Cross-Talk Between Nitrosative Stress, Inflammation and Hypoxia-Inducible Factor in Patients with Adrenal Masses

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Background: Adrenal masses are the most common of all human tumors. The role of nitrosative stress and inflammation in cancer development has already been demonstrated. However, it is not known whether they are involved in the pathogenesis of adrenal tumors. The aim of the study was to investigate a cross-talk between nitrosative stress, inflammation and hypoxia-inducible factor (HIF-1α) in 75 patients with different types of adrenal masses (non-functional incidentaloma, pheochromocytoma and Cushing’s/Conn’s adenoma).

Methods: The plasma concentrations of total nitric oxide (NO), S-nitrosothiols, peroxynitrite nitrotyrosine and the activity of serum myeloperoxidase (MPO) were measured spectrophotometrically, whereas concentrations of interleukin 1 beta (IL-1β), tumor necrosis factor α (TNF-α) and hypoxia-inducible factor 1 alpha (HIF-1α) were measured using commercial ELISA kits. The control group consisted of 50 healthy people matched by age and sex to the study group. The number of subjects was determined a priori based on our previous experiment (power of the test = 0.9; α = 0.05).

Results: We found significantly higher nitrosative stress (↑nitric oxide, ↑peroxynitrite, ↑S-nitrosothiols and ↑nitrotyrosine) in the plasma of patients with adrenal tumors, which was accompanied by increased inflammatory (↑myeloperoxidase, ↑interleukin 1 beta and ↑tumor necrosis factor α) and hypoxia (HIF-1α) biomarkers. Peroxynitrite and nitrotyrosine were positively correlated with aldosterone level. Nitrosative stress was also associated with inflammation and HIF-1α. Interestingly, plasma nitrotyrosine and serum MPO differentiated patients with adrenal tumor from healthy individuals with high sensitivity and specificity. Moreover, using multivariate regression analysis, we showed that ONOO− and IL-1β depended on cortisol level, while ONOO−, nitrotyrosine and HIF-1α were associated with aldosterone. Unfortunately, none of the assessed biomarkers differentiated between tumor types studied, suggesting that the severity of nitrosative damage and inflammation are similar in patients with incidentaloma, pheochromocytoma, and Cushing’s or Conn’s adenoma.

Conclusion: Adrenal tumors are associated with increased protein nitrilation/S-nitrosylation and inflammation.

Keywords: adrenal tumors, nitrosative stress, nitric oxide, peroxynitrite, S-nitrosothiols, nitrotyrosine, myeloperoxidase, interleukin 1 beta, tumor necrosis factor, α hypoxia-inducible factor

Introduction

Adrenal masses are usually benign neoplastic lesions detected accidentally during the diagnosis of other diseases. They are the most common of all human tumors, and the frequency of their detection continues to increase, especially with age. 1,2 Although
most of adrenal masses are hormonally inactive, some of them may contribute to the overproduction of cortisol, aldosterone or catecholamines.\textsuperscript{4,5} Even though, adrenocortical carcinomas are very rare, the risk of adrenal masses becoming malignant increases with tumor size. It is estimated that 25\% of adrenal tumors larger than 6 cm may become malignant.\textsuperscript{6} An adrenal masses is associated not only with the risk of malignancy, but also with the development of metabolic disturbances.\textsuperscript{7–9} In patients with phaeochromocytoma, excessive secretion of catecholamines (dopamine, adrenaline and noradrenaline) may lead to the development of obesity and disorders of lipid and glucose metabolism, insulin resistance, diabetes and hypertension.\textsuperscript{7} Cushing’s syndrome also leads to weight gain and, consequently, to the development of obesity and its metabolic complications, while Conn’s syndrome is the cause of treatment-resistant hypertension.\textsuperscript{10,11} Nevertheless, due to the variety of adrenal tumors, their clinical symptoms are not specific and their diagnosis is difficult. The pathogenesis of adrenal masses is still not fully understood. Probably genetic factors have the greatest impact on the development of adrenal tumors.\textsuperscript{12,13} Indeed, the mutation of VHL/HIF axis is the risk factor of pheochromocytoma development.\textsuperscript{14} Hypoxia-induced factor (HIF-1) has various effects on tumor growth and metabolism, as well as metastasis, especially in cancer secreting catecholamines.\textsuperscript{15}

Recent studies emphasize the crucial role of oxidative stress (OS) in the development of cancer.\textsuperscript{16–18} The production of reactive oxygen (ROS) and nitrogen (RNS) species is an inherent process of the oxygen metabolism of cells associated with many physiological and pathological processes in the human body.\textsuperscript{19} OS is closely associated to nitrosative stress.\textsuperscript{20} Nitric oxide (NO) reacts with molecular oxygen, superoxide anion and metal cations, causing the formation of further reactive oxygen species.\textsuperscript{21,22} The excessive formation of ROS and RNS leads to oxidative and nitrosative damage to lipids, proteins and DNA.\textsuperscript{19,23} Proteins are particularly sensitive to the action of ROS and RNS because oxidation or nitrosylation of amino acid residues can lead to their denaturation, fragmentation and aggregation, resulting in impairment of their (proteins’) function or even cell apoptosis.\textsuperscript{24,25} Peroxynitrite (ONOO\textsuperscript{−}) is one of the most cytotoxic reactive forms of nitrogen and the factor with the greatest potential for protein oxidation. It is formed in the reaction of NO with O₂\textsuperscript{−}.\textsuperscript{26,27} Interestingly, its formation is promoted by an increase in inducible NOS isoform (iNOS) activity.\textsuperscript{27} ONOO\textsuperscript{−} in reaction with amino acids leads to irreversible modifications of proteins, which results in the formation of carbonyl groups, as well as nitration, nitrosylation and dimerization of proteins.\textsuperscript{28–30} Moreover, both the formation of modified proteins and an increase in NO concentration can lead to the disruption of many signaling pathways and the initiation of inflammatory processes.\textsuperscript{21,22,31} Indeed, ONOO\textsuperscript{−} and protein nitration products activate the nuclear factor kappa B (NF-κB), which induces the secretion of interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 8 (IL-8) and tumor necrosis factor α (TNF-α), intercellular adhesion molecule 1 (ICAM-1), as well as nitric oxide synthase (NOS).\textsuperscript{32,33} This leads to self-driving further oxidative/nitrosative damage.\textsuperscript{34} NO is also the mediator of inflammation due to its enhancing effect of cyclooxygenases action.\textsuperscript{20} Up to 20\% of tumors are thought to be caused by inflammation. Cytokines produced in excess contribute to the transformation of healthy cells into neoplastic ones.\textsuperscript{35}

In our previous study, we found impaired enzymatic and non-enzymatic antioxidant barrier as well as increased oxidative damage to lipids, proteins, and DNA/RNA in plasma, serum and urine in patients with adrenal tumor.\textsuperscript{36} This indicates a lack of effective protection against oxidative stress in patients with adrenal masses. So far, nothing is known about the role of nitrosative stress and inflammation in the development of adrenal tumors. Moreover, the diagnosis of adrenal tumors is often difficult, therefore the aim of this study was also to search their new diagnostic biomarkers.

## Materials and Methods

The study was designed and conducted according to the Guidelines for Good Clinical Practice and the Declaration of Helsinki, as well as approved by the Bioethics Committee of the Medical University of Bialystok (permission code: R-I-002/66/2015, APK.002.341.2020). All participants of this study gave their informed consent.

75 patients (39 women and 38 men aged from 49 to 65 years) with adrenal masses diameter> 1 cm and <8 cm were qualified for this study. Patients had been diagnosed at internal medicine departments with an endocrinology profile and underwent elective endoscopic adrenalectomy at the 1st Department of General and Endocrine Surgery at the University Hospital in Bialystok, Poland. Depending on the diagnosis, patients were divided into 3 groups: patients with non-functional incidentaloma (n = 25), pheochromocytoma (n = 25) and Cushing’s/Conn’s adenoma (n = 25). In the adenoma subgroup, 14 patients suffered from Cushing’s syndrome and 11 from Conn’s syndrome. Patients diagnosed with Conn’s syndrome were taking potassium or spironolactone.
(aldosterone receptor blocker) in the preoperative period. Patients with phaeochromocytoma were treated with doxazosin (a selective alpha-1-adrenergic receptor blocker) for 10 to 14 days prior to surgery to avoid intraoperative hypertensive crisis. 26 patients had hypertension, that was diagnosed according to World Health Organizations guidelines. Hypertension was diagnosed if measuring on two different days the value of systolic (SBP) and/or diastolic (DBP) blood pressure was 140/90 (mmHg) or above. Blood pressure was measured on the non-dominant upper arm using Diagnosis UA-651 A&D Medical apparatus. The cuff was put on tightly to the exposed arm, about 2–3 cm above the elbow flexion. Two measurements at intervals of several minutes were taken.

The control group consisted of 50 healthy people (25 women and 25 men aged 50 to 65) who underwent a dental follow-up examination at the Specialist Dental Clinic at the Medical University of Bialystok. None of the volunteers had periodontal disease (probing pocket depth (PPD) ≤ 2; bleeding on probing (BOP) < 15) or active caries (decayed teeth < 1). Their full blood counts and biochemical blood parameters (INR, K⁺, Na⁺, creatinine ALT and AST) were within the reference values.

Participants for both the control and study groups were uniformly classified on the basis of a negative history of the following diseases: cardiovascular diseases (other than hypertension), neoplastic diseases, autoimmune diseases (Hashimoto’s disease, ulcerative colitis and Crohn’s disease), diseases of the respiratory, genitourinary and digestive systems, metabolic diseases (insulin resistance, type 1 diabetes, mucopolysaccharidosis, osteoporosis and gout), infectious diseases (HIV/AIDS, hepatitis A, B or C), acute inflammation, abusing alcohol and smoking as well as pregnancy in women. All participants of the study denied taking antibiotics, glucocorticosteroids, nonsteroidal anti-inflammatory drugs and antioxidant supplements (including iron preparations) for three months prior to collecting material for the study. The clinical and laboratory characteristics of the control and study groups are shown in Table 1.

### Blood and Urine Collection

The participants of the study declared, that they did not perform intense physical activity twenty-four hours before blood sampling. All blood samples from patients of control and study groups were collected in a fasting state into serum and EDTA tubes (SARSTEDT, S-Monovette) and centrifuged for 10 minutes at 4°C and 4000 rpm. The urine samples were collected from the first-morning portion of urine from the middle stream immediately after bedtime into a sterile

| Table 1 Clinical and Laboratory Characteristics of the Controls, Incidentaloma, Pheochromocytoma, and Cushing’s/Conn’s Adenoma Patients. Results are Presented as Median with 25% and 75% Percentiles |
|-----------------|-----------------|-----------------|-----------------|
| **Controls (n=50)** | **Incidentaloma (n=25)** | **Pheochromocytoma (n=25)** | **Cushing’s/Conn’s Adenoma (n=25)** |
| **Age** | 60 (51–65) | 25.3 (24.3–30.1) | 29.9 (28.3–31.7) | 26.9 (24.7–29.1) |
| **BMI (kg/m²)** | 23.1 (22.6–23.7) | 26.7 (25.7–27.7) | 29.9 (28.4–31.2) | 29.9 (28.9–31.6) |
| **Glucose (mg/dL)** | 71.7 (69.0–74.4) | 72.5 (70.0–75.0) | 76.0 (72.2–80.0) | 76.0 (72.5–80.0) |
| **Hemoglobin (g/dL)** | 12.5 (10.5–13.0) | 12.1 (10.5–13.0) | 12.5 (10.5–13.0) | 12.5 (10.5–13.0) |
| **Creatinine (µmol/L)** | 1.1 (1.0–1.2) | 1.1 (1.0–1.2) | 1.1 (1.0–1.2) | 1.1 (1.0–1.2) |
| **Urea (mg/dL)** | 20.2 (14.5–24.0) | 20.1 (14.5–24.0) | 20.2 (14.5–24.0) | 20.2 (14.5–24.0) |
| **K⁺ (mmol/L)** | 4.5 (4.3–4.8) | 4.5 (4.3–4.8) | 4.5 (4.3–4.8) | 4.5 (4.3–4.8) |
| **Na⁺ (mmol/L)** | 139 (119–139) | 139 (119–139) | 139 (137–139) | 139 (137–139) |
| **WBC (10³/μL)** | 9.6 (8.25–11.3) | 9.6 (8.25–11.3) | 9.6 (8.25–11.3) | 9.6 (8.25–11.3) |
| **RBC (10³/μL)** | 4.6 (4.3–4.6) | 4.6 (4.3–4.6) | 4.6 (4.3–4.6) | 4.6 (4.3–4.6) |
| **HGB (g/dL)** | 13.2 (13.0–13.4) | 13.2 (13.0–13.4) | 13.2 (13.0–13.4) | 13.2 (13.0–13.4) |

**Notes:** *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 indicate significant differences from the controls; ^ p<0.05, ^^^^ p<0.0001 indicate significant differences from the pheochromocytoma group; ~p<0.05, ~~~p<0.001 indicate significant differences from the Cushing’s/Conn’s group.

**Abbreviations:** BMI, body mass index; CRP, C reactive protein; HGB, hemoglobin; PLT, platelet count; RBC, red blood cell count; WBC, white blood cell count; UA, uric acid.
dihydrochloride). and NEDA·2 HCl (N-(1-naphthyl)-ethylenediamine measured spectrophotometrically method, using sulfanilamide measured spectrophotometrically at 450 nm. The sulfanilamide hexadecyl trimethyl ammonium, ortho-dianisidine dihydrochloride, and hydrogen peroxide were used as reagents. The concentrations of interleukin 1 beta (IL-1β), tumor necrosis factor α (TNF-α) and hypoxia-inducible factor 1 alpha (HIF-1α) were measured using commercial ELISA kits from EIAab, Wuhan, China. We followed the instructions provided by the manufacturer.

### Laboratory Measurements

Full blood count, as well as serum biochemical test (cortisol before 10 a.m., serum aldosterone, glucose Na⁺, K⁺, and urine metanephrine and normetanephrine) were determined using automated blood analyzers (Sysmex XN1000, Abbott Diagnostics, Wiesbaden, Germany). In both groups, the laboratory tests were performed in the Laboratory of Biochemical Diagnostics, University Hospital in Białystok, Poland.

### Nitrosative Stress

All reagents to this study were purchased from Sigma-Aldrich (Nümbrecht, Germany and/or Saint Louis, MO, USA). All determinations were conducted in duplicate samples and results were standardized to 1 mg of total protein. NO concentration was determined in triplicates. The total protein content was analyzed spectrophotometrically using the bicinchoninic acid (BCA) method. Commercial diagnostic kit (Thermo Scientific PIERCE BCA Protein Assay; Rockford, IL, USA) was performed according to manufacturer’s instructions. The absorbance and fluorescence were measured using BioTek Synergy H1 (Winooski, VT, USA) Microplate Reader.

The plasma concentration of total nitric oxide (NO) was measured spectrophotometrically method, using sulfanilamide and NEDA·2 HCl (N-(1-naphthyl)-ethylenediamine dihydrochloride). The plasma concentration of S-nitrosothiols was evaluated spectrophotometrically in the reaction of the Griess reagent with Cu²⁺ ions. The concentration of plasma peroxynitrite was estimated spectrophotometrically according to the reaction of peroxynitrite-mediated nitration resulting in the formation of nitrophenol. The absorbance of NO and S-nitrosothiols was measured at 490 nm, whereas peroxynitrite at 320 nm. The concentration of plasma nitrotyrosine was analyzed spectrophotometrically using ELISA method. Commercial diagnostic kit (Immundiagnostik AG; Bensheim, Germany) was conducted in accordance with manufacturer’s instructions.

The activity of serum myeloperoxidase (MPO) was measured spectrophotometrically at 450 nm. The sulfanilamide hexadecyl trimethyl ammonium, ortho-dianisidine dihydrochloride, and hydrogen peroxide were used as reagents. The plasma concentration of total nitric oxide (NO) was measured spectrophotometrically method, using sulfanilamide and NEDA·2 HCl (N-(1-naphthyl)-ethylenediamine dihydrochloride).

### Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 8.4.3 for MacOS (GraphPad Software, Inc. La Jolla, USA) according to Shapiro–Wilk test, Levene’s test, Kruskal–Wallis ANOVA test and post-hoc Dunn’s test. Statistical significance was established at p ≤ 0.05 and multiplicity-adjusted p-values were calculated for all comparisons. Data was expressed as median with 25% and 75% percentiles. Relations between various parameters were tested by Spearman correlation coefficient. Multivariate analysis of the simultaneous impacts of many independent variables on one quantitative dependent variable was conducted by means of linear regression. Cortisol, metanephrine, normetanephrine and aldosterone were included as independent variables. A 95% CI was reported along with regression parameters. Based on receiver operating characteristic (ROC) curves, the diagnostic value of the analyzed biomarkers, ie, sensitivity and specificity of the test, was evaluated. The number of subjects was determined based on our previous experiment, assuming that the power of the test = 0.9 and α = 0.05. ClinCalc online calculator was used to estimate the sample size.

### Results

Table 1 shows a comparison of the clinical and laboratory characteristics of the controls and patients with adrenal masses. BMI of patients with adrenal masses was higher than the controls. Serum glucose concentration was increased in incidentaloma and Cushing’s/Conn’s adenoma patients compared to the healthy controls. In Cushing’s/Conn’s adenoma patients the serum concentration aldosterone was greater than the controls. Additionally, aldosterone level was higher in Cushing’s/Conn’s adenoma in comparison with incidentaloma patients. Urinary concentrations of metanephrine and normetanephrine were higher in pheochromocytoma group as compared to other groups. The CRP level was greater pheochromocytoma than the controls and incidentaloma patients. We found content of PLT in patients with incidentaloma and Cushing’s/Conn’s adenoma as compared to the controls. Moreover, patients with Cushing’s/Conn’s adenoma have greater amount of PLT than pheochromocytoma one.
Total Plasma Nitric Oxide (NO)

NO total plasma concentration of Cushing’s Conn’s adenoma was increased (+131%, p= 0.0018) as compared to the controls. Additionally, we found significantly lower plasma concentration of total NO in incidentaloma (−71%, p=0.0015) and pheochromocytoma (−55%, p= 0.0174) than Cushing’s Conn’s adenoma patients (Figure 1A).

Plasma Peroxynitrite (ONOO⁻)

Plasma concentration of ONOO⁻ was increased only in Cushing’s Conn’s adenoma (+62%, p<0.0001) patients as compared to the controls. Moreover, the ONOO⁻ plasma concentration in Cushing’s Conn’s adenoma subgroup was higher than incidentaloma (+75%, p= 0.0002) and pheochromocytoma (47%, p= 0.0097) subgroups. We also observed elevated peroxynitrite concentrations in OB

Figure 1 Violin plots of plasma total NO (A), ONOO⁻ (B), S-nitrosothiols (C) and nitrotyrosine (D) of the control, incidentaloma, pheochromocytoma and Cushing’s Conn’s adenoma patients. Results are presented as median with 25% and 75% percentiles. **p<0.01, ****p<0.0001 indicate significant differences from the controls; ~p<0.05, ~~~p<0.001, ~~~~~p<0.0001 indicate significant differences from the Cushing’s Conn’s adenoma group; total nitric oxide (NO), peroxynitrite (ONOO⁻).
+HYP (+44%, p=0.0011) and OB+MS (+60%, p=0.0009) patients compared to OB ones (Figure 1B).

**Plasma S-Nitrosothiols**
We noticed greater plasma concentration of S-nitrosothiols in each group of adrenal masses patients: incidentaloma (+37%, p<0.0001), pheochromocytoma (+42%, p<0.0001) and Cushing’s Conn’s adenoma (+44%, p<0.0001) than the controls (Figure 1C).

**Plasma Nitrotyrosine**
Plasma nitrotyrosine concentration was greater in incidentaloma (+64%, p=0.0008), pheochromocytoma (+88%, p<0.0001) and Cushing’s Conn’s adenoma (+154%, p<0.0001) patients than the controls. Further on, we noticed that Cushing’s Conn’s adenoma (+55%, p=0.0218) patients had increased plasma concentration of nitrotyrosine than incidentaloma patients (Figure 1D).

**Serum Myeloperoxidase (MPO)**
We observed higher plasma concentration of MPO in each group of adrenal masses patients: incidentaloma (+39%, p<0.0001), pheochromocytoma (+41%, p<0.0001) and Cushing’s Conn’s adenoma (+42%, p<0.0001) in comparison with the controls (Figure 2A).

**Interleukin 1 Beta (IL-1β)**
We observed that plasma concentration of IL-1β was significantly increased in patients with adrenal masses: incidentaloma (+33%, p=0.0002), pheochromocytoma (+16%, p=0.0099) and Cushing’s Conn’s adenoma (+42%, p<0.0001) patients in comparison with the controls (Figure 2B).

**Tumor Necrosis Factor α (TNF-α)**
Plasma concentration of TNF-α was greater in each group of adrenal masses patients: incidentaloma (+39%, p<0.0001), pheochromocytoma (+22%, p=0.004) and Cushing’s Conn’s adenoma (+33%, p<0.0001) than the controls (Figure 2C).

**Hypoxia-Inducible Factor 1 Alpha (HIF-1α)**
Plasma HIF-1α concentration was markedly higher in incidentaloma (+12%, p=0.0002), pheochromocytoma (+13%, p<0.0001) and Cushing’s Conn’s adenoma (+18%, p<0.0001) patients than the controls (Figure 2D).

**Correlations**
We found positive correlations between ONOO− and aldosterone (R=0.337, p=0.004), as well nitrotyrosine and aldosterone (R=0.41, p<0.0001). NO total plasma concentration was associated positively with plasma ONOO− (R=0.717, p<0.0001), nitrotyrosine (R=0.434, p<0.0001) and S-nitrosothiols (R=0.313, p=0.006). Plasma ONOO− correlated positively with nitrotyrosine (R=0.711, p<0.0001) and S-nitrosothiols (R=0.314, p=0.006). Plasma nitrotyrosine was positively associated with S-nitrosothiols (R=0.23, p=0.047).

Nitrosative stress was also associated with inflammation. We observed positive correlations ONOO− with MPO (R=0.591, p<0.0001), IL-1β (R=0.356, p=0.002) and TNF-α (R=0.337, p=0.003), as well as nitrotyrosine with MPO (R=0.338, p=0.003), IL-1β (R=0.598, p<0.0001) and TNF-α (R=0.611, p<0.0001). The positive correlations were also seen between total NO and MPO (R=0.441, p<0.0001), and S-nitrosothiols and MPO (R=0.307, p=0.007).

Interestingly, we also found correlations between hypoxia and nitrosative stress, inflammation and clinical parameters. The positive correlations were observed between HIF-1α and ONOO− (R=0.389, p<0.0001), nitrotyrosine (R=0.636, p<0.0001), IL-1β (R=0.418, p<0.0001), TNF-α (R=0.514, p<0.0001) and aldosterone (R=0.274, p=0.019).

BMI was positively associated with serum concentration of glucose (R=0.253, p=0.033) and negatively with urine metanephrine (R=−0.339, p=0.003). Serum concentration of glucose correlated negatively with urine metanephrine (R=−0.346, p=0.003) and normetanephrine (R=−0.246, p=0.0039). We also observed positive correlations between aldosterone and urinary normetanephrine (R=0.261, p=0.026), metanephrine and normetanephrine (R=0.246, p<0.0001) and IL-1β and TNF-α (R=0.78, p<0.0001).

Correlations between nitrosative stress biomarkers and inflammation and clinical parameters are shown in Figure 3.

**Multivariate Regression Analysis**
Using multivariate regression analysis, we showed that concentration of ONOO− and IL-1β depend on cortisol level, while ONOO−, nitrotyrosine and HIF-1α varies with aldosterone. Results of multifactorial regression analysis are shown in Table 2.

**ROC Analysis**
We also analyzed diagnostic usefulness of the estimated nitrosative stress and inflammatory biomarkers. The
results of ROC analysis are presented in Table 3. Interestingly, plasma nitrotyrosine and MPO with high sensitivity and specificity differentiates adrenal tumor patients from the healthy individuals (Table 3).

**Discussions**

The etiology of adrenal tumors is not well understood. Recent studies indicate that some patients are characterized by chromosomal abnormalities, deficiencies in growth factor production as well as diminished antioxidant barrier and higher oxidative stress level. However, the role of NO metabolites and their association with inflammatory molecules is still unknown in patients with adrenal tumors. This study is the first to demonstrate systemic nitrosative stress and inflammation in different types of adrenal masses. Additionally, we have shown that plasma nitrotyrosine

Figure 2 Violin plots of serum MPO (A), plasma IL-1β (B), TNF-α (C) and HIF-1α (D) of the control, incidentaloma, pheochromocytoma and Cushing’s Conn’s adenoma patients. Results are presented as median with 25% and 75% percentiles. **p<0.01, ***p<0.001, ****p<0.0001 indicate significant differences from the controls; myeloperoxidase (MPO), interleukin 1 beta (IL-1β) and tumor necrosis factor α (TNF-α).
may be a potential biomarker differentiating healthy individuals from cases with incidentaloma, pheochromocytoma, and Cushing’s/Conn’s adenoma.

NO is an important signaling molecule involved in the regulation of many physiological processes. This compound also has a second face because, in a high concentration, NO exhibits strong cytotoxic properties. The biological activity of NO is mainly manifested by protein S-nitrosylation and tyrosine nitration. Therefore, in adrenal tumor patients, we observed not only increased NO formation, but also higher S-nitrosothiois and nitrotyrosine levels. NO may be a promoter of carcinogenic nitroamines, while nitrosylation of primary arylamines is directly responsible for its mutagenic effects. It is believed that nitrosative damage to DNA and disruption of replication and transcription processes represent an early stage of tumor development. Nevertheless, NO may also contribute to tumorigenesis by suppressing the immune response and inducing angiogenesis. Indeed, cytokine-activated macrophages produce large amounts of NO, which increase, on a positive feedback, the formation of cytokines, chemokines, and growth factors. This may be confirmed by the positive correlation between NO and MPO in the adrenal tumor patients. Furthermore, nitric oxide reacting with superoxide radical generates peroxynitrite, which is the most potent oxidizing and nitrating agents. In our study, this may be confirmed by the

Figure 3 Correlation heat map between the analyzed nitrosative stress, inflammation and clinical parameters in serum and plasma of the patients with adrenal masses; body mass index (BMI), C reactive protein (CRP), nitric oxide (NO), myeloperoxidase (MPO), interleukin 1 beta (IL-1β), tumor necrosis factor α (TNF-α) and hypoxia-inducible factor 1 alpha (HIF-1α).
| Biomarker   | β1: Cortisol |       | β2: Metanephrine |       | β3: Normetanephrine |       | β4: Aldosterone |       |
|-------------|-------------|-------|-----------------|-------|--------------------|-------|-----------------|-------|
|             | Estimate    | 95% CI | P value         | Estimate | 95% CI | P value         | Estimate | 95% CI | P value |
| Total NO    | −0.9032     | −1.950 to 0.1439 | 0.0897 | −0.01666 | −0.04279 to 0.009469 | 0.2076 | 0.002921 | −0.03319 to 0.03903 | 0.8722 | 0.1679 | −0.3978 to 0.7336 | 0.5556 |
| Peroxynitrite | −0.7573     | −1.481 to 0.03359 | 0.0405 | −0.003478 | −0.02154 to 0.01458 | 0.7019 | −0.00785 | −0.03281 to 0.01711 | 0.5323 | 0.5457 | 0.1547 to 0.9367 | 0.0069 |
| S-nitrosothiols | −0.1579     | −0.4363 to 0.1206 | 0.2619 | −0.0001034 | −0.007052 to 0.006846 | 0.9764 | −0.00007228 | −0.009675 to 0.009530 | 0.9881 | −0.0276 | −0.1780 to 0.1228 | 0.7155 |
| Nitrotyrosine | −0.1827     | −0.8154 to 0.4499 | 0.5663 | 0.0001806 | −0.001561 to 0.001597 | 0.9819 | −0.003021 | −0.02484 to 0.01880 | 0.7831 | 0.3514 | 0.009547 to 0.6932 | 0.0441 |
| MPO         | −0.000006629 | −0.0007959 to 0.0007827 | 0.9867 | −0.000001015 | −0.00002071 to 0.00001868 | 0.9184 | 0.000003794 | −0.000002343 to 0.000003101 | 0.7817 | 0.0000716 | −0.00003548 to 0.00004980 | 0.7386 |
| IL-1β       | −0.04327     | −0.08314 to 0.003399 | 0.0339 | 0.0001613 | −0.00008315 to 0.0001154 | 0.7467 | −0.001012 | −0.002385 to 0.0003614 | 0.146 | 0.01017 | −0.01149 to 0.03184 | 0.352 |
| TNF-α       | −0.0101      | −0.1585 to 0.1383 | 0.8924 | 0.0005452 | −0.003159 to 0.004429 | 0.7699 | −0.003281 | −0.008400 to 0.001838 | 0.2053 | 0.02296 | −0.05723 to 0.1032 | 0.5696 |
| HIF-1α      | −1.521       | −1.505 to 12.01 | 0.8232 | 0.09888 | −0.2388 to 0.4366 | 0.5609 | −0.3156 | −0.7823 to 0.1510 | 0.1816 | 11.16 | 3.846 to 18.47 | 0.0033 |

**Abbreviations:** NO, nitric oxide; MPO, myeloperoxidase; IL-1β, interleukin 1 beta; TNF-α, tumor necrosis factor α; HIF-1α, hypoxia-inducible factor 1 alpha.
concentration below the minimum level leads to a decrease in the level and disappearance of HIF-1α. The above statement may explain the positive correlations of HIF-1α with ONOO\(^-\) and nitrotyrosine in patients with adrenal tumors observed in our study. Additionally, we observed that HIF-1α was positively associated with IL-1β and TNF-α. Under hypoxic conditions, the activity of HIF-1α is closely related to inflammation. It regulates the secretion of cytokines, the differentiation of monocytes into macrophages accompanied by the secretion of cytokines such as IL-1β, IL-6, IL-12, IL-23 and TNF-α. It is important to note that HIF-1α may be stabilized by inflammatory cytokines, especially TNF-α, inducing positive feedback, stimulating further differentiation of monocytes into macrophages. Moreover, nuclear factor kappa B (NF-kB) plays a key role in the stabilization of HIF-1α by TNF-α, which intensifies HIF-1α-mediated inflammation under hypoxic conditions.

Metabolic abnormalities accompanying adrenal masses may be responsible for increased inflammation and nitrosative stress. Semi-quinone radicals (formed in the oxidation of dopamine, epinephrine, and norepinephrine) have been shown to cause glutathione oxidation, lipoperoxidation, and DNA oxidative damage. This promotes the formation of inflammatory mediators and enhances NADPH oxidase activity, which is not only an important source of free radicals but also cytokines and chemokines. Catabolism of catecholamines by MAO and COMT enzymes may also increase NO production and interleukin secretion. In our study, we also observed a higher BMI in patients with adrenal tumors compared to healthy controls. Although excessive catecholamine secretion leads to weight loss, it may also be involved in the development of obesity and metabolic disorders. Several studies have shown that catecholamines inhibit adiponectin secretion, which is significantly reduced in patients with adrenal masses. In transgenic mice with a deletion of the collagen domain of adiponectin, a lower concentration of circulating adiponectin was observed, which led to an increase in lipoprotein lipase activity and lipid removal. However, it should be noted that patients in the study group were only overweight but not obese.

Similarly, increased cortisol output may also disrupt redox homeostasis and inflammation. On the one hand, cortisol blocks cyclooxygenases (COX-1 and COX-2) responsible for the formation of prostaglandins and proteolytic enzymes (elastase and collagenase), impoverishing the phagocytosis process and protecting lysosomes against

| Table 3 Area Under the Curve of Nitrosative Stress and Inflammatory Biomarkers Between Adrenal Tumor Patients and the Controls |
|---|---|---|---|---|---|---|---|---|
| Biomarker | Study Group | Control Group | P value | AUC | 95% CI | Cut Off | Sensitivity% | Specificity% |
| NO | 22.72 (18.63–26.57) | 14.24 (12.06–15.93) | 0.0357 | 0.5755 | 0.4705 to 0.6805 | <15.40 | 68 | 50.19% to 90.33% |
| Peroxynitrite | 25.35 (22.08–27.9) | 25.35 (22.08–27.9) | 0.0019 | 0.6643 | 0.5698 to 0.7687 | <26.56 | 58 | 44.23% to 76.46% |
| S-nitrosothiols | 18.47 (14.11–21.87) | 18.47 (14.11–21.87) | <0.001 | 0.8739 | 0.7662 to 0.9805 | <31.19 | 77 | 82.32% to 96.79% |
| Nitrotyrosine | 12.43 (9.802–15.12) | 12.43 (9.802–15.12) | <0.001 | 0.8739 | 0.7662 to 0.9805 | <31.19 | 77 | 82.32% to 96.79% |
| MPO | 0.1608 (0.1535–0.1664) | 0.1608 (0.1535–0.1664) | <0.001 | 0.8739 | 0.7662 to 0.9805 | <31.19 | 77 | 82.32% to 96.79% |
| IL-1β | 2.109 (1.717–2.579) | 2.109 (1.717–2.579) | <0.001 | 0.8739 | 0.7662 to 0.9805 | <31.19 | 77 | 82.32% to 96.79% |
| TNF-α | 11.79 (10.62–13.63) | 11.79 (10.62–13.63) | <0.001 | 0.8739 | 0.7662 to 0.9805 | <31.19 | 77 | 82.32% to 96.79% |
| IL-1α | 710.7 (650.1–775.1) | 710.7 (650.1–775.1) | <0.001 | 0.8739 | 0.7662 to 0.9805 | <31.19 | 77 | 82.32% to 96.79% |

Note: Results are presented as median with 25% and 75% percentiles.
Abbreviations: NO, myeloperoxidase; IL-1β, interleukin 1 beta; TNF-α, tumor necrosis factor alpha; HIF-1α, hypoxia-inducible factor 1 alpha.
the release of degrading hydrolytic enzymes. However, this hormone also increases hepatocyte’s sensitivity to adrenaline and glucagon as well as intensifies gluconeogenesis by stimulating phosphoenolpyruvate carboxykinase and glucose-6-phosphatase activities. Cortisol also enhances adipose tissue lipolysis with accompanying release of glycerol and free fatty acids into circulation. Therefore, it may be responsible for hyperglycemias and hyperlipidemias in adrenal masses patients. In our study, although glucose level was generally within reference values it was also significantly higher compared to controls. The highest glucose values were observed in patients with incidentaloma and Cushing’s/Conn’s adenoma. Therefore, metabolic abnormalities caused by higher cortisol/catecholamine output may be associated with systemic inflammation and nitrosative stress. However, this hypothesis requires further research and clinical observations.

An important part of the study was also to determine the diagnostic utility of nitrosative stress and inflammatory biomarkers. Diagnosis of adrenal tumors is based on imaging studies in conjunction with evaluation of blood/urine hormones such as cortisol, metanephrine, and normetanephrine. Therefore, biomarkers for early and non-invasive diagnosis of adrenal cancer are still being sought. Using ROC analysis, we demonstrated that circulating S-nitrosothiols, nitrotyrosine, and MPO differentiate controls from adrenal tumor patients with very high sensitivity (>72%) and specificity (>83%). Of particular note is nitrotyrosine, whose levels correlate not only with inflammatory markers (MPO, IL-1β, TNF-α) but also with HIF-1α and aldosterone. In a multivariate regression model, we showed that nitrotyrosine is a predictor of aldosterone levels, which also supports the diagnostic value of this biomarker. It should be noted that nitrotyrosine determination is simple and inexpensive and can be performed in a routine diagnostic laboratory. Unfortunately, none of the assessed biomarkers did not differentiate between tumor types, suggesting that the severity of nitrosative damage and inflammation are similar in patients with incidentaloma, pheochromocytoma, and Cushing’s/Conn’s adenoma.

Our study is the first to evaluate the association of adrenal masses with nitrosative/nitrative stress, inflammation and HIF-1 expression. Although NO metabolites and the inflammatory response may be involved in adrenal masses development, the exact mechanism is still unknown. Due to the lack of ethics committee approval, we could not conduct animal studies to make the conclusions more convincing. Further studies evaluating the role of redox imbalance, inflammation, and apoptosis in adrenal masses progression are needed.

Conclusions
1. Adrenal tumors are associated with increased protein nitration/S-nitrosylation and inflammation.
2. There is a need for molecular studies to explain disturbances in redox homeostasis, nitrosative stress, and inflammation in patients with adrenal tumors.

Data Sharing Statement
The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

Ethics Statement
The studies involving human participants were reviewed and approved by the Bioethics Committee of the Medical University of Bialystok (permission code: R-I-002/66/2015, APK.002.341.2020). Written informed consent to participate in this study was provided by the participants.

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Disclosure
The authors declare no conflicts of interest.

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