

Research Communication

Myeloperoxidase Promoter Polymorphism –463G Is Associated With More Severe Clinical Expression of Cystic Fibrosis Pulmonary Disease

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The severity of cystic fibrosis (CF) pulmonary disease is not directly related to CFTR genotype but depends upon several parameters, including neutrophil-dominated inflammation. Identification of agents modulating inflammation constitutes a relevant goal. Myeloperoxidase (MPO) is involved in both microbicidal and proinflammatory neutrophil activities. The aim of this study was to evaluate whether the –463GA MPO promoter polymorphism is linked to clinical severity of CF-associated pulmonary inflammation. This polymorphism significantly affects the level of MPO gene expression in leukocytes and the G allele is more expressing than the A allele. We show that MPO genotype significantly influences the severity of pulmonary disease in early stages, prior to the development of chronic lung infections, with GG genotype being associated with more severe CF disease. Our findings indicate that the level of MPO gene expression influences the CF pathogenesis, presumably reflecting cellular damage by MPO-generated oxidants or other activity of MPO in airway inflammation.

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INTRODUCTION

Cystic fibrosis (CF) is a lethal autosomal recessive disorder which is caused by mutation of the cystic fibrosis transmembrane regulator (CFTR) gene [1]. Although CF is a multi-organ disease, most of the morbidity and the mortality are due to progressive pulmonary disease with lung parenchymal destruction and chronic pulmonary bacterial colonization [2, 3]. There is a hypersecretion and a massive influx of neutrophils within the airways which mediate deleterious effects [4, 5]. Moreover, the intensity of the airway inflammation is directly correlated with the clinical status as evidenced by a close correlation between respiratory scores and neutrophil-derived proteinases [6]. The airway inflammation typical of CF relies thus on the paradox of misdirected neutrophil microbicidal activity resulting in an exacerbation of neutrophil-mediated tissue damage and a concomitant failure in the antimicrobial system, since CF patients can become chronically infected with *Pseudomonas aeruginosa* (*P. aeruginosa*) [7, 8]. Activation of the neutrophil results in the oxidative

burst which occurs at the level of NADPH oxidase leading to the generation of superoxide anion which can dismutate into hydrogen peroxide (H₂O₂) [9, 10]. Myeloperoxidase (MPO), an enzyme contained in azurophil granules, catalyzes the H₂O₂-dependent oxidation of chloride (Cl⁻), which yields hypochlorous acid (HOCl) [11]. HOCl is a potent microbicidal agent and a toxic chlorinating oxidant. MPO released from activated neutrophils can also damage bystander cells at inflammatory sites. Airway secretions from CF patients contained high concentrations of active MPO [12] that could catalyze protein tyrosine oxidation and produce high levels of dityrosine, 3-chlorotyrosine, and 3-nitrotyrosine [13]. Levels of MPO were correlated with poor clinical conditions and sputum production [14], thus demonstrating that extracellular MPO could contribute to hypersecretion, bronchial injury, and respiratory failure in CF. MPO could be a critical parameter in the modulation of inflammation and/or infection because MPO is involved in both microbicidal and proinflammatory activities of neutrophils.

When we have studied purified circulating neutrophils in CF, we provided evidence of a significant increase in MPO-dependent oxidant formation in both CF children and their parents as compared to controls, whereas NADPH activity was similar to that of controls. This increased intracellular MPO activity observed in CF homozygotes and heterozygotes was observed at the basal level as well as following PMA or opsonized zymosan stimulation [15]. The fact that this increase was observed in the parents of CF children, which are heterozygous for CFTR mutation but are free of clinical symptoms, led us to the conclusion that the disturbance in MPO activity observed in CF was constitutive, independent from infection. These prior studies demonstrated that increased MPO activity could potentiate inflammation in CF patients.

In the present study, we asked whether a functional MPO promoter polymorphism influenced their clinical outcome. The $-463G/A$ polymorphism is linked to the differences in MPO expression levels [16, 17]: the $-463G/A$ promoter polymorphism significantly affects the level of MPO gene expression in bone marrow precursors and in descendant leukocytes. The G allele is more expressing than the A allele. This polymorphism is linked to incidence or severity of inflammatory diseases including atherosclerosis [18, 19], Alzheimer's [20–22], MPO-ANCA vasculitis [23], HCV-induced fibrosis [23], multiple sclerosis [24], periodontal disease [25], lung cancer [26, 27], and myeloid leukemia [24]. This polymorphism is in an Alu element, within a cluster of four hexamer half sites recognized by various nuclear receptors [17] and termed an Alu receptor response element (AluRRE). These hexamers are organized as direct repeats with spacing of two-four-two base pair. The $-463A$ is in hexamer 1 within an estrogen receptor binding site [16], while $-463G$ promotes binding by SP1 transcription factor [17]. The hexamer 3/4 pair is recognized by retinoic acid receptor and PPAR γ as heterodimers with retinoid X receptor (RAR-RXR), while the middle DR-4 pair (hexamers 2/3) is recognized by thyroid hormone receptor (TR-RXR). The $-463GG$ genotype has been linked to higher MPO mRNA and protein expression than GA/AA genotypes in primary myeloid leukemia cells and monocyte-macrophages [28], while in transfection assays, the $-463G$ promoter element supported higher expression of a reporter gene than $-463A$ [17]. In a recent study performed in human monocyte-derived macrophages, we have provided evidence that MPO gene expression was downregulated as iNOS (inductible nitric oxide synthase) was upregulated, thus suggesting a potential connection between these two enzymes [29]. Because NO has physiological importance in airways, a difference in MPO expression levels could be relevant in the pulmonary disease in CF.

MPO could be considered a candidate for potential genetic modifiers of disease severity in patients with CF not necessarily related to infection. Indeed, MPO can also play a significant proinflammatory role in the absence of infection, in atherogenesis, or in neurodegenerative diseases. The mechanisms leading to neutrophil-dominated inflammation in CF patients as well as the relationships with CFTR

mutations remain largely unknown. The course of lung disease in patients with CF varies considerably, even among patients with the same CFTR who are receiving standardized care [7]. This variation is largely unexplained. So far, several genes implicated in innate immunity and in the control of inflammation have been studied as potential modifiers of CF [30]. Among them, mannose-binding lectin [31], alpha-antitrypsin [32], glutathione S-transferase, TNF-alpha, IL-1 beta, and IL-1 RA have already been studied [33]. Interestingly, a possible link between the NOS1 gene locus and the rate of decline in lung function in CF patients has been described [34]. So far, there is no data available on the influence of MPO genotype on the clinical course of CF.

In the present study, we investigate the influence of the MPO promoter polymorphism on the clinical severity of CF disease. Our results clearly demonstrate that MPO genotype influences the severity of the lung disease in CF patients in the absence of infection, with the GG genotype associated with more severe CF disease. In infected CF patients, the GG genotype was associated with an increase in the circulating neutrophil inflammatory state.

METHODS

Study population

Eighty eight patients with CF including 45 males and 43 females (mean age: 17.8 ± 0.74 years) were recruited for the study. Informed consent was obtained from patients or their parents and the clinical research was conducted according to the guidelines of the French Institut National de la Santé et de la Recherche Scientifique (INSERM) and the French ethics committee. CF was diagnosed according to the standard criteria including a sweat chloride test. Patient's clinical status was evaluated in CF patients by the Shwachman-Kulczycki scoring system [35] which includes nutritional criteria, the highest value being 100. The pulmonary function was assessed by spirometry and expressed as percent-predicted FEV1 (forced expiratory volume in one second) and FVC (forced vital capacity) normalized for age and height [36]. The severity of lung disease was evaluated according to the chest radiograph and the number of radiologic lesions (obstructive lesions and segmental or diffuse bronchiectasis were counted).

The lung infection was determined in 77 CF patients on the basis of bacteriological analysis of sputum, which was collected according to the methodology previously described [36]. On the basis of sputum bacterial qualitative and quantitative analysis, bronchial infection was defined as bacterial counts $\geq 10^8$ colony forming units (CFU)/l in at least 3 cultures at one-month interval, and CF patients were stratified into three groups.

A first group designated "uninfected CF children" included 32 children without history or evidence of *P. aeruginosa* infection. At the time of testing, sputum cultures were all negative and all children were stable and clinically well.

The second group designated "infected CF children" included 45 CF children either infected with *Staphylococcus*

aureus (*S aureus*) ($n = 8$) or chronically colonized with *P aeruginosa* ($n = 37$).

Analysis of CFTR mutations by PCR and direct sequencing

Genotyping of CF patients was performed as previously described [37–39]. In the CF population tested, 73% carried the delta F508 mutation with 50% being delta F508 homozygote, 23% being delta F508 heterozygote with an unknown mutation, and 27% having mutations other than delta F508 at both alleles as previously described [36].

Determination of MPO genotype by allelic discrimination assay

DNA was isolated from leukocytes by proteinase K digestion followed by phenol extraction and ethanol precipitation. DNA (100 ng) was used in the ABI 7900 allelic discrimination assay using dual fluorophore probes which discriminate between alleles based on the single base mismatch. Primers and probes, designed using the Primer Express 2.0 software (ABI), are as follows: forward primer, 5'-AATCTTGGGCTGGTAGTGCTAAA-3'; reverse primer, 5'-GCCAGGCTGGTCTTGAAGTC-3'; -463A specific probe, 5'-FAM TCCACCTGCCTCAG MGB; and -463G specific probe, 5'-VIC TCCACCCGCCTCA MGB. Probes are labeled at the 5' end with the fluorophores FAM or VIC and are stabilized by a minor groove binding moiety (MGB). Endpoint allelic specific fluorescence was measured on the ABI prism 7900 using the Sequence Detection Systems 2.0 software for allelic discrimination.

Statistical analysis

Statistical analysis was performed using the Statview software. Comparisons were made by analysis of variance (ANOVA) or unpaired Student *t* test. Data are expressed as mean \pm standard error of the mean (SEM).

RESULTS

Frequencies of MPO genotype in CF patients as compared to control population

We determine the MPO genotype for 79 CF children. As shown in Table 1, there is no difference in the percentages of the GG, GA, or AA genotypes in the CF population as compared to the frequencies within normal European populations [16, 26, 40]. When the cases were stratified by gender, there was no statistically significant difference.

Influence of MPO genotype on clinical parameters of CF disease

Since clinical conditions in CF patients are greatly influenced by recurrent infections, it was relevant to evaluate the influence of MPO genotype within a homogeneous group of CF

TABLE 1: Frequencies of MPO genotype in the population of CF children.

	CF patients ($n = 79$)	Males ($n = 42$)	Females ($n = 37$)
GG	47/79 = 59.49%	28/42 = 66.67%	21/37 = 56.76%
GA	30/79 = 37.97%	13/42 = 30.95%	15/37 = 40.54%
AA	2/79 = 2.53%	1/42 = 2.4%	1/37 = 2.7%

patients according to infectious/inflammatory criteria. When comparing the respiratory scores between the first group of noninfected CF patients, the second group of *S aureus*, and the third group of *P aeruginosa*-infected patients, it appeared that the FEV was significantly different in the noninfected ($n = 32$, 79.3 ± 3.9) versus the group of *S aureus*-infected CF patients ($n = 8$, 56.75 ± 5.2 , $P = .009$) and versus the group of *P aeruginosa*-infected CF patients ($n = 37$, 60.55 ± 4.1 , $P = .002$). In contrast, there was no difference in the FEV in the group of *S aureus*- as compared to *P aeruginosa*-infected CF patients ($P = .674$). As a result, for further analysis, only two homogeneous groups, in terms of severity of CF disease, will be considered: noninfected CF patients and infected CF patients, mainly composed of *P aeruginosa* chronically infected CF patients. As shown in Table 2, the group of infected CF children clearly differed from the groups of noninfected CF children on the basis of respiratory scores (FEV or FVC) or infectious score measured as the number of antibiotic therapies during the past year. Although not statistically significant, there was a clear difference in the Shwachman and in the radiographic scores between noninfected and infected CF patients; the noninfected CF patients having more severe Shwachman and radio scores. Of note, the mean age of the noninfected CF patients was significantly lower than the age in the infected CF patients. This could be expected since infection occurs in the course of the disease and thus affects older patients.

Within each group, either the noninfected or the infected CF patients, we evaluated the influence of MPO genotype. As shown in Figure 1(a), in the group of noninfected CF patients, there is significantly lower FEV in CF patients with GG than GA genotype (73.5 ± 5.6 versus 95.7 ± 8.2 , $P = .03$). Likewise, FVC was significantly lower in CF patients with GG genotype than with GA (80.8 ± 4.0 versus 99.7 ± 6.7 , $P = .01$, resp). As expected, there was more lesions seen on chest radiography in GG than in GA (23.6 ± 8.6 versus 5.5 ± 1.5 , resp). Shwachman scores were decreased in the group of GG genotype as compared to GA (64.3 ± 9.1 versus 87.8 ± 3.4 , resp). The number of antibiotic therapies was higher in GG than in GA (3.5 ± 1.2 versus 2.4 ± 0.8 , resp). Of note, the mean age of the CF patients was not different between the groups of GG and GA providing evidence that there was no artifact of older patients in GG group explaining their more severe clinical expression (Table 3).

In contrast, in the group of infected CF patients, there was no significant difference in clinical parameters between GG and GA genotypes evaluated either by respiratory scores (FEV or FVC) (Figure 1(b)) or by radiography scores. There is no difference in FEV in CF patients with GG or GA

TABLE 2: Clinical characteristics of CF patients stratified with their infectious status. A total of 77 CF children were studied and two groups were made up according to the infectious status of the CF patients. The data are the mean \pm SEM. The radio score indicates the number of lesions observed on the chest radiography. The ATB refers to the number of antibiotherapy treatment during the preceding year.

	Age (y)	FEV %	FVC %	Shwachman	Radio	ATB
Noninfected CF $n = 32$	15.8 \pm 0.9	80.9 \pm 3.9	85.8 \pm 2.9	75.8 \pm 4.3	8.2 \pm 1.3	2.7 \pm 0.5
Infected CF $n = 45$	18.6 \pm 0.8	59.3 \pm 3.4	72.5 \pm 2.9	66.9 \pm 2.9	20.2 \pm 3.7	7.0 \pm 0.7
	$P = .02$	$P < .0001$	$P = .002$	$P = .08$	$P = .018$	$P < .0001$

genotype (61.0 \pm 5.4 versus 59.6 \pm 4.7, resp). Likewise, no difference was observed in FVC with GG or with GA genotype (73.1 \pm 4.6 versus 74.2 \pm 4.3, resp). It could be noted, however, that the Shwachman score was higher in the group of GA than in the group of GG phenotype, but this increase was not statistically significant. With regard to the number of antibiotherapies in the preceding year, there was no significant difference. Interestingly, this lack of influence of MPO genotype on clinical parameters was observed separately both in the subgroup of CF children chronically infected with *P aeruginosa* and in the subgroup of CF children infected with *S aureus* (data are not shown).

Taking together, our data strongly suggest that the MPO genotype influences the clinical course of the disease with less severity for the GA allele versus the GG allele in CF patients, in the absence of infection. In contrast, as far as there is an infection and especially a chronic *P aeruginosa* infection, MPO genotype is not as discriminative, and the GA genotype is not associated with better clinical status.

Influence of gender

Since a number of studies have shown that the influence of MPO genotype could be gender-specific [20, 23], we compared the influence of MPO genotype in CF females and CF males. No influence of gender was observed. We confirmed that, in the absence of infection, in either CF females or CF males, the GG genotype was associated with a more severe lung disease and worse clinical conditions. In the group of infected CF patients, there was no significant influence of MPO genotype in CF females or males, thus corroborating the results found in the mixed population.

Influence of the CFTR mutation

CF patients were stratified into two groups according to their CFTR mutation, either homozygotes $\Delta F508/DF508$ or heterozygotes $\Delta F508/others$. The group of patients having the CFTR genotype corresponding to other/other was so small that we could not perform analysis. Within the group of $\Delta F508/DF508$ or within the group of $\Delta F508/others$, we confirmed what we have found for the whole population, which is that the GA phenotype was associated with better clinical parameters as compared with GG, in the absence of infection. In infected CF patients, there was no effect of MPO genotype whatever the CFTR genotype. These results concerning the influence of CFTR mutation on the clinical severity of the

disease are not surprising since the CFTR genotype is not related to the inflammatory status of the patient, and it is now well documented that there is no correlation between CFTR genotype and the evolution of lung disease.

DISCUSSION

Our present findings establish for the first time that the GG MPO genotype is associated with more severe clinical expression of CF disease in the absence of infection. The G allele is the higher expressing allele, and the GG genotype is most often associated with worse disease. Thus, the findings in this paper linking GG genotype to worse outcome in CF is consistent with most prior studies.

In CF patients, the association of the higher expressing GG genotype with more severe disease in the absence of infection implies that MPO enzyme contributes to the inflammatory status in noninfected patients. Our data suggest a significant proinflammatory role for MPO in the early phase of CF, in the absence of infection. Several hypotheses can be made to explain the influence of MPO in airways in the absence of infection and the potential mechanisms resulting in the presence of MPO within airways. Firstly, noninfected CF patients means that these patients are non-chronically infected by *P aeruginosa* or *S aureus* and they do not have any detectable infection demonstrated by negative bacterial analysis of the sputum at the time of the clinical analysis. In our study, we differentiated between noninfected CF patients and chronically infected patients because their clinical status were significantly different. However, our classification does not rule out some subclinical infection, which could explain the presence of some neutrophils within the lung. Secondly, it has been described that there is a “constitutive” inflammatory process in CF epithelial cells with a basal activation of the transcription factor NF- κ B in the absence of infection [41]. It has been reported that epithelial cells from CF patients have the ability to secrete more IL-8, which is a potent chemoattractant for neutrophils [42, 43]. It can be hypothesized that inflammatory cytokines could attract neutrophils and/or upregulate MPO in macrophage in the absence of infection. The inhibitory role of MPO on the regulation of NOS might also explain the better clinical conditions associated with the GA genotype (producing less MPO). Indeed, epithelium-derived NO reduced *P aeruginosa* adherence and enhance killing of bacteria [44], and NOS expression decreased in CF as airway inflammation increases [45]. A combined study of NOS and MPO gene expression

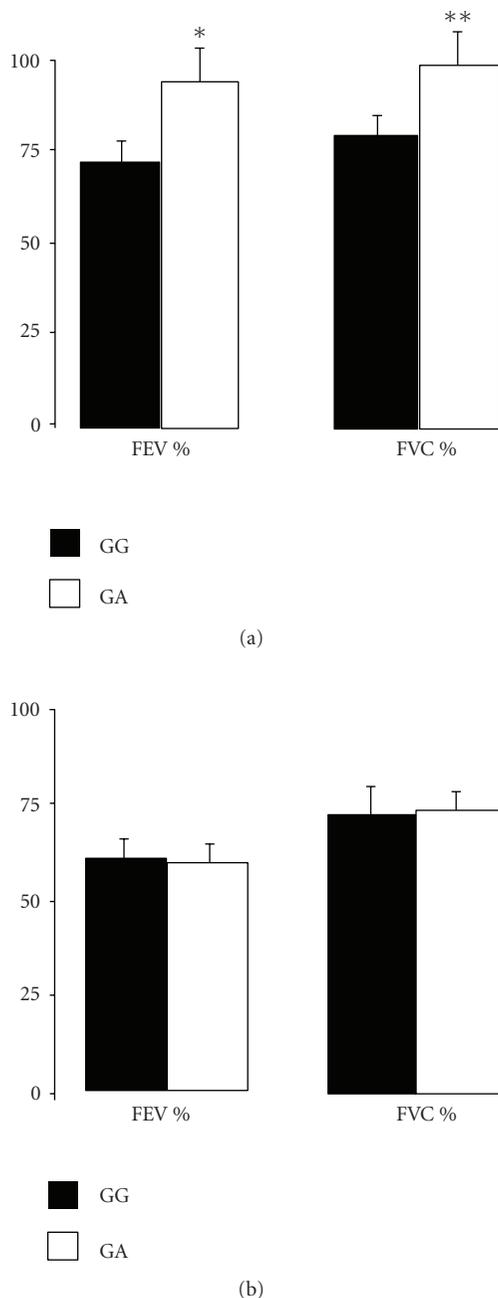


FIGURE 1: Influence of MPO genotype on respiratory scores in CF patients. FEV1 % (on the left) and FVC % (on the right) were measured in (a) 21 noninfected CF patients either with ($n = 14$) for the GG or ($n = 7$) for the GA genotype and (b) 37 chronically infected CF patients with ($n = 22$) for the GG or with ($n = 15$) for the GA genotype. The data are the mean \pm SEM. Differences were statistically significant (* $P = .03$ and ** $P < .001$).

and polymorphisms might be relevant to CF and would help to understand the variability of the clinical expression of the pulmonary disease.

In chronically infected CF patients, the GA genotype is not as beneficial as it is in noninfected patients. The observation that GG genotype is not associated with greater

resistance to *P aeruginosa* or fewer infection per year suggests that MPO does not play a critical role in the antimicrobial systems of CF neutrophils. In this situation, many neutrophils are recruited within airways and these fail to kill the bacteria (*S aureus* or *P aeruginosa*). One would expect that CF patients having more MPO (GG genotype) would be more efficient in killing bacteria and thus would have less severe pulmonary disease. We do not see any difference in the severity of the pulmonary disease between GG and GA genotypes who are chronically infected.

Our study thus emphasized that MPO contributes significantly to the proinflammatory effects of PMN. In the absence of infection, genotype GG may be more deleterious due to exacerbation of the inflammatory process and production of more oxidants which damage the airways and epithelium. When chronic colonization with *P aeruginosa* occurs, the increased production of chlorinated oxidants could be an advantage, counterbalancing the negative impact of oxidative damage to the epithelium, such that GG genotype may have both positive and negative impact; thus the difference between GG and GA is less noticeable. Moreover, additional physiopathological mechanisms are involved in the destruction of airways depending on the bacteria, thus masking the clear-cut influence of MPO genotype on the clinical expression of CF disease that we have observed in noninfected patients. Another interesting aspect of our results is the fact that MPO activity could result in the formation of inflammatory mediators by oxidizing mucus components, as it has previously been reported for plasma components in hemodialyzed patients [46]. Indeed, it has been reported that neutrophils components, especially elastase, can stimulate mucus secretion in airways [47] via an oxidant-dependent mechanisms. It would be pertinent to study whether MPO-modified mucins could have some inflammatory activities. This could fit with the data that MPO levels in neutrophils were correlated with sputum production [14], thus suggesting that MPO could be linked with CF mucus hypersecretion.

Several studies on the mechanisms of inflammation in CF have now shown that a genetic disease like CF is not as simple as originally envisioned. The cloning of the CFTR was a major milestone in understanding the molecular basis of CF, but dissecting the pathogenesis of CF has proven far more complicated. The challenge remains to select appropriate gene polymorphisms and investigate their effects in order to achieve a more complete understanding of the pathogenesis of a disease as complex as CF. The identification of a collection of modifying genotypes could lead to a more refined understanding of specific disease. In a study focused on potential genetic modifiers of disease severity in chronic granulomatous disease (CGD) patients, genotype of MPO and Fc gamma RIII were found to be strongly associated with an increased risk for gastrointestinal complications. There were, however, no data on MPO activity in PMN from CGD patients or addressing the possibility that MPO genotype could modulate levels of MPO activity [48].

Understanding the role of modifying genes has considerable potential for determining risk profile that might impact therapeutic decisions. By subclassifying disease, according

TABLE 3: Influence of MPO genotype on clinical parameters of CF disease. The $-463G/A$ MPO polymorphism was determined in 58 CF patients as described in the "methods." The influence of the polymorphism of MPO promoter was studied either in (a) noninfected or in (b) chronically infected CF children. The data are the mean \pm SEM. The radio score indicates the number of lesions seen on the chest radiography. The ATB means the number of antibiotherapy treatment during the preceding year. NS: $P > .05$.

(a)				
	Age (y)	Shwachman	Radio	ATB
GG ($n = 14$)	18.6 \pm 0.9	64.3 \pm 9.1	23.6 \pm 8.6	3.5 \pm 1.2
GA ($n = 7$)	18.0 \pm 1.3	87.8 \pm 3.4	5.5 \pm 1.5	2.4 \pm 0.8
	NS	$P = .08$	NS	NS
(b)				
	Age (y)	Shwachman	Radio	ATB
GG ($n = 22$)	18.7 \pm 0.8	63.3 \pm 4.8	19.2 \pm 4.5	6.9 \pm 1.0
GA ($n = 15$)	17.7 \pm 2.1	71.1 \pm 3.8	12.1 \pm 1.4	7.1 \pm 1.5
	—	NS	NS	NS

to secondary genetic risk factors, therapy could be adapted to individuals need. Indeed, the current study strongly suggest that pharmacological modulation of MPO activity could have different effects depending on MPO genotype and MPO inhibitors may have a more beneficial effect in CF patients having GG phenotype.

In summary, our hypothesis is that, in CF, the MPO promoter polymorphism $-463G/A$, which influences the amount of neutrophil MPO, could modulate inflammatory and/or infection processes, thereby modulating the severity of the pulmonary disease. MPO-HOCl is known to oxidize a variety of biological molecules, and can have significant impact at inflammatory sites, independent of infection. This is likely to explain the observed difference between the MPO genotypes in severity of pulmonary disease in noninfected CF patients.

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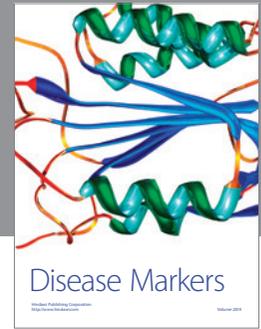
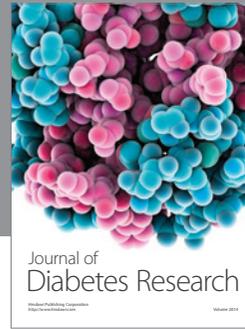
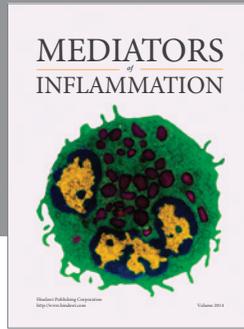
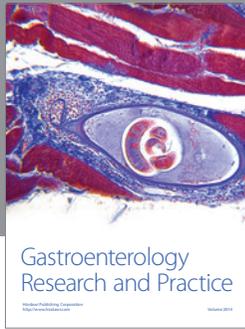
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