Hereditary Deficiency of gp91\textsuperscript{phox} Is Associated With Enhanced Arterial Dilatation
Results of a Multicenter Study

Francesco Violi, MD; Valerio Sanguigni, MD; Roberto Carnevale, PhD; Alessandro Plebani, MD; Paolo Rossi, MD; Andrea Finocchi, MD; Claudio Pignata, MD; Domenico De Mattia, MD; Baldassarre Martire, MD; Maria Cristina Pietrogrande, MD; Silvana Martino, MD; Eleonora Gambineri, MD; Anna Rosa Soresina, MD; Pasquale Pignatelli, MD; Francesco Martino, MD; Stefania Basili, MD; Lorenzo Loffredo, MD

Background—NADPH oxidase is believed to modulate arterial tone, but its role in humans is still unclear. The objective of this study was to evaluate whether NADPH oxidase is involved in flow-mediated arterial dilation (FMD).

Methods and Results—Twenty-five patients with hereditary deficiency of gp91\textsuperscript{phox}, the catalytic core of NADPH oxidase, (X-CGD), 25 healthy subjects, and 25 obese patients matched for sex and age were recruited. FMD, platelet gp91\textsuperscript{phox}, serum levels of nitrite and nitrate as markers of nitric oxide generation, oxidized low-density lipoprotein, and urinary excretion of isoprostanes as markers of oxidative stress were determined. Platelet gp91\textsuperscript{phox} expression was downregulated in X-CGD patients (1.0±0.8 mean fluorescence; \(P<0.001\)) and upregulated in obese patients (4.1±2.2 mean fluorescence; \(P=0.01\)) compared with healthy subjects (2.9±1.7 mean fluorescence). Urinary excretion of isoprostanes was reduced in X-CGD patients (41.7±33.3 pg/mg creatinine; \(P=0.04\)) and increased in obese patients (154.4±91 pg/mg creatinine; \(P<0.001\)) compared with healthy subjects (69.5±52.4 pg/mg creatinine). Obese patients had higher serum oxidized low-density lipoprotein than healthy subjects (35.3±6.7 versus 24.8±9.8 U/L; \(P<0.001\)) and X-CGD patients (28.5±7.2 U/L; \(P<0.001\)). X-CGD patients had significantly higher FMD (14.7±5.9%) compared with healthy subjects (7.9±2.5%; \(P<0.001\)); obese patients had lower FMD (5.3±3.0%; \(P=0.028\)) compared with healthy subjects. Serum nitrite and nitrate levels were significantly higher in patients with X-CGD (36.0±10.8 μmol/L; \(P=0.016\)) and lower in obese patients (9.3±11.0 μmol/L; \(P=0.001\)) compared with healthy subjects (27.1±19.1 μmol/L). Serum nitrite and nitrate levels significantly correlated with FMD (\(R_s=0.403, P<0.001\)) and platelet gp91\textsuperscript{phox} (\(R_s=-0.515, P<0.001\)). FMD inversely correlated with platelet gp91\textsuperscript{phox} (\(R_s=-0.502, P<0.001\)) and isoprostanes (\(R_s=-0.513, P<0.001\)).

Conclusion—This study provides the first evidence that, in humans, gp91\textsuperscript{phox} is implicated in the modulation of arterial tone. (Circulation. 2009;120:1616-1622.)

Key Words: atherosclerosis ▪ gp91\textsuperscript{phox} protein, human ▪ oxidative stress

Endothelial dysfunction is a hallmark of early atherosclerosis and predicts cardiovascular events in high-risk cohorts, including the elderly and patients with hypertension, coronary heart disease, or peripheral arterial disease.\(^1-4\) The mechanism accounting for endothelial dysfunction is not completely understood. Oxidative stress is believed to play an important role in that it can influence activity and generation of nitric oxide (NO), a potent vasodilator molecule produced by endothelial cells.\(^5\) Several reactive oxidative species (ROS) -generating enzymes, including myeloperoxidase, xanthine oxidase, and NADPH oxidase, may be implicated in arterial dysfunction.\(^6\) Accordingly, experimental studies performed in animal models suggest a pivotal role of NADPH oxidase in modulating arterial tone.\(^7-9\) In particular, overexpression of gp91\textsuperscript{phox}, the catalytic subunit of NADPH oxidase (Nox2), potentiates the hemodynamic response to angiotensin II.\(^7\)
We conducted a multicenter study in collaboration with the Italian Primary Immunodeficiency Network. Of the 60 patients with CGD registered in the national database, 35 were not included in the study because of the presence of acute infections, critical physical conditions, hereditary NADPH oxidase deficiency unrelated to gp91phox, or unwillingness to participate in the study. The remaining 25 patients who were gp91phox deficient and willing to participate in the study were included.

Diagnosis of X-CGD was performed as previously described. All X-CGD patients were receiving itraconazole, trimethoprim, and sulfamethoxazole.

Unpublished observations obtained by our department showed that X-CGD patients had serum cholesterol levels ~20% lower than healthy subjects. Because this could represent a confounding factor, 25 control subjects matched for serum cholesterol, sex, and age were identified and included in the study. Twenty-five patients affected by obesity who were matched for gender and age were randomly collected from the pediatric clinic and from the outpatient clinic of our division at the “I Clinica Medica” of the Sapienza University of Rome (the Table). Control populations were screened from routine visits. For subjects <20 years of age, obesity was defined according to body mass index–age-growth charts, which identify obesity as a body mass index ≥95th percentile. Adult patients with a body mass index >30 kg/m² were considered obese.

Subjects were excluded from the study if they had liver insufficiency, serious renal disorders (serum creatinine >2.8 mg/dL), cancer, myocardial infarction, unstable angina, acute cerebrovascular disease, or deep venous thrombosis; if they were being treated with statins or antioxidant vitamins; or if they were current smokers. The study was approved by the ethics committee. Each subject enrolled gave informed consent to participate in the study. A subgroup of X-CGD patients (n=7), healthy subjects (n=7), and obese patients (n=7) who were matched for age was performed to investigate platelet formation of NO and isoprostanes.

Blood Sampling
Between 8 and 9 AM, subjects underwent routine biochemical evaluations, including fasting total cholesterol and glucose. After an overnight fast (12 hours) and supine rest for at least 10 minutes, blood samples were collected in Vacutainers (Vacutainer Systems, Belliver Industrial Estate, Plymouth, UK) and centrifuged at 300g for 10 minutes to obtain supernatant, which was stored at −80°C until use. Total cholesterol was measured with a routine enzymatic colorimetric method on a Dimension RXL apparatus (Dade Behring AG, Ziegelbrucke, Switzerland).

### Methods

#### Study Population

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Urinary Collection
Morning spot urine samples were collected from all participants between 7 and 9 a.m. Morning urine collected for 8-iso-prostaglandins was stored in 10-mL aliquots at −80°C until analysis.

Urinary 8-Iso-Prostaglandin F₂α, Assays
Urinary 8-iso-prostaglandin F₂α (8-iso-PGF₂α) was measured by a previously described and validated enzyme immunoassay method (Cayman Chemical, Ann Arbor, Mich),15,16 and expressed as picograms per milligram of creatinine. Intra- and interassay coefficients of variation were 2.1% and 4.5%, respectively.

Oxidized Low-Density Lipoprotein and C-Reactive Protein
Serum levels of oxidized low-density lipoprotein (ox-LDL) and C-reactive protein were measured by commercially available immunoassays (Tema Ricercare, Bologna, Italy). Intra- and interassay coefficients of variation were 4.0% and 8.3% for ox-LDL and 9.5% and 9.0% for C-reactive protein.

Nitrite and Nitrate Measurement
A colorimetric assay kit (Tema Ricercare) was used to determine the NO metabolites nitrite and nitrate (NOₓ) in the serum and supernatant of platelet-rich plasma (platelet = 3 × 10⁶/mL) activated with collagen (7 μg/mL) at 37°C for 15 minutes as previously described.13 Intra-assay and interassay coefficients of variation were 2.9% and 1.7%, respectively.

Platelet gp91 Phox Expression
Platelet gp91phox expression was analyzed as previously described.18 Briefly, blood samples were incubated with the unconjugated antibody anti-gp91phox, followed by an FITC-labeled donkey anti-goat immunoglobulin G secondary antibody. For platelet detection, the monoclonal antibody CD61-PE was used.

Samples were analyzed on an Epics XL-MCL cytometer (Coulter Electronics, Hialeah, Florida) equipped with an argon laser at 488 nm. FITC was detected at 525 to 550 nm, PE at 675 to 900 nm. Analysis was stopped automatically after the measurement of 50,000 events. Platelet gp91phox was expressed as mean fluorescence, intra-assay and interassay coefficients of variation were 1.0% and 0.2%, respectively.

Platelet 8-ISO-PGF₂α Assay
Citrated blood samples (ratio of blood to sodium citrate, 9:1) were centrifuged for 15 minutes at 180g to obtain platelet-rich plasma. Platelet-rich plasma was then centrifuged for 20 minutes at 300g to obtain washed platelets, and the pellets were suspended in Tyrode buffer to obtain a final platelet concentration of 3 × 10⁶/mL.

Platelet suspensions were incubated with or without collagen (7 μg/mL) for 15 minutes at 37°C. After incubation, platelets were pelleted 3 minutes at 300g with acid citrate dextrose.

Supernatants from stimulated and unstimulated platelets were acidified to pH 3 with 1 mol/L HCl; 20 μg internal standard (8-iso-PGF₂α) was added. After addition of the internal standard, the mixture was vortexed and applied to a C₁₈ Sep-Pak column (Waters Corp, Milford, Mass) preconditioned with 5 mL methanol and 5 mL water (pH 3). The sample and subsequent solvents were eluted through the Sep-Pak column with a 10-mL acetonitrile/water (15:85 vol/vol), and 10 mL heptane. The isoprostanes were then eluted with 10 mL ethyl acetate/heptane (50:50 vol/vol).19 The eluted samples were completely evaporated under ultrahigh purity (99.9%). The samples, previously dissolved with 10 μL acetonitrile, were derivatized at various times (2 to 24 hours) with 40 μL N,O-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane.

The 8-iso-PGF₂α in derivatized samples was analyzed by a PerkinElmer gas chromatograph (PerkinElmer, Waltham, Mass) equipped with a flame ionization detector with a SPB5 wide-bore column (Supelco, Bellefonte, Pa). Detector responses for 8-iso-prostaglandin and internal standard (8-iso-PGF₂α) were calculated by use of an area integrator LC100 (PerkinElmer).

The carrier gas, helium, was set to a flow rate of 1 mL/min. Derivatized samples (0.5 μL) were injected into the gas chromatograph injection port. The column temperature was maintained at 170°C for 2 minutes, increased to 275°C at 15°C/min, and then held at 275°C for 11 minutes. Finally, the temperature was raised to 300°C at 25°C/min and held for 10 minutes. 8-iso-PGF₂α content was expressed as picomole per liter.

FMD and Carotid Intima-Media Thickness
Ultrasound assessment of FMD was investigated according to the recently reported guidelines20 as previously described.21 The coefficient of variation for FMD measurements, obtained on 3 separate occasions, was 12.5%.

Longitudinal ultrasonographic scans of the carotid artery were obtained on the same day as the studies of the brachial artery reactivity and included the evaluation of the right and left common carotid arteries 1 cm proximal to the carotid bulb. Three measurements of intima-media thickness (IMT) were obtained from the right and left carotid arteries, respectively, and were averaged to determine the mean IMT for both sides combined. The coefficient of variation for IMT measurements, obtained on 3 separate occasions, was 4.90%. FMD and IMT were performed with a 7.5-MHz linear-array transducer ultrasound system (Sonomed, Lake Success, NY).

Statistical Analysis
We used linear mixed-effects models to compare means across groups because the subjects in the study were matched by age and sex. We used subject-specific random intercepts that were assumed to arise from Gaussian distributions, with clusters of random effects identified by the matched triplets (X-CGD, healthy subjects, obese). The group indicators were included as fixed effects. Results were further confirmed by nonparametric tests with the rank transformation.

Data are presented as mean ± SD unless indicated otherwise. The correlation analysis was carried out by Spearman rank correlation test. Statistical significance was defined at P < 0.05. Statistical analysis was performed with SPSS version 13.0 for Windows (SPSS Inc, Chicago, Ill).

Sample Size Determination
On the basis of data from a pilot study,11 we computed the minimum sample size with respect to a 2-sample Student t test, considering a clinically relevant difference for FMD variation to be detected between the X-CGD patients and control subjects 1.5 ± 5%, SDs that were homogeneous between groups (SDs = 3%), and type I error probability of α = 0.05 and power 1 - β = 0.90. This resulted in a minimum sample size of 9 subjects for each group. Sample size calculations were performed with the nQuery Advisor software, version 5.0 (Statistical Solutions, Saugus, Mass).

Results
Platelet expression of gp91phox and urinary excretion of 8-iso-PGF₂α were downregulated in X-CGD patients (P < 0.001 and P = 0.04) and increased in obese patients (P = 0.01 and P ≤ 0.001) compared with healthy subjects (the Table). On the other hand, obese patients had higher serum ox-LDL than healthy subjects and X-CGD patients (P < 0.001), and X-CGD patients and healthy subjects had similar values of serum ox-LDL (the Table). Furthermore, X-CGD patients had significantly higher FMD (P < 0.001) and serum levels of NOx (P = 0.016), markers of NO generation,21 compared with healthy subjects (Table 1). Conversely, obese patients had lower FMD (P = 0.028) and serum NOx levels (P = 0.001) compared with healthy subjects (the Table). IMT was significantly lower in patients with X-CGD (P < 0.001) but significantly higher in obese individuals (P = 0.004) compared with healthy subjects (the Table).
No difference was found among the brachial resting vessel size of the 3 groups (X-CGD, 2.90 mm [median] [interquartile range 2.48 to 3.60]; healthy subjects, 3.27 mm [interquartile range, 2.69 to 3.75]; obese patients, 3.30 mm [interquartile range, 2.90 to 4.0]). However, we stratified patients according to the median value observed in healthy subjects (3.27 mm) and found that X-CGD patients with basal brachial artery diameter >3.27 mm still showed significantly higher values of FMD ($F_{\Delta} = 13.0, P<0.001$) than healthy subjects and obese patients (12.3±1.1% versus 7.2±17% versus 5.4±3.6%, respectively).

Correlation analysis carried out by Spearman test showed that FMD inversely correlated with platelet gp91phox expression ($R_s = -0.502, P<0.001$; Figure 1A) and urinary excretion of 8-iso-PGF$_{2\alpha}$ ($R_s = -0.513, P<0.001$). Moreover, FMD directly correlated with serum NOx levels ($R_s = 0.403, P<0.001$; Figure 1B). The latter, in turn, significantly correlated with platelet gp91phox ($R_s = -0.515, P<0.001$; Figure 1C). IMT significantly correlated with FMD ($R_s = -0.303, P=0.008$), platelet gp91phox ($R_s = 0.398, P<0.001$), NOx ($R_s = -0.433, P<0.001$), and urinary excretion of 8-iso-PGF$_{2\alpha}$ ($R_s = 0.334, P=0.003$).

Finally, a substudy of age-matched X-CGD patients ($n=7$), healthy subjects ($n=7$), and obese patients ($n=7$) was performed to investigate platelet formation of NO and isoprostanes. The clinical characteristics of enrolled subjects did not differ from those of each subgroup study. After platelet stimulation with collagen, patients with X-CGD had significantly higher NO platelet production (35.5±10.0 μmol/L) compared with healthy subjects (22.5±12.0 μmol/L, $P<0.05$), whereas obese individuals had lower NO production compared with healthy subjects (11.2±2.5 μmol/L, $P<0.001$; Figure 2A). Moreover, 8-iso-PGF$_{2\alpha}$ formation in platelets stimulated with collagen was lower in X-CGD patients (124.4±104.8 pmol/L; $P<0.05$) and higher in obese patients (851.8±222.8 pmol/L; $P<0.001$) compared with healthy subjects (373.4±59.2 pmol/L; Figure 2B).

**Discussion**

This study provides the first evidence of an increased FMD in patients with hereditary deficiency of gp91phox, suggesting a role for this ROS-generating pathway in modulating arterial tone. We also show that gp91phox is relevant for the isoprostane formation, given that both systemic and cellular formation of isoprostanes is significantly reduced in gp91phox-deficient patients.

Because NADPH oxidase is the most important cellular producer of superoxide anion,22 we investigated its role in the formation of oxidant species such as isoprostanes and ox-LDL. We found that only urinary excretion of isoprostanes was significantly reduced in X-CGD patients compared with healthy subjects. Isoprostanes are chemically stable free-radical catalyzed products of arachidonic acid.23 So far, the ROS-generating pathway eliciting isoprostane formation has not been fully clarified. Here, we provide evidence that gp91phox activation may play a pivotal role in the formation of isoprostanes because patients with gp91phox knockout had significantly reduced urinary excretion of isoprostanes. Monocytes and platelets express gp91phox and produce isoprostanes.26 Therefore, it is conceivable that the reduced formation of isoprostanes reflects functional deficiency of...
There is increasing evidence that cardiovascular disease such as those with obesity, the coexistence of high values of different markers of oxidative stress suggests that other ROS-generating enzymes are involved in generating this marker of oxidative stress. An implication of this finding is that the increase in ox-LDL in obese patients should be attributed to other ROS-generating enzymes, which is in agreement with our previously published observations of a significant association between serum ox-LDL and myeloperoxidase, which suggested a role for this enzyme in generating ox-LDL in vivo. Hence, in patients at risk of cardiovascular disease such as those with obesity, the coexistence of high values of different markers of oxidative stress suggests that >1 ROS-generating pathway is upregulated.

Several experimental studies have shown a pivotal role of NADPH oxidase in modulating arterial tone. In an animal model of NADPH oxidase knockout, an increased arterial dilation compared with wild-type animals was observed. Furthermore, individuals with impaired dilation show an overexpression of the NADPH oxidase subunit p47phox in endothelial cells, but a cause-effect relationship between FMD and NADPH oxidase activation was never demonstrated. The results achieved in our human knockout model of NADPH oxidase are consistent with animals studies in that X-CGD patients had a higher FMD compared with healthy subjects.

FMD is prevalently dependent on NO release from endothelium, as also suggested by the significant correlation between FMD and plasma nitroso compounds. Oxidative stress seems to play a pivotal role in modulating FMD, likely through altered NO bioavailability and biosynthesis. Accordingly, oxidative stress and FMD were inversely related in patients with or without metabolic syndrome, and a 1-time infusion of an antioxidant such as vitamin C was associated with rapid restoration of arterial function. In this study, NOx serum levels were higher in X-CGD patients and lower in obese individuals compared with healthy subjects. The direct correlation between NOx and FMD suggests a role for NO in the enhanced arterial dilatation observed in X-CGD patients. This suggestion is corroborated by the finding of a lower NOx production in obese patients who had, in accordance with a previous report, reduced FMD. We recognize, however, that determination of NOx may be an unreliable method to measure NO generation. This may depend on the fact that NOx is influenced by many endogenous and exogenous factors, including dietary nitrate uptake, inhalation of atmospheric nitrogen oxides, salivary formation, and renal function. Even if we cannot exclude that such factors may have influenced NOx serum levels, platelet production of NO paralleled NOx in serum; thus, platelet generation of NOx was increased in X-CGD, suggesting that cellular production of NO was upregulated.

A potentially intriguing observation is the striking difference in FMD between healthy subjects (8%) and X-CGD patients (15%). It is possible that during the evolution phase of human beings, maximization of innate immune defense mechanisms against infectious disease needed “upregulation” of NADPH oxidase. Because NADPH oxidase is also expressed in the vascular wall, this could result in higher vascular oxidative stress and ensuing lowering of the arterial relaxation capability.

IMT is a noninvasive diagnostic measure of atherosclerosis that correlates with histology and predicts cardiovascular events, including myocardial infarction and stroke. IMT is already increased in children with cardiovascular risk factors, suggesting that it may be a good tool to measure premature atherosclerosis. Previous studies have shown a direct correlation between oxidative stress and IMT, suggesting a role for oxidative stress in eliciting arterial damage. Indirect evidence in support of the role of NADPH oxidase in enhancing IMT has also been reported. In fact, in asymptomatic subjects free of overt atherosclerosis, phagocyte production of superoxide anion significantly correlated with IMT, but direct evidence of NADPH activation was not provided. In our X-CGD population, in whom gp91phox was functionally deficient, we observed a significant decrease in IMT compared with healthy subjects. However, these data should be considered cautiously because the difference was very small and may not firmly reflect a slower atherosclerotic progression in X-CGD patients.

This study has potential limitations. In particular, for ethical reasons, we did not have the possibility of discriminating whether the enhanced arterial dilatation was dependent on endothelium. Therefore, the role of NO in enhancing arterial dilatation was not fully elucidated. However, FMD is mostly dependent on endothelial release of NO. As a result, it is possible that enhanced NO generation and/or bioactivity resulting from low oxidative stress may be responsible for the enhanced arterial dilatation of X-CGD patients. This hypothesis is consistent with our previous report in which the administration of N-nitro-L-arginine methyl ester, an inhibitor of endothelial NO synthase, to adult X-CGD patients abolished the increase in FMD. Reduced isoprostane formation could be an alternative mechanism because isoprostanes are vasoconstrictor molecules, although it has recently been argued that isoprostanes may have such property in vivo.

Despite the increase in NO generation, X-CGD patients had normal arterial diameter and blood pressure. A possible interpretation of this finding is that a resting increase in NO (33% versus control) was insufficient to change such parameters. Thus, it is of note that in the group with obesity and high blood pressure, NO generation was reduced by 65% compared with control subjects.

Even if in obese patients gp91phox upregulation could contribute to lower FMD, it is possible that other mechanisms may contribute to lowering FMD in this setting. For instance, ox-LDL, which was not influenced by gp91phox, could play a role because its increase may directly affect NO and...
isoprostanes\(^9\); however, the relationship between ox-LDL and FMD is still unclear.\(^{40–42}\)

Finally, we must take into account the possibility that some antibiotics may favorably affect FMD either directly, as in the case of azithromycin,\(^{43}\) or indirectly through reduced serum cholesterol levels.\(^{44,45}\) Reduced serum cholesterol levels, in fact, are known to inversely correlate with FMD,\(^8\) but this potential bias was eliminated in our study by the inclusion of healthy subjects with comparable serum cholesterol. Moreover, no data on the effects of the antibiotics used by our X-CGD patients (itraconazole, trimethoprim, and sulfamethoxazole) on FMD have been reported so far.

**Conclusion**

In this human knockout model of NADPH oxidase, arterial dilatation was enhanced, thus providing the first evidence that this ROS-generating pathway is implicated in modulating arterial tone.

**Acknowledgments**

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**Disclosures**

None.

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28. Wolford RM, Stokes KY, Granger DN. CD4+ T lymphocytes mediate hypercholesterolemia-induced endothelial dysfunction via a NAD(P)H
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**CLINICAL PERSPECTIVE**

Arterial dysfunction is a hallmark of early atherosclerosis and predicts cardiovascular events. The mechanisms accounting for arterial dysfunction in humans are still unclear. NADPH oxidase is an enzyme that plays a major role in producing reactive oxygen species in the cells. Reactive oxidant species formation is associated with inhibition of nitric oxide activity and/or bioavailability and may have deleterious effects in arterial function. We conducted a multicenter study to measure flow-mediated dilation in 25 patients with chronic granulomatous disease (X-linked CGD) that is characterized by hereditary deficiency of gp91phox, the catalytic subunits of NADPH oxidase. Compared with 25 healthy subjects and 25 obese patients, X-CGD patients had lower oxidative stress, as assessed by urinary excretion of isoprostanes, and higher flow-mediated inhibition of NADPH oxidase. Gp91phox expression increased progressively from X-CGD to obese patients, who showed lower flow-mediated dilation compared with the other 2 groups. In the entire cohort, gp91phox expression inversely correlated with flow-mediated dilation. In a subgroup study, platelet formation of nitric oxide was also determined. This analysis showed a progressive decrease in nitric oxide from X-CGD to obese patients. Together, these findings suggest that vascular tone is modulated by NADPH oxidase–generating reactive oxidant species with a mechanism involving nitric oxide generation. Systemic and cellular measurement of NADPH oxidase expression could have a relevant impact on our understanding of the role of reactive oxidant species in the initiation and progression of atherosclerosis. This could be a useful tool for exploring whether inhibition of NADPH oxidase is able to halt the atherosclerotic process and its clinical sequelae.
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