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Oxidative stress and cystic fibrosis-related diabetes: A pilot study in children

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Abstract

Background: Cystic fibrosis (CF) is characterized by chronic inflammation with increased oxidative stress. We evaluated the relationship between glucose tolerance and oxidative stress in CF children.
Methods: Patients 10–18 years old underwent oral glucose tolerance testing ($n=31$). At 2-h, we assessed blood glutathione and 4-hydroxynonenal-protein adducts (HNE-P), and urine 1,4-dihydroxynonane-mercapturic acid conjugate (DHN-MA). Plasma fatty acid (FA) profile was performed. Patients with impaired glucose tolerance (IGT) were retested 6 to 24 months later and received additional nutritional recommendations (NR) when possible.
Results: Fifty-two percent of patients had normal glucose tolerance (NGT), 42% IGT and 6% cystic fibrosis-related diabetes (CFRD). HNE-P concentrations significantly increased with diabetes (109%). Two-h BG correlated positively with HNE-P and negatively with DHN-MA. FA profile was modified with IGT. Of retested IGT patients, 25% received no NR; they remained IGT at 6 months and progressed to CFRD. Of those who received NR, 67% normalized, 11% remained intolerant and 22% developed CFRD. HNE-P levels dropped (88%) in IGT patients reverting to NGT, increased (94%) in the IGT patients with NR developing CFRD, decreased (90%) with persistent IGT.
Conclusion: CF children showed evidence of increased oxidative stress with worsening of glucose metabolism. NR may delay the appearance of CFRD.

Keywords: Cystic fibrosis; Diabetes; 4-hydroxynonenal; Protein modifications; Glucose tolerance; Lipid peroxidation

1. Introduction

Cystic fibrosis (CF), the most common lethal genetic disease in Caucasians [1], is caused by mutations of a gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). The most frequent mutation is the deletion of a phenylalanine residue at position 508 (ΔF508). The CFTR protein, a chloride channel, is also involved in homeostasis of other ions and metabolites such as glutathione [2]. In CF, the defective water and chloride transport leads to viscous secretions causing scarring and destruction of organs. This may result in exocrine and endocrine pancreatic insufficiency as well as cystic fibrosis-related diabetes

Abbreviations: AA, Arachidonic acid; A1c, Glycosylated haemoglobin; BG, Blood glucose; CF, Cystic fibrosis; CFRD, Cystic fibrosis-related diabetes; CFTR, Cystic fibrosis transmembrane conductance regulator; DHA, Docosahexaenoic acid; DHN-MA, 1,4-Dihydroxynonane-mercapturic acid; EFAD, Essential fatty acid deficiency; FA, Fatty Acid; FEF_{25-75}, Forced expiratory flow; FEV_1, Forced expiratory volume in one second; GSH, Reduced glutathione; GSSG, Oxidized glutathione; HNE, 4-Hydroxynonenal; HOMA, Homeostasic Model Assessment; IGT, Impaired glucose tolerance; OGTT, Oral glucose tolerance test; PA, *Pseudomonas aeruginosa*; PUFA, Polyunsaturated fatty acids; LA, Linoleic acid; NGT, Normal glucose tolerance; NR, Nutritional recommendations; ROS, Reactive oxygen species.

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(CFRD). The largest U.S. survey of CF patients revealed a prevalence of treatment-requiring diabetes of 17% in females and 12% in males aged ≥13 years [3].

The pathophysiology of abnormal glucose metabolism in CF patients is multifactorial. Endocrine pancreatic dysfunction may be due to impairment of β-cell function with progressive fibrosis of Langherans islets, resulting in distortion, ischemia, cell death, a decrease in number of islets and disturbances of other islet cell functions. Other contributory factors may include defects in insulinotropic gut hormone secretion and changes in insulin sensitivity and clearance rate [4–7]. Islet cell antibodies, the ΔF508 genotype and hepatic as well as peripheral insulin resistance may also be involved [8,9].

Oxidative stress has been implicated in the pathophysiology of several chronic diseases. CF is characterized by chronic inflammation with increased oxidative stress and glutathione imbalance [10,11], possibly by abnormal glutathione transport across the CFTR. The resulting glutathione deficiency has been associated with changes in other antioxidant levels, increased oxidative stress with hepatic and pancreatic damage, increased lipid peroxidation, and enhanced protein oxidation, denaturation and aggregation [12–15]. However, this systemic glutathione deficiency is not related to defects in glutathione synthesis nor in the transferase, redox or recycling systems [11].

Evaluation of oxidative stress requires accurate quantification of secondary products such as isoprostanes, aldehydes and oxidized proteins [16]. Furthermore, polyunsaturated fatty acids (PUFA) are major targets of free radical attacks leading to lipid peroxidation and production of several toxic metabolites [17]. Compared to isoprostanes, the longer half-life of aldehydes makes them good candidates for propagation and amplification of effects elicited by free radicals. Amongst aldehydes, the 4-hydroxy-2-alkenals, including 4-hydroxynonenal (HNE), a normal constituent of mammalian cell membranes, are considered the most reactive species. HNE reacts with thiol and amino residues of proteins, peptides, lipids and nucleic acids [18]. Accumulation of modified proteins, particularly HNE-protein adducts, depends on the degree of insult and can lead to increased production of reactive oxygen species (ROS). Growing evidence supporting HNE's role as a (patho)physiological modulator of signal transduction, transcriptional regulation and protein activities, targets HNE in the development and progression of diseases [19]. In diabetes, the production of reactive oxygen and nitrogen species appears to be a consequence of hyperglycemia, the effects of which are partly mediated through increased protein glycation and oxidation [20]. Molecular rearrangements occurring in such reactions result in the formation of more ROS [21], which can decrease antioxidant capacity and activate redox-sensitive transcription factors [22]. Moreover, the evaluation of *in vivo* HNE metabolism has been shown to be possible with the HNE urinary end-product, 1,4-dihydroxynonane-mercapturic acid adduct (DHN-MA) [23,24].

Abnormal fatty acid composition and metabolism have been associated with the development of CF. It has been estimated that an essential fatty acid deficiency (EFAD) occurs in up to 85% of CF patients [25], characterized by increased plasma levels of oleic (C18:1n-9) and palmitoleic (C16:1n-7) acids [26,27] and low levels of arachidonic (C20:4n-6; AA), linoleic (C18:2n-6; LA) and docosahexaenoic (C22:6n-3; DHA) acids [28]. In CF, EFAD is clinically more severe in infants and may occur even before the condition is diagnosed [29]. Although many factors related to fat malabsorption [30] or altered utilisation of fatty acids in cells [31] can explain the modification in the fatty acid profile, peroxidation of PUFA [32,33] seems to be more relevant.

Given that increased oxidative stress has been implicated in the progression of both CF and diabetes, this raises the possibility of its involvement in the development of CFRD. Our goals were as follows: 1) To evaluate glucose metabolism in CF patients from 2 pediatric centers, 2) To determine whether markers of oxidative stress, such as glutathione, HNE-P adducts and DHN-MA, are correlated to and possibly predictive of glucose metabolism in CF patients, and 3) To evaluate whether plasma FA profiles are related to markers of oxidative stress or glucose tolerance in CF.

2. Methods

2.1. Patients

Patients were recruited through the pediatric CF clinics at the CHU Sainte-Justine and the Montreal Children's Hospital. Informed consent was obtained after institutional review board approval. Inclusion criteria were a documented diagnosis of CF, age >10 years, previously normal glucose metabolism and follow-up by a collaborating physician. Patients taking medications known to interfere with glucose metabolism and those with psychological problems, acute illness or other ongoing medical problems were excluded. During the recruitment period (2 months), 2 patients attending the clinics refused to participate. Two patients had CFRD and a comparable number of patients for normal and impaired glucose intolerance were included: 16 and 13 for NGT and IGT respectively, for a total of 31 patients.

2.2. Oral glucose tolerance test (OGTT)

After an overnight fast, patients underwent oral glucose tolerance testing using 1.75 g/kg (maximum 75 g) of dextrose solution. Blood was taken at time 0 (fasting) for evaluation of glucose, insulin and A1c, and at 2 h for glucose, insulin and oxidative stress markers. Glucose tolerance was classified according to the Canadian Diabetes Association guidelines [34]. Patients with CFRD were referred to an endocrinologist. At the CHU Sainte-Justine, IGT patients received additional nutritional recommendations (NR) from a dietician to avoid simple concentrated sugars and high glycemic index foods. All IGT patients underwent repeat OGTT after 6 to 24 months.

2.3. Pulmonary function

Pulmonary function was assessed using forced expiratory volume in one second (FEV_1) and forced expiratory flow$_{25–75}$

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($FEF_{25–75}$) and was expressed as percent predicted FEV_1 and percent predicted $FEF_{25–75}$.

2.4. Pseudomonas aeruginosa

Sputum cultures were performed to identify *Pseudomonas aeruginosa* (PA) colonization at the time of the OGTT.

2.5. Analyses of oxidative stress

To avoid measurement bias, 2-h samples were coded and glucose tolerance status was revealed after analyses were completed.

2.5.1. Glutathione assay

Oxidized glutathione (GSSG) and total glutathione (GSH+GSSG) measurements were assessed by a spectrophotometric method using vinylpyridine as described [35] in aliquots (500 µl) of whole blood (heparinized tubes). Reduced glutathione (GSH) concentration was calculated as the difference between total and oxidized glutathione.

2.5.2. HNE-protein adducts assay

Chemicals and organic solvents were supplied by Laboratoire Mat (Montreal, QC, Canada), Sigma Chemical Co. (St. Louis, MO, USA) and J.T. Baker (Phillipsburg, NJ, USA). Biomol (Plymouth Meeting, PA, USA) provided unlabeled HNE while Regis Chemical (Morton Grove, IL, USA) supplied N-methyl-N-(*t*-butyldimethylsilyl)-trifluoroacetamide. NaB^2H_4 and trans-4-hydroxy-2-nonenal-[5,5,6,6,7,7,8,8,9,9,9-$^2H_{11}$]diethyl acetal were purchased from Cambridge Isotope Laboratories (Andover, MA, USA) and CDN Isotopes (Pointe-Claire, QC, Canada), respectively. Quantification of HNE bound to proteins in whole blood samples (400 µl, EDTA tubes) [36] was assessed as follows: After addition of 200 µl of NaB^2H_4 (1 M) reducing HNE to its corresponding alcohol chemically stable [^{2}H]DHN, proteins were precipitated with saturated sulfosalicylic acid (200 µl). The protein pellet was extracted twice with mixed methanol:chloroform (3 ml; 2:1, vol:vol), rinsed thrice with water (1 ml) before resuspension in guanidine buffer (0.5 ml; pH 7.2) and addition of internal standard [$^2H_{11}$]1,4-dihydroxynonene (DHN; 0.1 nmol). After treatment with Raney Nickel, extraction and evaporation, the residue was treated with N-methyl-N-(*t*-butyldimethylsilyl)-trifluoroacetamide (75 µl), heated (90 °C, 3 h) and left overnight (70 °C) for derivatization.

Two µl aliquots were injected into a Hewlett Packard 6890 Series GC System version A.02.14 (Hewlett Packard, Palo Alto, CA, USA) equipped with HP-5 capillary column (50 m × 0.2 mm × 0.33 µm) coupled to a Mass Spectrometer (Agilent Technologies Mass Selective Detector 5973 Network) operated in ammonia positive chemical ionization mode. Two ion sets were measured: *m/z* 389, 390 and 400, corresponding to the molecular ion and 257, 258 and 268, resulting from fragmentation, for the analyses of DHN, [^{2}H]DHN and internal standard [$^2H_{11}$]DHN respectively. The MS source and quadrupole were set at 180 °C–126 °C and 300 °C–176 °C for the 2 ion sets respectively. The chromatograph temperature program was: 170 °C for 1 min, increased by 10 °C/min up to 210 °C, increased by 5 °C/min up to 280 °C and by 20 °C/min up to 320 °C for 10 min. Levels of HNE-proteins were calculated over a minimum of duplicate injections.

2.5.3. DHN-MA assay

Levels of the conjugate, dihydroxynonane-mercapturic acid, were determined in aliquots (400 µL) of 2h-urine samples when available by enzyme immunoassay using polyclonal antibodies as described by Gueraud et al. [37]. Concentrations were calculated and expressed relative to the creatinine levels assayed in the same urine samples.

2.6. Fatty acid analysis

Fasting free fatty acids were extracted from plasma following the method previously described by Lepage et al. [38] and analyzed by gas chromatography using nonadecanoic acid (C19:1) as internal standard.

2.7. Calculations

BMI *z*-scores were calculated using the *Epi Info* software from the Centers for Disease Control and Prevention (www.cdc.gov/epiinfo/).

Peripheral insulin resistance was evaluated using the Homeostasic Model Assessment [39] (HOMA) where HOMA = ([fasting BG (mM)] × [fasting insulin (pM)])/22.5.

Percentage of functional β-cells was estimated as calculated by Tofe et al.:

β-cell (%) = [fasting insulin (pM) × 3.33]/[fasting BG (mM) — 3.35] [40].

Mean Plasma Glucose was estimated using the equation validated by Brennan et al.:

MPG = (1.47 × A1c) — 1.15 [41].

2.8. Statistical analyses

Data are expressed as means ± SE. Statistical differences were assessed by factorial ANOVA, unpaired or paired Student's *t*-test after verification of the normal value distribution of the populations. When variances were significantly different (*F* test), Welch's correction was used. Correlation analyses were performed with determination of the Pearson correlation coefficient. Statistical significance was considered when $p < 0.05$.

Note that theoretically, taking into account all the patients (tested once and re-rested) should give a total of 47 sets of data with 22 NGT, 18 IGT and 7 CFRD. However, for several parameters, the data set was not always complete because of missing samples (not available; i.e. pulmonary function, genotype).

3. Results

3.1. Metabolic parameters and pulmonary function

For the 31 patients (18 males), initial tests showed 16 (52%) normal, 13 (42%) intolerant and 2 (6%) diabetic. Of the IGT

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patients, one was transferred to adult care and the remaining 12 were retested 6 to 24 months later. Twenty-two patients classified as NGT, 13 as IGT and 7 as CFRD at some point during the study. We have analysed our data in different ways: (i) taking into consideration the patients only tested once in comparison with those who have been retested and who changed in terms of their glucose tolerance over time; (ii) taking into account the evolution of patients with IGT (longitudinal approach: 13 IGT but only 12 who have been retested, of whom 6 became NGT, 5 became CFRD and 1 remained IGT), (iii) taking into account only the patients who have been tested once (16 NGT, 13 IGT and 2CFRD) in comparison with data obtained from all tests performed. For all the parameters considered in this study, there was no evidence of statistical difference between patients tested once and those who changed their glucose tolerance. Therefore, data are presented and analysed taking into account all OGTT performed. Table 1 describes the patients' demographic, genetic and anthropometric data according to glucose tolerance taking into account the patients only tested once and all tests performed. Average age of the whole population was 14.0±0.2 years. There was no significant difference when taking into account only the patients tested once *vs* including data from IGT patients who have been retested. No significant difference was observed for age or BMI *z*-score between females and males. Most patients were homozygous for the ΔF508 mutation. BMI *z*-score tended to be lower in the intolerant and diabetic groups but without statistical significance. Among the 12 retested IGT patients, 6 (50%) reverted to NGT, 1 (8%) remained IGT and 5 (42%) progressed to CFRD.

Table 1 also presents OGTT results, A1c levels and estimation of insulin resistance and functional β-cell mass. Fasting BG values were comparable across all groups. The groups differed significantly by their 2-h BG values ($p<0.001$). Fasting insulin levels were similar among the groups. The 2-h insulin levels tended to be higher in intolerant and diabetic groups compared to the NGT group and did reach significance ($p<0.05$) in the diabetic group. A1c trended upwards from normal to intolerant to diabetic status without statistical significance. A similar trend was observed for the estimate of insulin resistance with glucose tolerance but did not reach significance nor did the functional β-cell mass (15 and 23% decrease with IGT and CFRD respectively), suggesting no significant β-cell loss. A recent study has shown a strong correlation between A1c and mean plasma glucose in CFRD [41]. Using the authors' equation, we observed a significant difference in mean plasma glucose between NGT and IGT patients when considering only the patients tested once: 6.78±0.10 ($n=12$), 7.15±0.12 ($n=13$) and 7.52 ($n=2$) mM for NGT, IGT and CFRD patients respectively ($p<0.05$ using unpaired *t*-test) but also between

Table 1
Demographic, genetic and anthropometric data and evaluation of glucose metabolism and pancreatic function

	NGT	IGT	CFRD
M/F	9/7–**13/9**	9/4–**9/4**	0/2–**4/3**
Total (%)	16 (52)–**22 (52)**	13 (42)–**13 (31)**	2 (6)–**7 (17)**
Age (years; *n*)	13.9±0.6 (16)	14.0±0.5 (13)	13.5 (2)
	14.0±0.5 (22)	**14.0±0.5 (13)**	**14.4±0.8 (7)**
Mutations ΔF508 (%, *n*)			
Homozygous	50.0 (7)–**52.6 (10)**	55.6 (5)–**55.6 (5)**	– **–100 (2)**
Heterozygous	42.9 (6)–**36.8 (7)**	33.3 (3)–**33.3 (3)**	–
Other mutations	7.1 (1)–**10.5 (2)**	11.1 (1)–**11.1 (1)**	–
Not genotyped	12.5 (2)–**13.6 (3)**	30.8 (4)–**30.8 (4)**	100 (2)–**71.4 (5)**
BMI *z*-score	−0.35±0.17 (16)($p=0.49$ *vs* all)	−0.17±0.28 (13)($p=0.46$ *vs* all)	−0.63 (2)
	−0.18±0.16 (22)	**−0.47±0.28 (16)**	**−0.57±0.39 (7)**
Glucose (mM, *n*)			
0-h	5.0±0.1 (16)	4.9±0.1 (13)	5.1 (2)
	5.0±0.1 (22)	**4.9±0.1 (18)**	**4.9±0.2 (7)**
2-h	6.0±0.2 (16)	9.2±0.2 (13)[d]	12.4 (2)
	5.9±0.2 (22)	**9.3±0.2 (18)[b]**	**12.5±0.5 (7)[bc]**
Insulin (pM, *n*)			
0-h	52±7 (15)	53±11 (11)	31 (2)
	53±6 (21)	**47±8 (18)**	**41±7 (7)**
2-h	286±60 (16)	479±89 (13)($p=0.07$ *vs* NGT)	312 (2)
	298±54 (21)	**412±69 (18)**	**476±143 (7)[a]**
A1c (%, *n*)	5.47±0.10 (13)	5.65±0.08 (13)	5.90 (2)
	5.54±0.08 (18)	**5.65±0.08 (17)**	**5.86±0.10 (7)**
Pancreatic function			
HOMA (*n*)	11.6±1.6 (15)	12.1±2.5 (11)	7.8 (2)
	11.9±1.3 (20)	**10.6±1.8 (16)**	**9.2±1.8 (7)**
β-cell (%, *n*)	122±18 (15)	116±20 (11)	68 (2)
	127±16 (20)	**109±14 (16)**	**98±13 (7)**

Data are means±SE; *n*=number of tests performed. The percentages of ΔF508 mutation were calculated from the genotyped patients. Insulin resistance was evaluated using the HOMA model. NGT: Normal Glucose Tolerance; IGT: Impaired Glucose Tolerance; CFRD: Cystic fibrosis-related diabetes. Data in bold are obtained from all OGTT performed whereas the others are obtained from the patients tested only once (no re-testing of IGT patients). [a]$p<0.05$; [b]$p<0.001$ *vs* NGT; [c]$p<0.001$ *vs* IGT using factorial ANOVA; [d]$p<0.001$ *vs* NGT using Student unpaired *t*-test.

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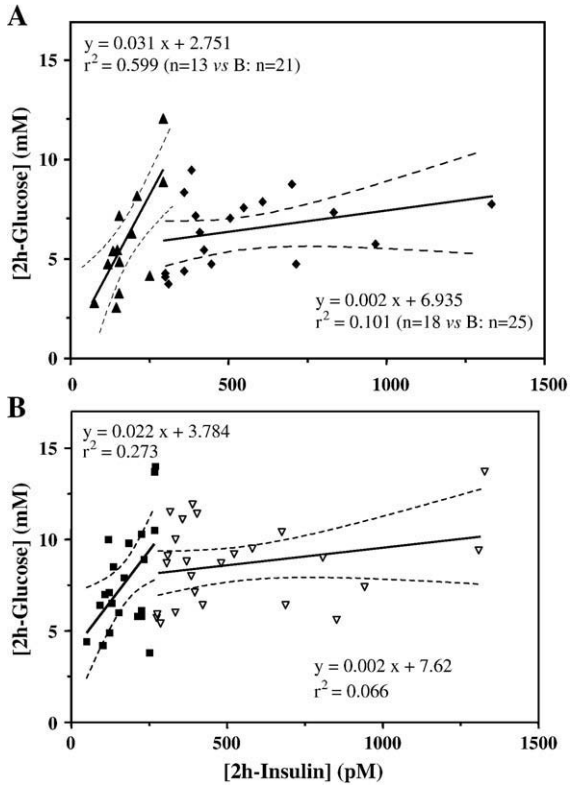

Fig. 1. Relationship between plasma 2h-insulin and glucose. Plasma 2h-insulin concentrations were plotted in relation to plasma 2h-glucose levels. A: using the data of the patients tested once and B: all OGTT performed. In both cases, the analysis of the insulin-glucose correlation (r^2=0.10 and 0.09 for A and B respectively, $p<0.05$) revealed two subgroups of patients: one with a significant correlation (insulin ≤270 pM; r^2=0.60 and 0.27; p=0.002 and 0.015 for A and B respectively) and one without any significance (insulin >270 pM; r^2=0.10 and 0.07; p=0.20 and 0.21 for A and B respectively). The dotted lines represent the 95% confidence interval.

NGT and CFRD patients when considering all OGTT performed: 6.96±0.11 (n=18), 7.15±0.12 (n=17) and 7.46±0.14 mM (n=7) for NGT, IGT and CFRD patients respectively ($p<0.05$ using factorial ANOVA).

A close analysis of the positive correlation between 2-h insulin and glucose levels (r^2=0.09, $p<0.05$, not shown) outlined 2 subgroups of patients. In the first subgroup there was a strong positive correlation between 2-h insulin and BG concentrations (insulin level ranging from 49 to 270 pM; r^2=0.60 and r^2=0.27, $p<0.05$, for taking into account only the patients tested once or the whole population, respectively), whereas this correlation disappeared in the second subgroup (insulin level >270 pM; r^2=0.10 or 0.07, ns, for taking into account only the patients tested once or the whole population, respectively). Fig. 1 shows the relationship between 2-h insulin and 2-h BG concentrations. At 2 h after the glucose challenge, insulin and BG increased until glucose reached 7.7 mM on average. Then insulin levels rose further to maintain the same glucose concentrations. At that point, the linear relationship between insulin and glucose levels disappeared; more insulin had to be secreted to maintain similar BG values. Furthermore, insulin levels were significantly higher in females: at baseline: 60±9 (n=15) *vs* 40±3 pM (n=28, $p<0.05$ using unpaired t-test) and at 2-h: 529±87 (n=15) *vs* 293±41 pM (n=31) for females and males respectively ($p<0.01$ using unpaired t-test, n=number of tests for all OGTT performed). Consequently, the evaluation of insulin resistance using the HOMA model was significantly higher in females compared to males: 14±2 (n=15) *vs* 9±1 (n=28; $p<0.05$) as well as the evaluation of functional β-cell mass (135±18 (n=15) *vs* 98±8 (n=28) % for females and males respectively; $p<0.05$, n=number of tests for all OGTT performed). Females also tended to have higher BMI z-score: −0.05±0.24 (n=15) and −0.39±0.17 (n=28) for females and males respectively.

Table 2
Pulmonary function in relation to glucose tolerance and gender

	Predicted FEV_1 (%)	Predicted FEF_{25-75} (%)
Glucose tolerance		
NGT (n)	89.3±3.7 (22)	69.5±6.2 (22)
IGT (n)	83.6±4.5 (16)	63.0±7.4 (16)
CFRD (n)	81.5±8.6 (6)	63.4±12.0 (6)
Gender		
Males (n)	90.0±3.2 (30)	72.3±5.0 (30)
Females (n)	79.6±4.4 (15)	55.8±7.4 (15)

Data are means±SE; n=number of tests performed. Pulmonary function is expressed as percentage predicted forced expiratory volume in one second: FEV_1 and forced expiratory $flow_{25-75}$: FEF_{25-75}. NGT: Normal Glucose Tolerance; IGT: Impaired Glucose Tolerance; CFRD: Cystic fibrosis-related diabetes.

Table 3
Blood glutathione levels in relation to glucose tolerance and gender

	Total glutathione (μM)	GSSG/total	GSSG/GSH
Glucose tolerance			
NGT (n)	637±36 (19)	4.7±0.9 (16)	5.0±1.0 (16)
IGT (n)	647±52 (12)	7.0±1.7 (12)	7.9±2.1 (12)
CFRD (n)	795±230 (4)	4.2±2.1 (3)	4.4±2.2 (3)
Gender			
Males (n)	585±25 (20)	6.4±1.2 (18)	7.2±1.5 (18)
Females (n)	740±74 (14)*	4.2±0.9 (13)	4.5±1.0 (13)

Data are means±SE; n=number of tests performed. Whole blood glutathione levels (oxidized glutathione (GSSG), total glutathione) were measured and reduced glutathione (GSH) calculated. NGT: Normal Glucose Tolerance; IGT: Impaired Glucose Tolerance; CFRD: Cystic fibrosis-related diabetes. *$p<0.05$ *vs* males using unpaired t-test.

Percent predicted FEV_1 and FEF_{25-75} were slightly diminished with IGT and CFRD (8.9%; Table 2). Females tended to have lower % predicted FEV_1 (12% decrease compared to males; p=0.060 using unpaired t-test) and % predicted FEF_{25-75} (23% decrease compared to males; p=0.08 using unpaired t-test).

3.2. Lung inflammation and infection

In order to evaluate the status of patients for lung inflammation and infection, colonization by PA in sputum cultures was tested. When considering only the patients tested once, 9 of the 16 NGT patients were PA positive (56%), 1 was identified as scanty and 6 (37%) were PA negative; of the 13 IGT patients, 8 tested positive (62%) and 5 negative (38%) whereas 1 of CFRD patients was identified PA positive and 1 scanty. Of the 12 retested IGT patients, 3 of the 6 patients who

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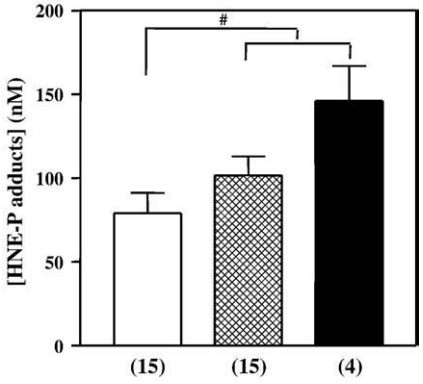

Fig. 2. Concentrations of blood HNE-protein adducts with glucose tolerance. HNE-protein levels were measured in whole blood of CF patients with normal (NGT, white bars), impaired (IGT, hatched bars) and diabetic (CFRD, black bars) glucose tolerance. Results are expressed as means±SE (n=number of tests) when available at 2-h. Data were analyzed by ANOVA after a natural logarithm transformation to meet homoscedasticity (Bartlett's chi squared) and showed a significant difference: #: $p<0.05$ *vs* NGT.

had their glucose tolerance reverted to normal were PA negative and 2 remained PA positive (no data for 1 patient) whereas 5 of the patients who became CFRD were PA positive. Therefore, taking into consideration all the data, there is a trend for an increase in PA positivity from NGT to CFRD: 52% (11 positive, 9 negative, 1 scanty and 1 NA), 72% (13 positive, 5 negative) and 86% (6 positive, 1 scanty) and for the NGT, IGT and CFRD groups respectively. There is no evidence of difference between males and females for colonization by PA (data not shown).

3.3. Oxidative stress markers

3.3.1. Glutathione assay

Total, reduced and oxidized glutathione levels were not significantly different between groups (Table 3). The analyses of glutathione according to gender, regardless of glucose tolerance, revealed a trend for a more reduced environment in females: redox ratio=2 GSSG/(GSH+2GSSG) (9.7±2.1 (n=13) *vs* 13.8±3.0 (n=18)) with a significant increase in total glutathione levels: GSH+GSSG (Table 3) and GSH levels: 740±74 (n=14) *vs* 585±25 μM (n=20) for females and males respectively ($p<0.05$, n=number of measurements for all OGTT performed).

3.3.2. HNE-Protein adducts assay

HNE-P adduct levels increased significantly with abnormal glucose tolerance (Fig. 2, 45% increase with IGT and 109% with

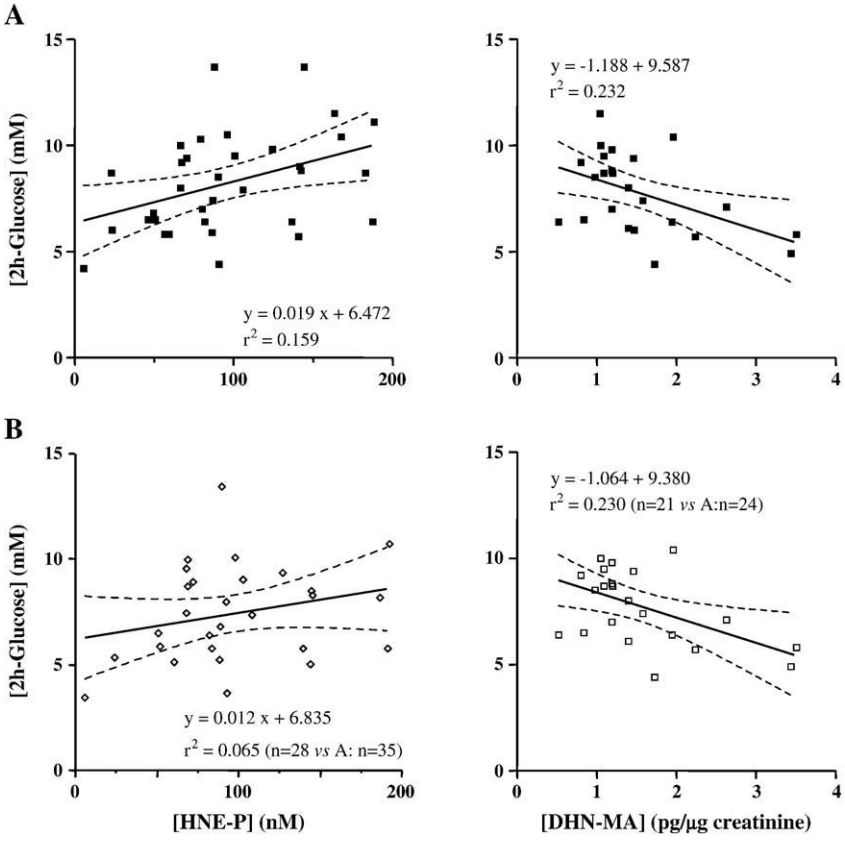

Fig. 3. 2h-blood glucose and levels of blood HNE-protein adducts and urine DHN-MA conjugates. A: Taking into consideration all OGTT performed, the positive correlation (r^2=0.16, $p<0.05$, n=35 tests performed) between the 2h-BG and the levels of HNE-protein adducts suggests that 16% of the increase in glucose levels is associated with oxidative stress. The analysis of the urine concentration of the conjugate DHN-MA with the 2h-BG revealed a negative correlation (r^2=0.23, $p<0.05$, n=24 samples measured). The dotted lines represent the 95% confidence interval. B: When taking into account the data obtained only from the patients tested once, the correlation between blood HNE-P and 2h-BG did not reach significance (p=0.19) whereas it was very similar to what observed with the whole population for urinary DHN-MA (r^2=0.23, p=0.028).

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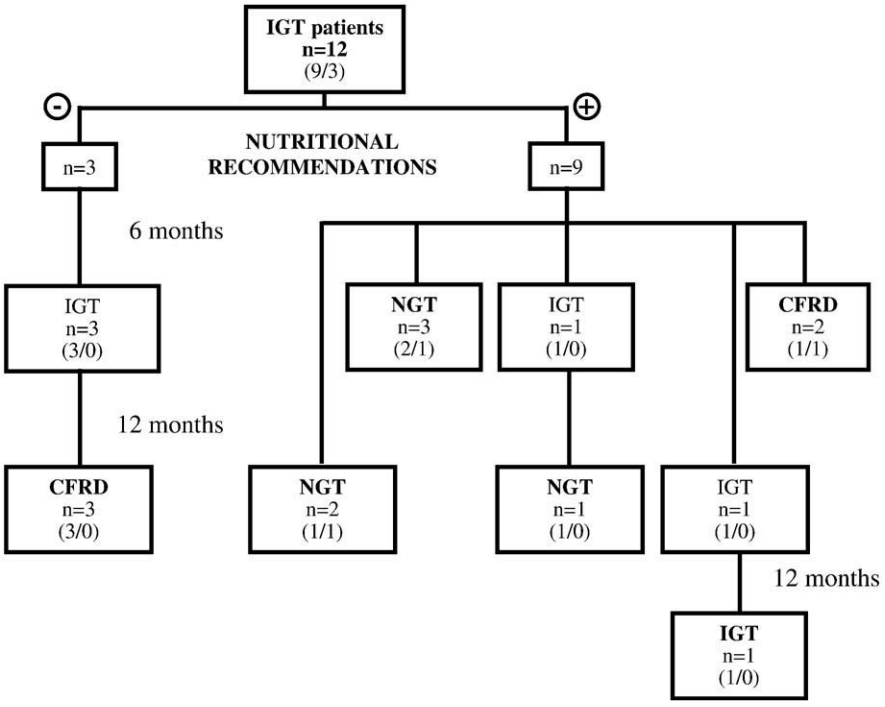

Fig. 4. Outcome of patients with Impaired Glucose Tolerance. IGT patients were retested 6 to 12 months later. The numbers in brackets indicate the male/female ratio.

CFRD; $p<0.05$ using factorial ANOVA). Results were similar if concentrations were expressed in pmol/mg protein (results not shown). HNE-P levels correlated positively with 2h BG concentrations ($r^2=0.16$, $p<0.05$, Fig. 3A), attributing 16% of the increase in glucose levels in CF patients to increased oxidative stress. There was no correlation between HNE-P levels and glutathione (reduced, oxidized or ratios). No significant change was observed between male and female levels of blood HNE-protein levels (97.6±15.8 ($n=13$) *vs* 88.2±10.4 nM ($n=23$) for females and males respectively; n=number of tests performed).

3.3.3. DHN-MA assay

The HNE fraction bound to GSH was estimated with its urinary end-product concentration, DHN-MA, measured in urine samples of some patients (14 NGT, 11 IGT and 1 CFRD). Urine concentrations of DHN-MA conjugate were significantly decreased in IGT patients compared to NGT: 1.22±0.09 ($n=11$) *vs* 1.83±0.23 ($n=14$) pg/μg creatinine ($p<0.05$ using unpaired *t*-test). These levels were not associated with measured blood concentrations of HNE-P adducts but negatively correlated with 2h-BG concentrations ($r^2=0.23$, $p<0.05$, Fig. 3A).

Of the 12 IGT patients retested, 3 did not receive NR (Fig. 4). These patients remained IGT at 6 months and progressed to CFRD at 12 months. Of the 9 patients who underwent NR, 2 progressed to CFRD (22%), 1 remained intolerant (22%) and 6 normalized (67%). Table 4 presents the HNE-protein levels in some patients with NR. Three of the 6 IGT patients reverting to NGT had their HNE-protein adduct levels repeated, showing a significant drop (88%) once glucose metabolism normalized ($p<0.05$, paired *t*-test), while levels increased (94%) in the 2 IGT patients with NR progressing to CFRD. The patient who received NR and remained intolerant had an initial drop of HNE-protein level of 84% and then 44% compared to the original measurement. These observations were unaccompanied by changes in BMI *z*-score, pulmonary function and glutathione levels (data not shown).

3.4. Fatty acid profiles

Of the 31 patients enrolled in the study, samples from only 12 patients (8 NGT and 4 IGT) were available for determination of plasma fatty acid profile. None of the IGT patients were re-analyzed when retested for glucose tolerance. In general, total fatty acid levels were 26% lower with IGT compared to NGT. As presented in Table 5, saturated and monounsaturated fatty acids were decreased in patients with impaired glucose tolerance

Table 4
Blood levels of HNE-protein adducts with changes in glucose tolerance following nutritional recommendations

Patient	HNE-protein adducts (nM)	
	IGT	NGT
#1	105.9	1.9
#2	66.7	2.5
#3	182.8	56.7
	IGT	CFRD
#4	66.7	163.5
#5	100.6	144.5
	IGT	IGT
#6	142.4	23.1
		79.1

Whole blood HNE-protein levels (nM) were measured in some IGT patients who received NR and repeated when they were retested for glucose tolerance.

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Table 5
Fatty acid profiles in CF patients with normal and impaired glucose tolerance

Fatty acids (*n*; male:female)	NGT (8; 4:4)	IGT (4; 3:1)
Saturated (SFA; mM) (% variation vs NGT)		
C16:0	3.2±0.5	2.1±0.2 (33%)
C18:0	0.59±0.04	0.49±0.04 (17%)
Sum	4.3±0.6	2.9±0.3 (32%)
Monounsaturated (MUFA; mM) (% variation vs NGT)		
C16:1n-7	0.44±0.08	0.28±0.06 (36%)
C18:1n-9	2.1±0.2	1.4±0.1 (31%)
SUM	2.9±0.3	2.0±0.2* (31%)
Polyunsaturated (PUFA) (% variation vs NGT)		
n-6 (mM)		
C18:2n-6	2.2±0.2	2.0±0.2 (10%)
C20:4n-6	0.49±0.03	0.42±0.04 (14%)
n-3 (μM)		
C18:3n-3	61.6±14.0	46.5±6.5 (25%)
C20:5n-3	55.3±10.2	41.3±8.8 (25%)
C22:6n-3	94.5±19.8	43.0±6.9* (55%)
n-9 (μM)		
C20:3n-9	20.4±3.7	14.6±5.5 (29%)
SUM (mM)	3.2±0.3	2.8±0.2 (13%)
Total (SFA-MUFA-PUFA; mM)	10.4±1.1	7.7±0.5 (26%)
Ratio (% variation vs NGT)		
∑n-3/∑n-6	0.080±0.009	0.061±0.005 (25%)
C22:6n-3/Total	0.009±0.002	0.006±0.001 (35%)
C20:3n-9/C20:4n-6	0.043±0.009	0.034±0.013 (21%)

Plasma fatty acid profile was determined in some patients with normal (NGT) and impaired (IGT) glucose tolerance. Data are means±SE; *n*=number of patients analyzed. *$p<0.05$ *vs* NGT using unpaired *t*-test.

compared to those with normal tolerance but the difference reached significance only in the concentration of monounsaturated fatty acids (sum of saturated+monounsaturated fatty acids: 32% decreased; $p=0.055$ using unpaired *t*-test). Compared to NGT patients, levels of n-6 fatty acids as LA (C18:2n-6) and AA (C20:4n-6) as well as n-3 fatty acids were found to be decreased in IGT patients. Nevertheless, the significance was observed for DHA only (C22:6n-3; $p<0.05$). EFAD has been reported in CF patients with pancreatic insufficiency. Its evaluation through the ratio (C20:3n-9)/(C20:4n-6) showed no deficiency and no significant difference between the two groups.

The levels of n-3 (C18:3n-3, C20:5n-3, C22:6n-3), n-6 (C18:2n-6) and total polyunsaturated fatty acids were significantly increased in females ($n=5$) compared to males ($n=7$) (104, 28 and 32% for n-3, n-6 and PUFA respectively; $p<0.05$, unpaired *t*-test). Furthermore, the ratio n-3/n-6 was significantly higher in females compared to males (59%, 0.094±0.001 ($n=5$) *vs* 0.059±0.003 ($n=7$) for females and males respectively; $p<0.05$). No gender difference was observed for EFAD.

4. Discussion

This study is the first involving a cohort of pediatric CF patients investigating glucose metabolism and oxidative stress. The ΔF508 mutation was the most common genotype in our population as previously published [42]. We were unable to draw conclusions regarding genotype and risk for abnormal glucose metabolism given the small number of IGT and CFRD patients genotyped.

Studies have shown that deterioration in pulmonary function, weight loss, decreased BMI and increased need for pancreatic enzymes precede CFRD [43]. However, a large Canadian study (5.2% CFRD) showed no change in pulmonary function or survival 5 years before and after diagnosis [44]. Our patients with CFRD tended to have diminished pulmonary function and lower BMI *z*-scores. However, these trends were subtle. This may be explained by early diagnosis of abnormal glucose metabolism in our cohort.

Normal fasting BG levels do not predict glucose tolerance in CF patients (Table 1). This underscores the importance of routine oral glucose tolerance testing in this population. A1c, though trending upwards in IGT and CFRD groups, was not significantly different and is not indicative of glucose metabolism in CF as previously published [45]. However, the evaluation of the mean plasma glucose using A1c values [41] showed a significant difference between NGT and CFRD patients. A1c may therefore be a useful tool for this estimation in the follow-up of CF patients. This finding remains to be confirmed in a larger cohort of pediatric patients.

Impaired insulin secretion has been demonstrated in CF and may be secondary to β-cell dysfunction [46]. Reports of reduced and increased insulin secretion in CFRD imply that both insulinopenia and insulin resistance may be involved in abnormal glucose metabolism [9,47]. We found no frank insulinopenia in children with IGT or CFRD. In our patients, 2-h insulin correlated positively with 2-h BG levels. Insulin levels increased with BG up to a plateau after which significant levels of insulin were required to maintain glucose concentration, suggesting a component of insulin resistance (Fig. 1). Evaluation of insulin resistance did not reveal any change but the HOMA model does not take into account area under the curves of insulin secretion and/or BG. CF patients may also present a decrease and/or delay in peak insulin secretion [48]. Estimation of functional β-cell mass was not significantly decreased (15%) in patients with CFRD. This may be explained by the calculation method which relied solely on fasting insulin and glucose levels. However, these data are not compared to those found in a healthy pediatric population. Similar observations have been recently reported on glucose tolerance and insulin secretion in children [49].

Insulin levels in females were significantly higher. Studies have suggested that females are intrinsically more insulin resistant, possibly because of specific sex-linked gene expression and differences in metabolic controls, such as signaling pathways, substrate shuttling and receptor function [50]. This is confirmed by our observation with a significant higher average value of the HOMA for females when compared to males. Furthermore, insulin resistance increases with higher BMI *z*-score, and percentage of body fat is greater in females [51,52].

Our female patients had a 23% decrease in % predicted FEF_{25-75} compared to males regardless of glucose status. Reduced pulmonary function in CF female adults has been reported [53], possibly due to reduced energy intake and higher resting energy expenditure but also to genetic factors [54]. Studies have shown that female CFRD patients have a poorer prognosis [55]

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and Sims et al. recently revealed that their percent predicted FEV_1 is 20% lower compared to CF patients without CFRD [56]. However, females with newly diagnosed CFRD did not have significant reduction in pulmonary function compared to matched controls. This suggests that early intervention in females with CFRD may prevent worsening of pulmonary function. We were unable to demonstrate a difference in pulmonary function in females with CFRD versus those with NGT but numbers were small (2 and 9 respectively). Also, patients were newly diagnosed and may not yet have shown a decline in pulmonary function [56]. Of the 4 retested female IGT patients, 3 became NGT and 1 CFRD. All showed an improvement in pulmonary function (data not shown) over the study period.

Nutritional recommendations given to IGT patients appeared to have a beneficial impact on progression to diabetes. CFRD is preceded by a variable period of glucose intolerance [57]. Ours is the first study to consider nutritional intervention as a means of delaying progression to diabetes. However, given our small numbers, the lack of objective measures of compliance to NR and of meticulous evaluation of nutritional status, further studies investigating the effects of nutritional intervention in patients with glucose intolerance on the progression to CFRD are warranted.

CF is characterized by chronic inflammation, lung infection and is associated with increased oxidative stress [10,11]. Our data show a trend for an increase of PA colonization with impairment of glucose tolerance but that remains to be confirmed in a larger population of patients. Reduced antioxidant capacity in CF also suggests a state of increased oxidative stress [11,58] due to production and release of reactive oxygen and nitrogen species by inflammatory processes [59] and/or CFTR dysfunction. Similarly, diabetes has been associated with increased oxidative stress. The formation of ROS consequent to hyperglycemia is likely mediated through nonenzymatic glycation [60] and oxidation of proteins [20] and molecular rearrangements with production of more ROS [21] which can result in diminished antioxidant capacity and activation of redox-sensitive transcription factors [16]. The positive correlation between blood HNE-P and glucose levels suggests that 16% of the increase in glucose levels in this group of patients can be associated with an increase in HNE-P concentrations related to increased oxidative stress.

Erythrocyte GSSG appeared to be greater in the IGT group although a small decrease was observed in the CFRD group. However, due to the small number of patients and the high variability in the latter group, no conclusion can be drawn. Also, there were no significant group differences in GSSG/GSH ratios. As previously described in children [61], we observed a difference in glutathione levels between males and females, with significantly higher total glutathione levels, GSH levels and a more reduced environment in females. This may be related to different hormonal environments [62].

Under normal conditions, cells contain basal levels of HNE (<1 μM), which may act as a signaling molecule [63]. Under oxidative stress conditions, the increased HNE production can lead to modifications of biological molecules and proteins [19]. We measured HNE-P adducts in whole blood to provide an accurate assessment of ongoing oxidative stress in CF. CFRD patients had increased (109%) HNE-P levels compared to NGT patients, suggesting further oxidative stress with CFRD. Exogenous HNE altered glucose-induced β-cell insulin secretion in an animal model [64]. It is likely that such HNE processes have not significantly modified β-cell function in newly diagnosed patients given the absence of frank insulinopenia in our patients. To the best of our knowledge, this study is the first to identify a direct link between 2-h BG and oxidative stress evaluated by HNE-P adducts. Moreover, in 3 patients, we measured similar levels of HNE-P at fasting and 2-h post OGTT (data not shown). This suggests that HNE-P may represent a long-term index of oxidative stress associated with chronic hyperglycemia. However, this remains to be further investigated. Differences observed in glutathione levels between males and females may suggest that females have better defence mechanisms against oxidative stress (i.e. more GSH). However, concentrations of HNE-P did not differ significantly between females and males.

DHN-MA is the major urinary metabolite of HNE and presents at physiological levels [23]. In conditions of induced oxidative stress, increased levels of DHN-MA levels have been reported in animals [24]. Although this metabolite originates from the conjugation of HNE with GSH, no decrease in GSH levels was observed in IGT patients. This may represent a GSH synthetic response to ongoing oxidative stress, as represented by the increasing HNE-P adduct concentrations in the IGT and CFRD groups. NGT patients showed higher DHN-MA levels compared to IGT whereas they presented lower concentrations of HNE-P (Fig. 3). Assuming that the nutrition and intake of exogenous HNE are similar across groups, we can raise two possibilities that bridge these results. Firstly, one can speculate that the increased HNE production is associated with a higher formation of conjugates with GSH but the malfunction/absence of CFTR could also affect the DHN-MA conjugates, decreasing their levels in urine. Another possibility is that the HNE produced by n-6 FA peroxidation is mainly diverted toward proteins as glutathione imbalance may also affect HNE metabolism in CF. However, not much is known about these metabolic pathways and they remain to be investigated.

Notably, among the IGT patients retested, HNE-P levels significantly decreased in those reverting to NGT whereas levels increased in patients who became diabetic. These results confirm the link observed between BG and oxidative stress evaluated by HNE-P adducts. It may be speculated that the decrease in HNE-P levels in the IGT patient who remained intolerant preceded an improvement in glucose tolerance. This would support the hypothesis that changes in oxidative stress precede disturbances in glucose metabolism.

Among the metabolic abnormalities in CF, disturbances in FA profile were reported in children and adults [65]. However, a large number of studies have characterized the FA composition in different compartments (i.e. serum *vs* plasma *vs* erythrocyte phospholipids) [66–68], using different methods (i.e. capillary GC, GCMS) making comparisons and data interpretation difficult. The most common observation concerns a decrease in both the n-6 fatty acids [69,70] and DHA (C22:6n-3) [71]. Moreover, the severity of the perturbations has been related to pancreatic status [30], genotype [72] and pulmonary function with increased oxidative stress [32]. In our patients, fatty acid

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abnormalities are probably already present as stated in previous studies [69,73]. Moreover, using a recently validated tool that could be an efficient diagnostic marker of CF [74], the product of plasma levels of (C18:2n-6)×(C22:6n-3) revealed that all our patients are within the expected range for CF (~20: 18.4±3.2; $n=12$ *vs* ~40 for healthy controls [74]) without significant change with glucose tolerance. The absence of EFAD evaluated using the ratio (C20:3n-9)/(C20:4n-6), is consistent with others reports [30,75] and may be due to the fact that no pancreatic insufficiency was reported in our patients. Not much information is available related to FA and glucose metabolism in CF. Our results showed significant decreases for the sum of monounsaturated fatty acids, DHA and the ratio (sum FAn-3)/(sum FAn-6) with impairment of glucose tolerance. However, further studies including diabetic CF patients and controls are needed to confirm our findings.

HNE results from the lipid peroxidation of n-6 unsaturated fatty acids and lipoprotein phospholipids [76,77]. Although IGT patients exhibited an increase in HNE-P levels (43%) compared to NGT patients, levels of n-6 fatty acids such as LA (C18:2n-6) and AA (C20:4n-6) were found to be only slightly decreased (~12%). Notably, concentrations of HNE-P were measured in whole blood, referring to a contribution of erythrocyte membrane status, whereas fatty acid profile was analyzed in plasma. Since FA of the n-3 and n-6 families are essentially structural components of membrane phospholipids, analyses of the fatty acid composition in erythrocyte membrane could be more appropriate to relate with HNE-P levels and the major lipids ongoing the peroxidation. Fatty acids such as AA and the precursor of DHA, EPA (C20:5n-3), are involved in the production of eicosanoids such as prostaglandins, prostacyclins, thromboxanes and leukotrienes which affect inflammation in CF. One can speculate that the observed decrease in n-3 fatty acid levels with IGT may be explained by a suppressive anti-inflammatory effect of n-3 on the increased formation of proinflammatory products by n-6 fatty acids.

During the course of CF, it has been shown that female patients were more prone to inflammation and had decreased pulmonary function (percent predicted FEV_1) compared to males [53]. Increased levels of LA and decreased levels in major n-3 fatty acids were found to be positively correlated with pulmonary function in pre-adolescent CF children [78]. Consistently, our female patients exhibited significantly lower levels of n-3 FA compared to males, which could potentially be linked to lower pulmonary function.

5. Conclusion

In summary, this study is the first showing a direct link between blood levels of glucose and oxidative stress as evaluated with blood levels of HNE-P in patients with CF. Previously published methods for measuring HNE-protein adducts using immunohistochemistry are mostly semi-quantitative. Further documentation of the relationship between glutathione, HNE-protein adducts and HNE metabolism in healthy controls and diabetic patients is required. Our preliminary data regarding the potential role of nutritional intervention for the prevention or delay of CFRD in patients with IGT suggest the need for larger clinical studies designed specifically for proper documentation of diet implementation and compliance, effect on growth and clinical status, quality of life, as well as impact on the development of CFRD. Finally, our data on FA profile with glucose intolerance suggest that inflammation and oxidative stress could be related to the development of CFRD and mediated through changes in lipid peroxidation.

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