ASSOCIATON BETWEEN NITRITE LEVELS AND SURVIVAL IN OLDER ADULTS

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Short running title: Nitrite and Older Adults Survival

Abstract

Aim: A previous study suggested that nitric oxide plasma levels might be associated with aging and survival in older adults participants; however a need for further evidence from complementary studies was identified. We assessed levels of plasma nitrite, an indicator of nitric oxide metabolism, other biochemical, anthropometric and physiological variables and other health and lifestyle indicators in the older adults of a free-living Amazonian community.

Methods: We assessed 588 participants in two categories, younger elderly (>60 to < 69 years old, n=274) and older elderly (> 70 years old, n=314) over three years; the main outcome was survival rate.

Results: After three years 74 (12.6%) participants had died. As expected, age was associated with mortality independent of other variables [OR= 2.262, 95% CI-1.296-3.947]. In older elderly with higher nitrite values (> 42 µmol/L) were associated with increased mortality. In Kaplan–Meier analyses of mortality older elderly in 4th quartile of the nitrite level distribution had a worse prognosis than those in the lower quartiles. Cox multivariate regression analysis showed that this association was independent of gender, hypertension, type 2 diabetes, obesity, smoking, previous cardiovascular and other morbidities, daily intake of medicine, education level and marital status.

Conclusions: The results corroborate the hypothesis that nitrite has potential as a predictive indicator of survival and suggest that nitric oxide may play a considerable role in aging processes and hence in age-related diseases and deficits.

Key Words: Aging; Cardiovascular risk; Older Adults; Nitric oxide; Prognostic marker.

Resumo

Objetivo: Um estudo anterior sugeriu que os níveis plasmáticos de óxido nítrico podem estar associados ao envelhecimento e à sobrevivência de pessoas idosas; no entanto, foi identificada a necessidade de mais evidências de estudos complementares. Avaliamos os níveis de nitrito plasmático, um indicador do metabolismo do óxido nítrico, outras variáveis bioquímicas, antropométricas e fisiológicas e outros indicadores de saúde e estilo de vida em pessoas idosas de uma comunidade amazônica de vida livre.

Métodos: Foram avaliados 588 participantes em duas categorias, pessoas idosas mais jovens (>60 a < 69 anos, n=274) e pessoas idosas mais velhas (> 70 anos, n=314) ao longo de três anos; o principal resultado foi a taxa de sobrevivência.

Resultados: Após três anos, 74 (12,6%) participantes faleceram. Como esperado, a idade foi associada à mortalidade independente de outras variáveis [OR= 2,262, IC 95%-1,296-3,947]. Em pessoas idosas mais velhas, valores mais elevados de nitrito (> 42 µmol/L) foram associados ao aumento da mortalidade. Nas análises de mortalidade de Kaplan-Meier, as pessoas idosas mais velhas no 4º quartil da distribuição do nível de nitrito tiveram um pior prognóstico do que aqueles nos quartis inferiores. A análise de regressão multivariada de Cox mostrou que essa associação era independente de sexo, hipertensão, diabetes mellitus tipo 2, obesidade, tabagismo, morbidades cardiovasculares e outras morbidades prévias, ingestão diária de medicamentos, escolaridade e estado civil.

Conclusões: Os resultados corroboram a hipótese de que o nitrito tem potencial como indicador preditivo de sobrevivência e sugere que o óxido nítrico pode desempenhar um papel considerável nos processos de envelhecimento e, portanto, nas doenças e défices relacionados com a idade.

Palavras-chave: Envelhecimento; Risco Cardiovascular; Idosos; Óxido Nítrico; Criador de prognóstico.

Introduction

Although a number of risk factors for cardiovascular morbidity and mortality in young and middle-aged adults have been identified, is well known that the prevalence and predictive value of these factors in older participants is less clear.[1] Aging has a remarkable effect on the heart and arterial system; it is an independent risk factor for development of atherosclerosis and is associated with impaired angiogenesis and endothelial dysfunction.[2] In this context the epidemiological evidence that some older adults persons do not develop important cardiovascular dysfunction and diseases is relevant to the search for biomarkers of survival to advanced age, such as plasma nitrite level, an indicator of nitric oxide (NO) metabolism.

NO is synthesized by three different enzymes depending on cell and tissue type: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) nitric oxide synthases. NO derived from endothelium is a potent vasorelaxant and participates in highly metabolically active and regulatory functions including control of hemostasis, fibrinolysis, platelet and leukocyte interactions with the arterial wall, presentation of histocompatibility antigens, regulation of vascular tone and regulation of blood pressure.[3] It is possible to carry out quantitative analysis of nitrite and nitrate (NOx), the major stable metabolites of endogenous NO.[4]

Some investigations have reported that higher plasma NO concentration is associated with severity of heart failure in cardiovascular disease (CVD) patients.[5] These results suggest that NO imbalance affects multiple aging processes and consequently the health status of older adults people. In view of the potential relevance of NO to human aging processes, the purpose of this study was to investigate potential associations between plasma nitrite levels and cardiovascular risk factors and other health and lifestyle indicators, as well as their association with older adults mortality, in a sample of elderly in a free-living Amazonian community over a three-year period.

Materials and Methods

Sample

The study is part of an epidemiological research project titled 'Amazon Rainforest Elderly Project' which is investigating gene-environment interactions in aging and age-related diseases among self-identified 'caboclos'. The caboclos are a riverine population living in a Brazilian tropical region; they are the main population group in Amazonia, a region with mixed genetic and cultural ancestry (African, indigenous, and European). [6] Due the relative geographical isolation and homogeneity of socioeconomic, cultural and lifestyle variables in this population we believe that it is a suitable substrate for researching the biology of human aging in a relative non-industrialized environment. The study was carried out in Maués city, Amazonas State (3° 23' 0.996"S 57° 43' 6.9954"W), because it has a relatively high older adults population - particularly participants ≥ 80 years old[7] - in comparison with other Amazonas cities, and almost all the older adult people living there were born in Maués (73.3%) or in neighboring cities in which similar conditions prevail (20.2%).

This study was approved by the Ethical Committee of the Universidade do Estado do Amazonas and each participant provided written or fingerprint indication of informed consent. Details of the data collection procedures have been published elsewhere. [8,9] In countries such as Brazil, and particularly in undeveloped areas such as the Amazonian region, individuals are considered 'older adult' when they reach 60 years. We grouped our sample into two age categories: younger elderly (≥60 < 70 years old) and older elderly (≥70 years old).

Study Design

The design includes both cross-sectional and longitudinal components. In July 2009 Maués had a total of 45.284 habitants. We enrolled 637 of the population of 1742 older adults (≥ 60 years) living in Maués's urban area in this study, however data including NO levels were collected from only 588 individuals (men = 271,46.3%; women = 317, 53.7%) with a mean age of 71.96 years ± 7.96 (range: 60-99 years).

The composition of the sample by age category was as follows: younger elderly = 274 (46.6%); older elderly = 314 (53.4%).

Variables and Data Collection

Demographic data (education, income, marital status, occupation) as well as data on lifestyle (smoking), CVD risk factors (hypertension, type 2 diabetes, obesity, dyslipidemia and metabolic syndrome), history of previous chronic diseases and indicator variables were collected using structured interviews. Details of data collection processes have been published elsewhere.[9] Briefly, all subjects included in the study were previously analyzed (2009 July) by a research team that included physicians, nurses, nutritionists, pharmacologist, biologist and physical educators. In the occasion, the general health conditions as well as anthropometric and other physiological analysis were performed in the sample analyzed. All subjects included in the study were free-living community, healthy or with controlled diseases.

Systolic and diastolic blood pressure (SBP and DBP respectively) were measured using a standard desk mercury sphygmomanometer (Wanross®) and stethoscopes (Littman®) at least 30 minutes after caffeine intake or cigarette smoking. The anthropometric variables measured were height (meters) and weight (kilograms). Body mass index (BMI) was calculated as weight divided by the square of the height. Waist circumference was measured with a tape measure midway between the lowest rib and the iliac crest whilst participants were standing. Metabolic syndrome (MS) was diagnosed in participants who met three or more of the following criteria (NCEP ATP III)[10]: abdominal obesity (waist circumference ≥102 cm or ≥88 cm in men and women respectively); triglyceride level ≥150 mg/dl; low level of high-density lipoprotein-cholesterol (HDL-c) (men ≤40 mg/dl; women ≤50 mg/dl); blood pressure ≥ 130/85 mm Hg); fasting glucose ≥110 mg/dl.

Peripheral blood samples from all participants were collected after an overnight fast using the venous puncture technique. Samples were collected into Vacutainer® (BD Diagnostics, Plymouth, UK) tubes with EDTA or no anticoagulants and were routinely centrifuged. Total cholesterol, HDL-C, triglycerides and glucose were determined by enzymatic colorimetric methods performed using standard equipment

(Labtest, Lagoa Santa-MG, Brazil). Low-density lipoprotein-cholesterol (LDL-c) levels were calculated using the Friedwald formula.[11] Nitrite levels were measured using the Griess method[12] with the Cobas Mira® automated analyzer (Roche Diagnostics, Basel, Switzerland), used as indirect measure of nitrite levels. Only one analysis of nitrite levels was performed in cohort investigated here due difficulties related to geographic access of the population. The blood sample used in the analysis was obtained after 12 h fasting, and therefore represent the NO levels in the morning.

Prospective Protocol

Participants were followed for three years to evaluate survival in riverine elderly. Official death records with dates and specific causes of death were available for all deceased participants. The strength of the association between plasma nitrite levels and mortality was evaluated and additional analyses were performed to uncover other associations between older adults survival and other demographic, lifestyle and health variables.

Statistical Analysis

Continuous variables are presented as mean ± standard deviation (SD) and categorical variables as number and percentage. In the cross sectional analysis we initially carried out group comparisons, taking into consideration socioeconomic and cultural factors. Student's *t* test was used for comparisons of quantitative variables and the chi-square test for categorical variables. Analysis of variance followed by Bonferroni *post hoc* tests was used to compare different nitrite level quartiles. Nitrite level quartile boundaries were as follows: quartile 1 = 7-14 µmol/L (158 participants); quartile 2 = 15-24 µmol/L (141 participants); quartile 3 = 24-42 µmol/L (142 participants) and quartile 4 = 42-77 µmol/L (147 participants). Additional multivariate logistic regressions (Backward Wald Method) were used to assess the influence of intervening variables on associations between sex, socioeconomic, cultural, health and lifestyle indicators and mortality. Survival of younger and older elderly was calculated using the Kaplan-Meier method and a multivariate Cox proportional hazards model

based on time from 3-year follow up to death. All tests were two-tailed. Odds ratios (OR) and 95% confident intervals (95% CIs) were calculated to estimate the mortality risk associated with age group status, health variables, nitrite levels. The criterion for statistical significance was $p < 0.05$ in all tests.

Results

The baseline characteristics of younger and older elderly are shown in Table 1. The older elderly group had a smaller proportion of men, had less education and higher proportions of retired or widowed participants than the younger elderly group. The prevalence of CVD risk factors, previous CVD and other morbidities and smoking was similar in the two groups. A higher proportion of older elderly reported daily use of medicine. The groups were similar in terms of biological and biochemical variables except that the older elderly group had lower HDL-c levels and higher plasma nitrite levels.

Age was correlated with nitrite level (Figure 1-A). Multivariate analysis showed that the association between HDL-c and age was independent of gender, smoking, hypertension, type 2 diabetes, obesity, previous CVD and daily intake of medicine (OR = 0.985, 95%CI = 0.974-0.976, p = 0.0028). The association between nitrite levels and age was also independent of these variables (OR = 1.019, 95%CI = 1.010-1.028).

Separate comparisons of plasma nitrite level quartiles were performed in the two groups. All biological and biochemical variables except LDL-c and HDL-c were similar across nitrite quartiles in both the younger and older elderly groups (Figure 1-B). In the younger elderly group LDL-c levels were higher ($p = 0.022$) in the 3rd and 4th quartiles (149.5 ± 42.5 mg/dL and 154.9 ± 42.1 mg/dL respectively) than the 1st and 2nd quartiles (130.9 ± 47.6 mg/dL and 131.2 ± 34.7 mg/dL respectively). Multivariate analysis showed that gender influenced the association between LDL-c and plasma nitrite level. In younger elderly women but not men LDL-c levels were higher in the 3rd and 4th nitrite level quartiles than the lower quartiles (p = 0.003).

HDL-c levels were lower in the 3rd and 4th nitrite quartiles than the 1st and 2nd quartiles (p = 0.0001) (Figure 2). Multivariate analysis showed that association

between HDL-c levels and nitrite level was independent of gender, obesity, hypertension, diabetes and smoking habit (p = 0.0001). Participants in the 3rd and 4th nitrite level quartiles were approximately twice as likely as participants in the lower nitrite quartiles to have HDL < 50 mg/mL (OR= 2.682, CI 95% = 1.904-3.779). The prevalence of CVD risk factors and levels of other health indicators were similar across nitrite quartiles in both younger and older elderly groups. The results were maintained when a complementary multivariate analysis was performed to assess the influence of gender.

Seventy-four participants (12.6%) had died after three years. The primary cause of death was recorded in only 38% of cases, as 62% had died without medical attendance. In the cases for which a primary cause was specified 53% died from CVD or cerebrovascular morbidities, 32% from infectious lung diseases, mainly pneumonia and 16% from other causes.

As expected there was an association between age and mortality independent of other variables. After three years 18 participants (7.1%) in the younger group and 56 (14.7%) in the older group had died (p = 0.003; OR = 2.262, 95% CI- = 1.296-3.947). The results of the adjusted Cox regression assessing the associations between survival and nitrite quartiles, CVD risk factors or other health indicators are presented in Table 2. In the older elderly group, we found association between survival and nitrite levels (Figure 3). Older elderly participants with higher nitrite levels (quartile 4) were more likely to have died during the study period than those with lower nitrite values (quartiles 1, 2 and 3). Cox regression multivariate analysis showed that the association between higher nitrite levels and mortality in older elderly was independent of gender, hypertension, type 2 diabetes, obesity, smoking, previous CVD and other morbidities, daily intake of medicine, education level and marital status; the other variables investigated were not associated with mortality in older elderly.

Discussion

Plasma nitrite levels were higher in older Amazonian riverine elderly than younger Amazonian riverine elderly. Having nitrite levels in the upper quartile of the distribution was associated with higher three-year mortality in older elderly participants

independent of other CVD risk factors and health indicators. Aluzic et al.[13] reported that NOx levels were higher in older than younger participants. Osawa et al.[14] assessed NOx in Japanese patients over about three years and found that mortality was higher in the upper quartiles of the distribution. Our replication of this finding has important implications in the context of the differences between the two populations; participants in the Japanese study were living in a highly industrialized and urbanized region whereas our participants were Amazonian riverine older adults living in a non-developed area in a tropical rainforest. It should be noted however that in our study the association between plasma nitrite levels and mortality was limited to older elderly (> 70 years old).

NO production is cell- or tissue-specific and depends on physiological conditions. Differential regulation of NO is orchestrated by changes in the expression of the three isoforms of NO synthase which use arginine and molecular oxygen as substrates and require some cofactors. In young people and adults iNOS is not usually constitutively produced by cells; its expression is regulated by internal and external stimuli such as bacterial lipopolysaccharide, cytokines and other immunological agents. Induction of the iNOS enzyme allows cells to produce large amounts of NO which act as a cytotoxin. During aging iNOS expression becomes continuous and extends to cardiovascular tissues[3]. A review by Cau et al.[15] summarized evidence of increased iNOS expression in aged vessels, mainly in the intima layer.

The increase in plasma nitrite levels in older people may be related to chronic inflammation processes which are widespread in this population. In the light of the relationship between inflammatory states and aging Jenny[16] recently suggested that aging could be regarded as a progressive degenerative process that contributes to modulation of both inflammation and age-related deficits and impairments.

NO mediates the inflammatory response via activation or inactivation of various molecules on several pathways. NO levels determine whether this molecule acts as a pro-inflammatory or ant-inflammatory mediator. In many cases the formation of peroxynitrite is thought to be responsible for some of the pro-inflammatory actions of NO.[17] Under these conditions, chronic inflammation is established if the inflammatory

stimulus is not removed; this causes alterations in homeostatic processes and results in metabolic dysfunction.

Previous research has shown that upregulation of NO caused by induction of iNOS may cause a pathophysiological depression in myocardial contractile function and have a cytolytic effect on cardiac myocytes.[18] Production of a large amount of NO by iNOS has also been associated with oxidative myocardial damage in myocarditis.[18] Elevated levels of NO produced by iNOS also contribute to the formation of peroxynitrite, which causes lipoperoxidation and may promote electrophysiological remodeling resulting in contractile dysfunction and energetic impairment due to oxidative damage.[18] These findings suggest that iNOS expression accounts for nitrosative stress and disrupted vascular homeostasis.[15]

Before we discuss the potential role of plasma nitrite as predictive biomarker of survival in older elderly it is important to consider our negative findings with respect to the classical CVD risk factors. The lack of association between CVD risk factors and mortality in riverine elderly is consistent with previous research. Epidemiological studies have consistently identified LDL-c and HDL-c as independent CVD risk factors.[19] Several lines of evidence suggest that increasing HDL-c decreases the risk of developing CVD as it has a number of important effects on endothelial cells, including reducing the numbers of adhesion molecules and increasing endothelial NO production.[20,21]

Other investigations have suggested that the relative risk of HDL-c and LDL-c decreases with advancing age.[22] Our results corroborate this hypothesis since we did not find an association between lipid levels and mortality in riverine elderly. We did however find an important independent negative association between HDL-c and nitrite levels when we analyzed associations between plasma nitrite levels and other biochemical variables. This association is biologically plausible as previous research has reported interactions between lipid levels and eNOS generation, especially in relation to atherosclerosis. Chikani et al.[23] observed that eNOS activity and the consequent generation of NO was influenced by several lipids including HDL-c. At present the pattern of associations among HDL-c and other plasma lipids, plasma

nitrite and iNOS activity are unclear. Our results suggest there may be an association between HDL-c and nitrite levels, but further research will be needed to confirm this.

Hypertension is an important CVD risk factor and is prevalent in elderly people due to the age-related increase in blood pressure. The marked increase in prevalence of hypertension in elderly populations is largely attributable to age-related changes in arterial structure and function. Hypertension is associated with mortality in young and middle-aged people but the association is attenuated in the elderly population. Analysis of data from a National Health and Nutrition Examination Survey that included 2340 participants aged > 65 years suggested elevated BP in the elderly was associated with increased mortality in fast- but not slow-walking older adults.[24] We cannot disregard the possibility that other intervening variables, mainly related to frailty, in our population may have contributed to the lack of association between hypertension or blood pressure levels and mortality observed in this study.

The main methodological limitation of this research is related to potential under-estimation of the prevalence of certain chronic diseases and the inability to determine cause of death in many of our elderly participants owing to the lack of clinical diagnostic and medical assistance in Maués-AM. Despite these limitations notwithstanding we believe that the results described here contribute to our understanding of the role of NO in aging and mortality risk in the elderly. These data provide support for McCann's hypothesis[25,26] and Afanas'ev's suggestion[27] that NO is one of the molecules largely responsible for aging processes and has an important role in age-related diseases due to its role in the control of physiological functions throughout the body. The 'acceptable' range of plasma nitrite concentration remains an open question.

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Disclosure statement

The authors have no financial disclosures or other conflicts of interest to report.

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Tables

Table 1 Biochemical, anthropometric and physiological characteristics of Amazonian riverine elderly in two age categories.

Variables		Younger Elderly (60-69 years inclusive)	Older Elderly ≥70 years)	*p*
n (%)				
Sex (n, %)	Male	100 (36,5)	171 (54,4)	0.053
	Female	174 (63,5)	143 (45,6)	
Education	Illiterate	149 (61.3)	258 (74.8)	
	< 4 years	59 (17.1)	54 (22.2)	0.0001
	4-8 years	15 (4.3)	18 (7.4)	
	> 7 years	13 (3.8)	22 (.1)	
Marital status	Married	146 (53.9)	180 (53.3)	0.0001
	Widowed	80 (29.5)	92 (29.0)	
	Single	37 (13.7)	41 (12.3)	
	Divorced	08 (3.0)	14 (5.0)	
Occupation	Retired	174 (68.2)	302 (96.3)	0.0001
Income	≤ U$ 250/monthly	146 (54.3)	213 (55.8)	0.709
Smoking habit		32 (12.5)	44 (11.5)	0.694
HAS		110 (43.1)	187 (49.0)	0.149
Obesity		32 (12.5)	47 (12.3)	0.409
Diabetes type 2		35(13.7)	51 (13.4)	0.927
Metabolic syndrome		27 (10.6)	33 (8.6)	0.892
CVD		20 (7.8)	22 (5.8)	0.299
Other morbidities		87 (34.1)	135 (35.6)	0.751
Smoking		32 (12.5)	44 (11.5)	0.694
Daily intake of medicine		111 (43.5)	203 (53.1)	0.017
Mean ± SD				
Age (years)		64.5 ± 2.9	77.3 ± 5.81	0.0001
BMI (Kg/m^2)		25.4 ± 4.9	25.1 ± 4.6	0.441
Waist circumference (cm)		87.0 ± 15.4	89.2 ± 13.2	0.069
SBP (mmHg)		127.6 ± 29.8	129.6 ± 24.5	0.387
DBP (mmHg)		72.4 ± 15.9	73.5± 13.6	0.370
Glucose (mg/dL)		126.8 ± 57.7	120.7 ±44.9	0.124
Cholesterol total (mg/dL)		209.3 ± 50.4	205.6 ± 53.7	0.121
Triglycerides (mg/dL)		158.9 ± 68.9	169.8 ± 92.8	0.213
LDL-cholesterol (mg/dL)		139.3 ± 43.7	143.9 ± 53.3	0.621
HDL-cholesterol (mg/dL)		54.2 ± 39.4	47.3 ± 3.4	0.031
Nitrite (µmol/L)		27.1 ± 20.2	34.8 ± 24.4	0.0001

BMI= body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HAS= hypertension; CVD= cardiovascular disease. Results with $p \leq 0.05$ were considered significant.

Table 2 Multivariate analysis of variables predicting overall survival of riverine elderly

Variables		Younger		Older	
		Multivariate-adjusted hazard ratio†	***p***	Multivariate-adjusted hazard ratio	***p***
Sex	Male	1.883 (0.743-4.772)	0.180	1.159 (0.685-1.959)	0.583
	Female	0.863 (0.511-1.459)		0.531 (0.210-1.345)	
Hypertension		1.309 (0.519-3.297)	0.568	1.425 (0.837-2.426)	0.216
Obesity		0.397 (0.131-1.207)	0.104	0.859 (0.505-1.460)	0.574
Diabetes type 2		0.712 (0.206-2.459)	0.591	1.434 (0.569-3.616)	0.445
Metabolic syndrome		1.269 (0.367-4.384)	0.706	1.469 (0.742-2.912)	0.270
Smoking		0.727 (0.210-2.511)	0.614	0.853 (0.504-1.444)	0.554
Cardiovascular disease (CVD)		2.525 (0.731-8.724)	0.143	0.863 (0.510-1.459)	0.582
HDL-cholesterol		0.549 (0.072-4.154)	0.561	0.868 (0.444-1.697)	0.680

Nitrite	Quartile 1	5.354 (0.631-45.392)	0.089	0.303 (0.123-0.743)	0.019
	Quartile 2	3.364 (0.371-30.542)	0.259	0.423 (0.203-0.883)	0.022
	Quartile 3	2.773 (0.286-26.859)	0.360	0.605 (0.319-0.883)	0.124
	Quartile 4	0.263 (0.034-2.049)	0.202	2.190 (1.291-3.717)	0.004

† Sex and the other CVD risk factor variables (hypertension, obesity, diabetes type 2, smoking), education, marital status, history of CVD and HDL-cholesterol were included in the multivariate model as covariates.

Figure Legends

Figure 1 Significant Pearson correlations between (A) Nitrite levels and age and (B) Nitrite levels and LDL-cholesterol levels.

Figure 2 Comparison of HDL-cholesterol levels in younger (60-69 years inclusive) and older (≥ 70 years) elderly according to nitrite quartile. Different symbols (†, ‡, §) indicate statistical differences evaluated by analysis of variance followed by Bonferroni post hoc tests.

Figure 3 Cox regression survival analysis of circulating levels of nitrite measured in (A) younger and (B) elderly from Maués, Amazonas, Brazil. For the purposes of this analysis arbitrary quartile boundaries for circulating nitrite level were set at registration.