 20)	Serum	Pediatric	53 circulating miRNAs were increased (27) or decreased (26) in CeD versus controls. 8/53 miRNAs differed significantly between controls and samples taken <1 year before TGA positivity: miR-21-3p, miR-374a-5p, 144-3p, miR-500a-3p, miR-486-3p let-7d-3p, let-7e-5p and miR-3605-3p. 6/26 downregulated miRNAs reconstituted upon GFD, including miR-150-5p/-3p

Table 1 (continued)

First author	Year	miRNAs	Number	Tissue/ blood	Pediatric/ adult population	Main findings
Felli	2022	13 miRNAs (miR-192-5p, miR-215-5p, miR-125b-5p)	Active CeD, n = 40 GFD, n = 40 Controls, n = 40	Serum	Pediatric	Accurate discrimination of CeD and controls: miR-192-5p (AUC = 0.854), miR-215-5p (AUC = 0.842), miR-125b-5p (AUC = 0.803)
Domsa	2022	miR-192-5p, miR-194-5p, miR-449a and miR-638	Active CeD, n = 15 GFD, n = 33 Controls, n = 10	Blood	Adult	Among miRNAs, the closest to a statistically significant value was miR-194-5p (CeD vs. controls: p = 0.051 and GFD vs. controls: p = 0.067)

in the small intestine of CeD children [22]. The maintenance of an adequate number of functional goblet cells is required for the homeostasis of the intestinal mucosa, as deficiency in the mucin composition makes the mucosa more susceptible to damaging agents in the lumen [27–29], and an altered NOTCH1 and KLF4 expression could reduce goblet cells in the small intestine of CeD patients.

In order to evaluate whether, in patients with different clinical manifestations of CeD, a differential duodenal miRNAs expression is present, Vaira et al. [17] examined untreated adult CeD patients with classical symptoms (CC), patients with iron-deficiency anemia (CA), patients on a GFD (NT-C), and non-CeD subjects with normal duodenal mucosa. Compared to control subjects, both CC and CA had a significant downregulation of miR-31-5p and miR-192-3p and upregulation of miR-1290, miR-638, and miR-551b-5p. Moreover, CC patients had reduced levels of miR-551a, while CA patients showed a downregulation of miR-194-5p compared to subjects without CeD. The downregulation of miR-194-5p and the overexpression of miR-638 appeared to be typical of CeD patients with anemia compared to CeD patients with classic symptoms. Conversely, the downregulation of miR-31-5p and miR-192-3p and the overexpression of miR-1290 appeared to be related to CeD regardless of the clinical presentation [17].

miRNAs could modulate innate and adaptive responses to gluten in CeD patients. In a study including 56 subjects (Marsh 3A-B, Marsh 3C, and controls), seven miRNAs were identified as significantly downregulated in CeD patients compared to controls [15]. A decreased expression of miR-192-5p, miR-31-5p, miR-338-3p, and miR-197 was demonstrated, particularly in patients with more severe histological lesions (Marsh 3C). Experiments to discover possible miRNA targets revealed that these miRNAs modulate the expression of several genes involved in innate and adaptive immunity. Among these genes, CXCL2 and NOD2 showed significantly increased mRNA and protein levels in Marsh 3C patients and a significant inverse correlation with miR-192-5p. In addition, FOXP3, Run-related transcription factor 1, and interleukin-18 (targets of miR-31-5p, miR-338-3p, and miR-197, respectively) were upregulated in CeD patients [15]. The trigger action of gliadin in determining these alterations (differential expression of CXCL2, NOD2, FOXP3, miR-192-5p, and miR-31-5p) has been demonstrated [15]. Therefore, this study showed that miRNA expression is significantly altered in the duodenal mucosa of CeD patients, leading to an increased expression of molecules involved in an immune response. To assess whether age of CeD presentation could influence the deregulation pattern of miRNAs, a study on duodenal biopsies of Marsh 3A-B and 3C in a pediatric cohort of CeD patients as compared to controls was performed [23]. A decrease in miR-192-5p (as in adults) and overexpression of MAD2L1 (a protein related to cell cycle control) were demonstrated but without variations in NOD2 and CXCL2. miR-31-5p and miR-338-3p were downregulated, and their respective targets, FOXP3 and RUNX1, involved in Treg function, resulted upregulated in CeD patients. Moreover, in CeD patients, an increased expression of miR-21-5p was detected, possibly caused by a regulatory loop with its putative target STAT3. Therefore, this study demonstrated that miRNAs with altered expression in the duodenal mucosa differ between pediatric and adult CeD patients.

To maintain homeostasis of the intestinal epithelium, the proliferation/differentiation of epithelial cells should be finely balanced with enterocyte turnover. Therefore,

perturbation in the autophagic process could be important in CeD pathogenesis, as demonstrated by a study investigating the expression of ATG7 and BECN1 and of their negative regulators, miR-17 and miR-30, involved in the autophagy process [24]. The association between autophagy-related genes and miRNAs and CeD was demonstrated.

Circulating miRNAs and Celiac Disease

Until recently, almost all researchers studying miRNAs in subjects with CeD focused on their modulation/profiling and examined miRNA expression levels in the intestinal mucosa (tissue miRNAs). By contrast, the role of cell-free miRNAs in CeD has been poorly investigated. Recently, miRNAs have been demonstrated to circulate in different body fluids, such as serum and plasma, either free, bound to proteins, or enclosed in vesicles and released in the extracellular space [30]. These circulating miRNAs may act as autocrine or paracrine mediators, conveying their message to other cells [31]. Since circulating miRNAs are highly stable in circulation and also in different laboratory conditions (e.g., pH and temperature, repeated free-thaw cycles), circulating miRNAs may represent promising and reliable biomarkers for diagnosis, prognostic prediction, and monitoring of response to treatment in different conditions [32–34].

Circulating miRNAs correlate with different stages of CeD and with adherence to GFD. To investigate if miRNAs found deregulated in tissue were also differently expressed in circulation and demonstrated their effectiveness as biomarkers, Buoli Comani et al. assessed the circulating levels of miRNAs previously identified in duodenal biopsies of Marsh 3A-B and 3C pediatric CeD patients [23]. The authors found that plasma miR-192-5p remained downregulated in GFD patients compared to controls, while miR-486-5p resulted in upregulated compared to controls, although not reaching a statistically significant difference. miR-31-5p, 21-5p, and 21-3p in GFD, circulating levels tended to return to control levels. However, some limitations typical of works on circulating miRNAs emerge in this study: a limited number of patients and controls were included (i.e., 12 controls, 17 CeD patients at first diagnosis, 7 CeD patients on GFD for at least 1 year); miRNAs were evaluated in plasma, which may contain a higher number of contaminants, and without adequate quality controls (hemolysis may release red blood cell miRNAs). Despite some limitations, the results of this study suggest that a wider panel of plasmatic miRNAs should be analyzed to provide reliable information on intestinal mucosal status [23].

Tan et al. [35] investigated miRNAs in >200 serum samples from 53 participants included in the study at 3 months of age, 33 of whom developed CeD during follow-up. Following inclusion in the study, samples were collected at predefined ages, diagnosis (first anti-transglutaminase antibody [TGA] positivity or diagnostic biopsy), and after the start of a GFD. Among the 53 circulating miRNAs differentially expressed compared to controls, several (miR-21-3p, miR-374a-5p, 144-3p, miR-500a-3p, miR-486-3p, let-7d-3p, let-7e-5p, and miR-3605-3p) were found to differ significantly compared to controls in samples collected >1 year before TGA positivity. In addition, 6 miRNAs downregulated in patients with CeD, including miR-150-5p/-3p, started to normalize upon GFD [35]. Another study conducted in pediatric patients revealed that miR-192-5p, miR-215-5p, and miR-125b-5p (alone or in combination) were able to discriminate between CeD at diagnosis, CeD on GFD, and healthy controls with high accuracy and specificity [36].

In a small group of adult patients (n = 10 active CeD; n = 10 CeD in GFD; n = 10 controls), the expression of miRNA-146a (AUC 0.91, 95% CI: 0.83–0.99) and miRNA-155 (AUC 0.92, 95% CI: 0.86–0.99) in peripheral blood mononuclear cells demonstrated the highest sensitivity and specificity for the presence of CeD. Similar results were obtained when miR-146a and miR-155 expression was evaluated in monocytes, while in plasma only miR-155 maintained a high accuracy (AUC 0.98, 95% CI: 0.95–1.00) [16]. In another study conducted in adult CeD patients (n = 15 CeD patients at diagnosis, n = 33 CeD patients on GFD for at least 1 year, and n = 10 controls) by Domsa et al., among the circulating miRNAs evaluated (miR-192-5p, miR-194-5p, miR-449a, and miR-638), a trend toward statistically significant differences was demonstrated only for miR-194-5p (p = 0.051 for CeD at diagnosis vs. controls and p = 0.067 for CeD in GFD vs. controls) [37].

miRNAs could also be useful in identifying patients with complicated CeD. A study conducted in patients with complicated CeD (refractory coeliac disease (RCD) type 1 and RCD type 2) compared to intestinal T-cell lymphoma and peripheral T-cell lymphoma revealed that intestinal T-cell lymphomas have a unique miRNA profile compared to other lymphomas [17]. Moreover, miR-192/215 and miR-200 were consecutively lost in RCD type 2 and intestinal T-cell lymphoma, whereas miR-19/92 and C19MC miRNAs were upregulated [17]. It is interesting to note that some miRNAs deregulated in RCD type 2 are associated with the inflammatory state. In contrast, others could be potentially useful in predicting

the progression toward intestinal T-cell lymphoma related to oncogenesis (through activation of STAT3 and c-Myc). A recent study identified several upregulated miRNAs characterizing RCD type 2 that could be monitored in patient serum: miR-770-5p, miR-181b-2-3p, miR-1193, and miR-1226-3p [38]. These miRNAs were involved in pathways of response to TGF-β, cell-cell response to Wnt, epigenetic regulation, and inflammatory profiles.

Oxidative Stress

An altered balance between reactive oxygen species (ROS) production and the efficiency of antioxidant mechanisms causes oxidative stress [39]. ROS are produced as a consequence of physiological cellular metabolism. Low levels of ROS are essential in different physiological processes, such as protein phosphorylation, transcription factor activation, cellular differentiation, apoptosis, and cellular immunity. By contrast, excessive ROS levels have a detrimental effect on specific cell components such as DNA, lipids, and proteins [40]. Excessive and long-lasting oxidative stress can cause chronic inflammation that mediates most chronic diseases, including cancer, diabetes, cardiovascular, neurological, and pulmonary diseases. Indeed, oxidative stress can activate several transcription factors, including NF-kB, AP-1, p53, HIF-1α, PPAR-γ, Wnt/β-catenin, and Nrf2, that can regulate the expression of several genes, including those for growth factors, inflammatory cytokines, chemokines, cell cycle regulatory molecules, and anti-inflammatory molecules [41].

In nuclear and mitochondrial DNA, 8-hydroxy-2′-deoxyguanosine (8-OHdG) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) are the principal markers of oxidative damage caused by ROS and have been widely used as biomarkers for oxidative stress and oxidative stress-mediated cancerogenesis [42]. Specifically, 8-OHdG is one of the major products of oxidative DNA damage, and it is generated following the oxidation of deoxyguanine (gG), which is among the normal DNA nucleosides [43]. 8-OHdG has been extensively evaluated as a biomarker of oxidative stress in several diseases, including cancer.

Interaction between Oxidative Stress and miRNAs in Celiac Disease

It is well known that miRNAs can regulate the expression of several genes, including those involved in the response to oxidative stress. Indeed, oxidative stress is among the factors that can dysregulate cell signaling and cellular homeostasis, thus affecting miRNA expression [44]. Some specific miRNAs, called ROS-miRs or redoximiRs, are regulated by oxidative stress and can modulate genetic targets in response to ROS [45]. In particular, the oxidative stress/NF-kB axis induces the expression of miR-9/9*, while miR-21 regulates oxidative homeostasis by inhibiting the antioxidant response in human endothelial cells [45].

Recently, a correlation between oxidative stress and miR-200a has been demonstrated [46]. In keratinocytes, a reduction of a critical enzyme in the process of 8-OHdG adduct reparation (OGG1-2a) is due to the overexpression of miR-200a. Moreover, the overexpression of miR-200a causes the upregulation of inflammasomes NLRP3 and IL-1β. These data suggest that miR-200a plays a fundamental role in favoring oxidative stress, inhibiting the reparation of DNA oxidative damage, and therefore, ROS may be both regulators and effectors of miRNA activity [45].

According to what is currently known, ROS regulates the expression and biogenesis of miRNAs in different ways (Fig. 1a) [47]: (1) intracellular redox homeostasis could directly modulate DGCR8 (a key regulator of the maturation of canonical miRNAs) activity, thus regulating miRNA biogenesis [48–50]. Dicer has been identified to be suppressed by oxidative stress [51, 52]. (2) The expression of miRNAs is regulated by several transcription factors, such as NF-kB, c-Myc, and HIF-1α [47]. (3) another important mechanism through which ROS regulates miRNA expression is through epigenetic alterations. The activities of DNA methyltransferases (DNMTs) and histone deacetylases (HDACs), which play essential roles in the epigenetic regulation of gene transcription, including miRNA transcription, can be modulated by oxidative stress and therefore represent a crucial regulating mechanism by which ROS can control miRNA expression and biogenesis. As an example of this modulation mechanism, promoter regions of miR-125b and miR-199a are hypermethylated by DNMT1 following the exposition to H_2O_2 [45, 47].

In addition to being modulated by oxidative stress, miRNAs, in turn, can modulate ROS production by targeting multiple signaling pathways (Fig. 1b) [47]. Several miRNAs, including miR-144, miR-28, miR-200a, miR-155, and miR-93, can modulate the transcription factor nuclear factor erythroid-derived 2-like 2 (Nrf2) and its inhibitor Kelch-like ECH-associated protein 1 (Keap1), which are important regulators of response to oxidative stress [47]. Indeed, under oxidative stress, the complex Nrf2/Keap1 separates, and Nrf2 is transferred to

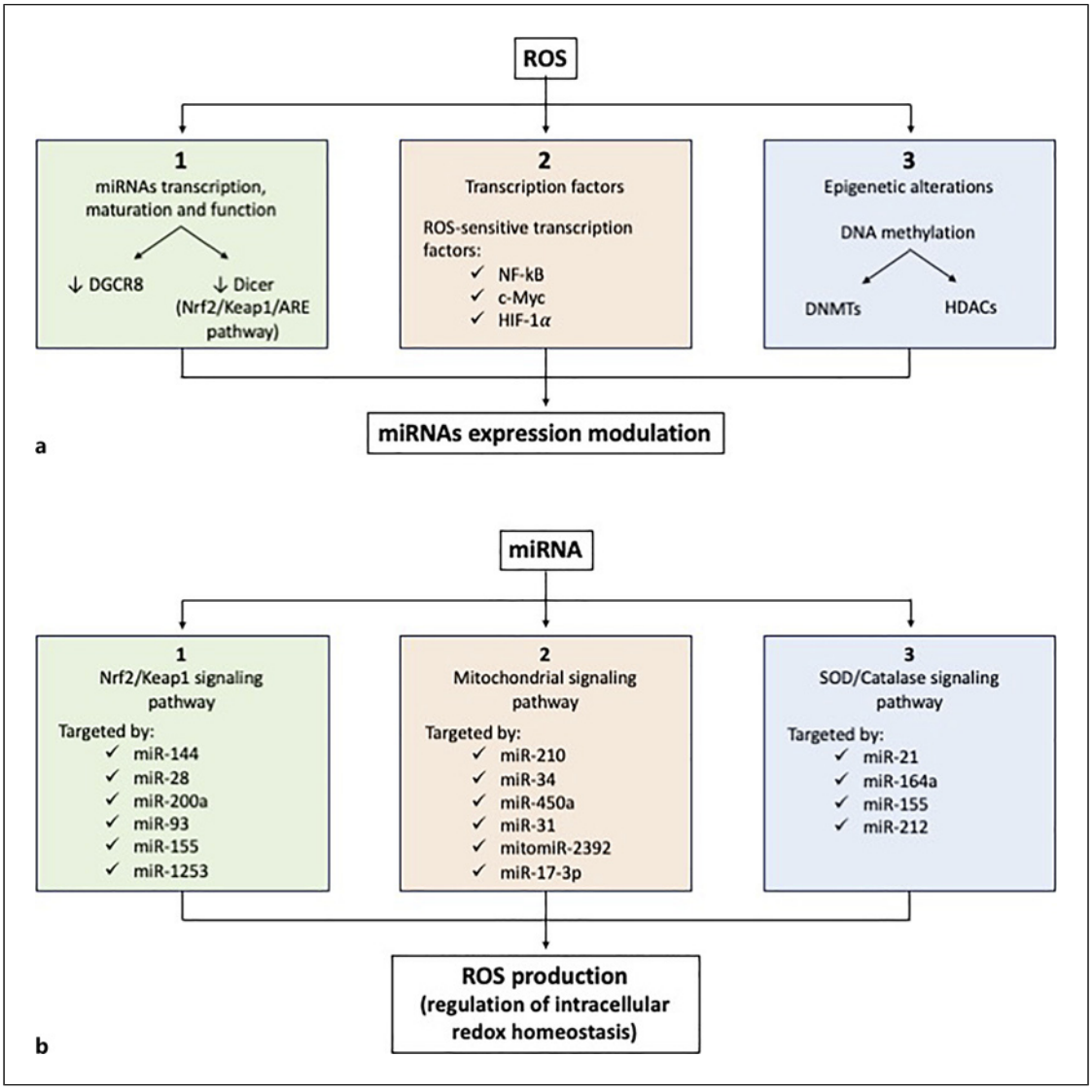

Fig. 1. The crosstalk of miRNAs and oxidative stress. **a** ROS regulates the expression and biogenesis of miRNAs through the control of proteins involved in post-transcriptional events, through the upregulation of transcription factors and through epigenetic alterations. **b** miRNAs modulate ROS production through regulating several redox signaling pathways, thus regulating intracellular redox homeostasis.

the nucleus, which enhances the expression and activity of several antioxidant genes that inhibit cell apoptosis and promote cancer cell survival and tumorigenesis [53, 54]. miRNAs regulate ROS homeostasis, modulating mitochondrial signaling pathways and regulating the activity and expression of several mitochondrial proteins, which are involved in the maintenance of redox homeostasis [47, 55]. Moreover, miRNAs can regulate oxidative stress through the superoxide dismutase (SOD)/catalase pathway. SOD, including Fe/Mn-SOD, Cu/Zn-SOD, and Ni-SOD family members, is metal-ion cofactor-requiring enzymes that promote specific biochemical reactions to remove accumulated ROS [56]. The majority of SOD family members are targets of specific miRNAs, among which miR-21 can suppress SOD2 and SOD3 by targeting TNFα generation, thus suppressing the dismutation of superoxide to the less damaging molecule of H_2O_2 [57–60].

The relationship between miRNAs and ROS has been studied, particularly in carcinogenesis (including gastrointestinal tract cancers, such as gastric, esophageal, and colorectal tumors). At the same time, the data regarding

this crosstalk in the pathogenesis of CeD are still poor [55]. miR-155, which has been demonstrated to be upregulated in CeD patients [16], has been implicated in the regulation of apoptosis and inflammation. In particular, in cardiovascular diseases and psoriasis, miR-155 can stimulate the production of Th17 lymphocytes, activate NK cells, and, when overexpressed, contribute to oxidative stress development [61, 62]. miR-146, another miRNA that is associated with the development of CeD, is involved in the regulation of apoptosis, autophagy, and inflammatory responses through the modulation of Treg, monocytes, and macrophages [62]. miR-21 is a candidate diagnostic biomarker in patients with CeD [35, 37, 63], and it is associated with oxidative stress and anti-ROS cellular responses [46, 47, 55]. miR-125b is also significantly associated with CeD [36]. It has a role in the modulation of an inflammatory response [61] and as a modulator of oxidative stress [64, 65]. Lastly, miR-451 is significantly downregulated in patients with CeD compared to healthy controls [30], and a recent preclinical study in mice demonstrated that the downregulation of this miRNA aggravates sepsis-induced oxidative injury of the lung epithelial cells, among other mechanisms, reducing the activities of catalase and glutathione peroxidase 1 [66]. Despite these data that indicate a potential and speculative involvement of several miRNAs in modulating response to oxidative damage, we lack studies specifically designed to investigate the crosstalk between miRNAs and oxidative stress.

miRNAs and Oxidative Stress: Results of a Preliminary Study

A total of 55 subjects were retrospectively included in a pilot study to evaluate the role of miRNAs and oxidative stress in adult CeD patients and to analyze their potential interactions. Eighteen CeD patients at the time of diagnostic upper GI endoscopy; 24 CeD asymptomatic patients after at least 1 year on a gluten-free diet; 3 patients with complicated CeD (2 females with enteropathy-associated T-cell lymphoma [EATL] and 1 female with RCD type 1); 10 control patients undergoing upper GI endoscopy for dyspepsia and/or gastro-esophageal reflux. In all these subjects, a blood sample and duodenal biopsies were collected. Based on a literature search, we selected several relevant miRNAs involved in the pathogenesis of CeD and with a known relationship with oxidative stress (miR-155, miR-200, miR-125, miR-192, miR-21, miR-451, miR-146, and miR-1226) which were quantified by qRT-PCR in plasma. Oxidative stress was evaluated by measuring the levels of 8-OHdG adduct in the DNA extracted from the duodenal tissue and peripheral blood using high-performance liquid chromatography with electrochemical detection (HPLC-EC).

Despite an evident trend towards higher levels for several miRNA in patients with CeD not on a GFD, probably due to small sample size and high biologic variability, no statistically significant difference in circulating levels among the evaluated miRNAs was demonstrated between groups (Fig. 2). On the other hand, we observed a particularly high level of miRNA-1226 in both 2 patients with EATL (22.3 and 27.1, respectively). Plasma and tissue levels of 8-OHdG did not differ among groups. No correlations were demonstrated between tissue 8-OHdG levels and miRNAs. However, in CeD-GFD blood, 8-OHdG levels showed a trend toward a statistically significant positive correlation with miR-200 ($r = 0.37$, $p = 0.07$) and a negative correlation with miR-1226 ($r = -0.38$, $p = 0.07$).

Discussion

Evidence is accumulating on the role of miRNAs, fine regulators of gene expression, in the pathogenesis of this disease. In particular, miRNAs seem to be involved in the activation of the inflammatory and immune pathways. However, available data are still poor and come from small studies which are hardly comparable (both for differences in miRNAs investigated and differences in methodology). Moreover, the majority of available studies have been conducted in pediatric populations of CeD patients, and it is not obvious that the information deriving from these studies can be transferred to adult patients considering, for instance, the different duration of damage at the intestinal level. Intriguingly, also oxidative stress could have a role in the pathogenesis of CeD, and it could be a marker of chronic inflammation and damage present in CeD intestine. An interplay between miRNAs and oxidative stress in the development of CeD has been postulated. However, to the best of our knowledge, no studies specifically directed at investigating the relationship between miRNAs and oxidative stress have been conducted, but only indirect evidence of this interplay has been derived by showing that several miRNAs can regulate the expression of genes involved in the response to oxidative stress. Our pilot study did not find any statistically significant differences in the levels of miRNAs and oxidative stress markers between the different groups evaluated, as well as no correlations between different miRNAs and 8-OHdG in all groups (except for a positive correlation with miR-200 and a

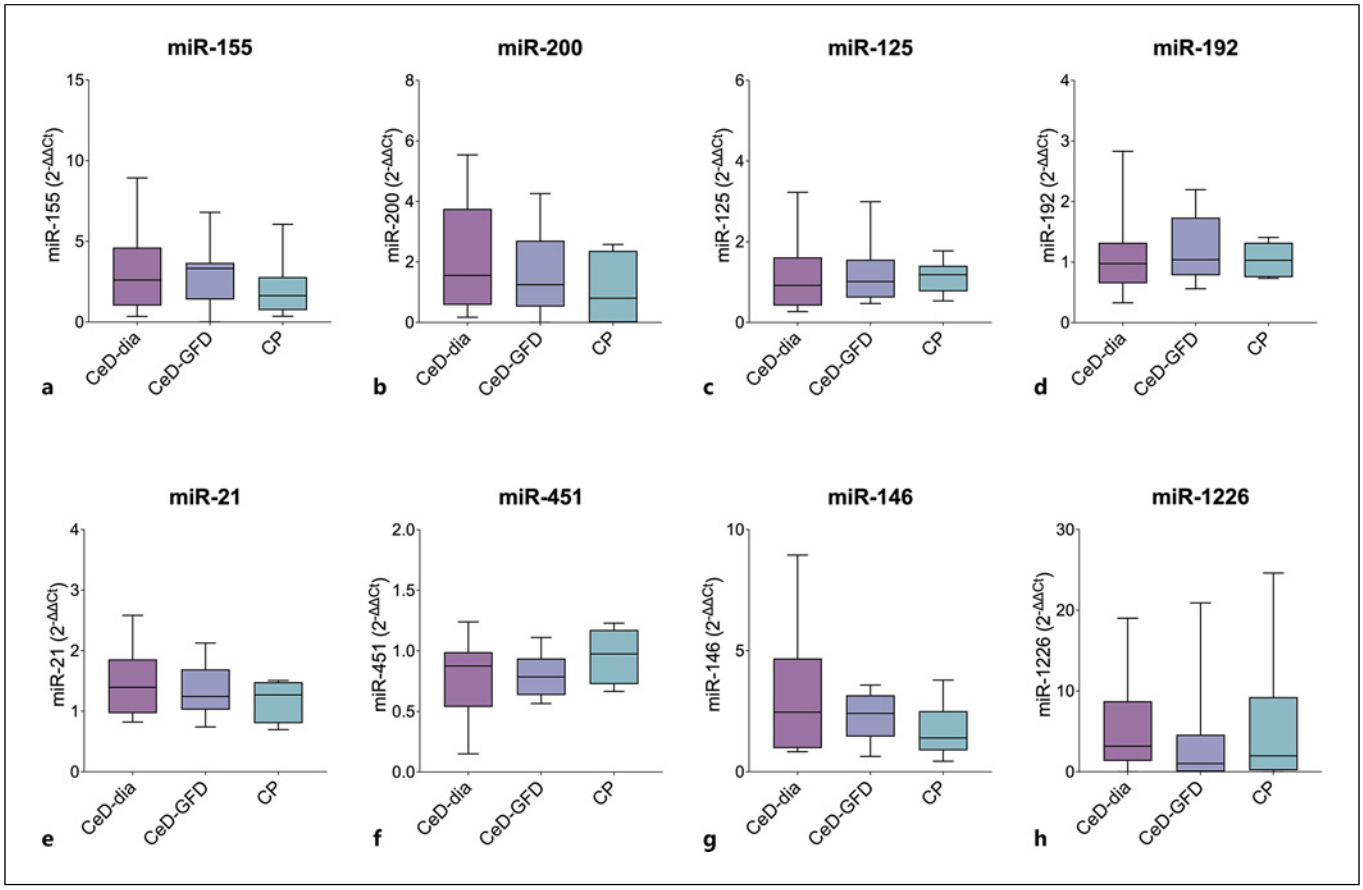

Fig. 2. miRNAs levels in CeD patients at diagnosis (CeD-dia), CeD patients after at least 1 year of GFD (CeD-GFD), and control patients (CP) (**a–h**).

negative correlation with miR-1226 in CeD-GFD as a trend toward significance). Interestingly, we found particularly high levels of miR-1226 in patients with complicated CeD, similarly to what has been found in other studies [38], but no conclusions can be drawn from our findings due to the very limited number of patients evaluated. In order to demonstrated the role of miRNAs and oxidative stress in the pathogenesis of CeD and that these two influence each other. Some points need to be considered: technical issues should be resolved (1); the methodology for miRNA evaluation should be standardized (2); among the many that can be measured, the right miRNAs, which play a role in CeD, must be identified, in order to obtain which, larger studies are necessary in order to find definitive conclusions. Once these challenges have been addressed and overcome, it will be possible to demonstrate whether and to what extent miRNAs and oxidative stress are interconnected in the pathogenesis of CeD, and possibly new therapeutic targets will be identified.

Conflict of Interest Statement

Matteo Fassan has a consulting or advisory role for Astellas Pharma, GlaxoSmithKline, Roche, MSD Oncology, Amgen, Lilly, Incyte, Novartis, AstraZeneca, and Pierre Fabre, and research funding for Astellas Pharma, QED Therapeutics, Macrophage Pharma, and Diaceutics. Fabiana Zingone has served as a speaker for Werfen, EG Stada Group, Fresenius Kabi, Kedrion, Janssen, Pfizer, Takeda, Unifarco, Malesci, and Galapagos and has served as a consultant for Galapagos and Takeda. Filippo Pelizzaro served as a consultant for EISAI and MSD. Romilda Cardin, Giulia Sarasini, Milena Minotto, Chiara Carlotto, Michela Palo, and Fabio Farinati have no conflicts of interest to declare.

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Author Contributions

Fabiana Zingone designed the research study and analyzed the data; Fabiana Zingone and Filippo Pelizzaro coordinated the research; Romilda Cardin, Giulia Sarasini, Milena Minotto, Chiara Carlotto, Matteo Fassan, Michela Palo, and Fabio Farinati collected the data; Filippo Pelizzaro wrote the first draft of the paper; Matteo Fassan and Romilda Cardin contributed to the design of the study; and Filippo Pelizzaro, Romilda Cardin, Giulia Sarasini, Milena Minotto, Chiara Carlotto, Matteo Fassan, Michela Palo, Fabio Farinati, and Fabiana Zingone revised and approved the final version of the article.

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