Evaluation of the Blood and Serum Biomarkers for the Prognosis and Progression of the ALS Disease by Eliminating the Effects of Aging

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is the most common motor neuron disease. There are no pathognomonic tests for ALS prognosis; clinical diagnosis of the disease takes time and is usually difficult. Prognostic biomarkers are urgently needed for rapid and effective ALS prognosis. Male albino rats were divided into ten groups based on age as 0 (40–45 days old), A (70–75 days old), B (90–95 days old), C (110–115 days old), and D (130–135 days old). Each group is divided into two subgroups according to their mutation status as wild type ($SOD1^{WT}$) and mutated ($SOD1^{G93A}$). Serum and blood biomarkers were measured of 90 rats to evaluate possible biomarkers for ALS prognosis. Weight loss, cholesterol, creatinine, Alkaline phosphatase (ALKP), glucose, total bilirubin (TBIL), blood urine nitrogen (BUN), c-peptide, glucagon, PYY, MCP-1, white blood cell (WBC), lymphocyte (LYM), monocyte (MID), granulocyte (GRAN), red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width with standard deviation (RDW-SD), red cell distribution width with the coefficient of variation (RDW-CV), platelet (PLT), mean platelet volume (MPV), platelet distribution width (PDW) and procalcitonin (PCT) levels were changed in the $SOD1^{G93A}$ rats compared to the $SOD1^{WT}$ rats. First time in the literature, we showed promising blood and serum biomarkers in the pre-symptomatic and symptomatic stages of ALS by eliminating the effects of aging that can be used for early diagnosis of ALS.

Highlights

- Wide range of serum and blood biomarkers were evaluated in the ALS disease
- Pre-symptomatic and symptomatic phases were compared by eliminating aging factor
- Our data help to rapid and accurate diagnosis of ALS

Significance

We have reported potential biomarkers in the pre-symptomatic and symptomatic stages of amyotrophic lateral sclerosis (ALS) by eliminating effects of aging first time in the literature. Our study represents serum biochemistry, blood parameters, animal weight, hormone parameters, trace element and minerals in the serum samples of SOD1G93A mutated rats divided by age to five groups and each group compared with wild-type rat group. Our data may help early diagnosis and progression biomarkers to improve quality of life and survival time patients with ALS.

Introduction

Amyotrophic lateral sclerosis (ALS) is the most frequent adult-onset motor neuron disease 1.7–2.1 per 100.000 people per year worldwide, and 90–95% of ALS cases are sporadic (sALS), where 5–10% of patients account for familial ALS (fALS) [1] Since there are no pathognomonic tests for diagnosis and

prognosis of ALS, clinical diagnosis of the disease takes time and is usually difficult. Additionally, the presentation of symptoms can vary between patients regarding disease progression. However, the diagnosis should be as rapid and effective as possible since median survival is only 2–3 years, and approved drugs can only slow the progression of the disease at the early stages. Since ALS diagnosis takes 12–15 months, which is extremely late for the drugs to work effectively, prognostic biomarkers are urgently required to diagnose ALS rapidly and effectively [2,3].

ALS follows as a hypermetabolic state leading to malnutrition, lower BMI, impaired blood metabolism, and muscle mass in patients [4]. Hypermetabolic state can be evaluated using various serum biochemical biomarkers, including pre-albumin, cholesterol, albumin, creatinine, retinol-binding protein, transferrin, fibronectin, high-density lipid (HDL), and insulin-like growth factor [5]. Transferrin and albumin refer to the insufficient protein intake in 15–20 days; on the other hand, pre-albumin, fibronectin, and retinol-binding protein indicators of temporary nutrient deficiency [6]. On the other hand, blood tests can help evaluate disease progression since blood is easily sampled and a potential biomarker source for ALS. Neuroinflammation, elevated oxidative stress, protein aggregation, and mitochondrial dysfunction are hallmarks of ALS. Therefore, blood and serum biomarkers addressing indicated hallmarks can be used as possible biomarkers, for instance, neurofilaments, MCP-1, interleukins, and hemoglobin [7,8].

In this study, we have evaluated various blood and serum biomarkers in G93A mutated rats ($SOD1^{G93A}$), expressing mutated human $SOD1^{G93A}$ protein compared to wild-type rats ($SOD1^{WT}$) for the early prognosis and progression of the disease by eliminating the effects of the aging first time in the literature.

Methods

Ethical Statement

All experimental procedures and animal use were approved by the Ethics Committee of Koc University with the number 2019.HADYEK.006. All procedures were performed by personnel trained in the techniques according to Koc University Animal Laboratory guidelines (Istanbul, Turkey).

The facility operates according to the Guide for the Care and Use of Laboratory Animals, and the requirements of the Animal Welfare Act and Regulations and the Public Health Service Policy on Humane Care and Use of Laboratory Animals. All methods were performed in accordance with the relevant guidelines and regulations.

All animals were sacrificed by servical dislocation following overdose isofluorane. Our report follows the recommendations in the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. No human subjects were used during the experiments.

Animal studies

Six male and six female SOD1^{G93A} mutated albino rats were purchased from Taconic with catalog number NTac: SD-Tg(SOD1G93A)L26H. Animals were inbred at the Animal Research Facility of Koc University, and 70 male rats weighing 140–650 g were used during the experiments. Isoflurane was used as an anesthetic, and cervical dislocation under anesthetic was performed to sacrifice each animal. Isofluorane was used to avoid interfering with the biochemical measurements and minimize animal suffering.

The sample size was calculated via power analysis. Animals were housed as described previously in detail [9]. Animals were divided into ten groups based on age as 0 (40-45 days old), A (70-75 days old), B (90-95 days old), C (110-115 days old), and D (130-135 days old), and each group was divided into two subgroups according to their mutation status respectively as SOD1^{WT} and SOD1^{G93A}. Group C accounts for the early stage, and group D refers to the late stage of the ALS disease, where groups 0, A, and B represent the pre-symptomatic stages of ALS (Supplementary file 1). All experimental procedures and animal use were approved by the Ethics Committee of Koc University with the number 2019.HADYEK.006.

Genotyping of SOD1^{WT} and SOD1^{G93A} Rats

Rat tails of each rat were collected, and 25 mg of tissue was used to isolate DNA via DNeasy Blood & Tissue Kit (QIAGEN, Germany). 20ng/ul of DNA was used to perform PCR with forward and reverse primers (Forward primer: 5' GTG GCA TCA GCC CTA ATC CA 3' and Reverse primer: 5' CAC CAG TGT GCG GCC AAT GA 3'). Cycling conditions were denaturation at 95 ºC for 1 min, extension at 95 °C 15s, annealing 62,1□C 15s and then at 72□C 20s, final extension 72□C for 8 min 34 cycles. After PCR was done, products were loaded into the 2% agarose gel to evaluate the corresponding bands. NDAL Laboratory conducted genotyping of SOD1 rats at the Koc University Hospital.

Blood and serum biochemical analysis

Blood samples were collected in the EDTA tubes to prevent coagulation. Blood analysis was immediately performed with PROKAN PE-6800Vet and serum biochemistry parameters were evaluated via VetTest 8008 using VetTest serum biochemistry kits (General Health Profile).

Magpix Luminex analysis

MAGPIX® System of Luminex was used to evaluate hormone levels in the serum samples of SOD1^{WT} and SOD1^{G93A} rats via Milliplex kit (MERCK, USA, # RMHMAG-84K). Blood samples were collected from rats and assembled in the plastic whole blood tube with spray-coated K2EDTA (367842). Blood samples were centrifuged at 400 x g for 3 min to collect serum stored at – 80 °C until analysis was performed. Serum samples were pipetted into a 96 well plate as duplicate, and the experimental procedure was followed according to the kit instructions.

Microwave digestion of serum samples and inductively coupled plasma mass spectrometry (ICP-MS)

A microwave digestion system (Milestone START D) only equipped with the temperature control sensor was used to dissolve serum samples as described previously [10]. Trace and mineral element levels in the rat serum samples were evaluated using Agilent 7700x ICP-MS (Agilent Technologies Inc., Tokyo, Japan) as described previously [9,10].

Statistical analysis

Graphpad Prism (8.0) Software was used to analyze the data. Statistical analysis was always performed by comparing mutated and wild-type groups using the non-parametric Mann-Whitney test. All data were represented as mean ± SD. MILLIPLEX® Analyst 5.1 software was used to analyze raw data acquired by MILLIPLEX® MAP kit # RMHMAG-84K.

Results

Animal weight and relative organ weights as % body weight

Animals were weighed just before sacrification, and our data showed that the bodyweight of $SOD1^{G93A}$ rats decreased in groups C and D compared to the $SOD1^{WT}$ rats (Fig. 1). Relative organ weight (ROW) was calculated as % of total body weight (Table 1). ROW of liver, brain, heart, and kidney organs significantly increased in the $SOD1^{G93A}$ rats of groups C and D compared to the $SOD1^{WT}$ rats of the indicated groups. ROW of tissue and lung increased in the $SOD1^{G93A}$ rats of groups C and D compared to the $SOD1^{WT}$ rats; however, this increase was only significant in group D between WT and G93A rats. No significant changes have been observed between splenic weights of $SOD1^{WT}$ and $SOD1^{G93A}$ rats belonging to any groups (Table 1).

Serum biochemistry parameters of $SOD1^{WT}$ and $SOD1^{G93A}$ rats

Albumin, ALT, amylase, calcium, globulin, and TP levels in the serum did not significantly change between $SOD1^{WT}$ and $SOD1^{G93A}$ rats (Table 2). Cholesterol levels increased in the $SOD1^{G93A}$ rat of each group compared to the $SOD1^{WT}$ rats; on the other hand, this increase was only significant for groups 0 and D (Figure 2). ALKP and creatinine levels decreased in the $SOD1^{G93A}$ rats compared to the WT ones for groups A, B, C, and D; however, this decrease was significant for groups C and D for ALKP, significant in group D for creatinine (Fig. 2). Phosphate concentration increased in the $SOD1^{G93A}$ rats of all groups except groups D, in which phosphate levels significantly decreased in $SOD1^{G93A}$ rats compared to the $SOD1^{WT}$ ones (Fig. 2). TBIL levels increased dramatically in the $SOD1^{G93A}$ rats of groups C and D compared to the $SOD1^{WT}$ rats (Fig. 2). BUN levels increased in the $SOD1^{G93A}$ rats compared to the $SOD1^{WT}$ rats in all groups except group B; however, this increase was only significant for group D (Figure 2).

Serum hormone and glucose levels

C-peptide levels increased in the G93A rats compared to the WT in groups A and B; however, they decreased in groups C and D. This decrease was almost double in the G93A rats (Figure 3). Glucagon levels of G93A rats have started to increase compared to WT rats in group B, and this trend has continued in groups C and D (Figure 3). Serum glucose levels were evaluated in the rat serum samples. Glucose levels in the $SOD1^{G93A}$ rats increased compared to the $SOD1^{WT}$ rats in groups A and B (Figure 3). Controversially, glucose levels in the $SOD1^{G93A}$ rats decreased compared to the $SOD1^{WT}$ rats, and this decrease was significant (Fig. 3). PYY levels decreased in the G93A rats compared to the WT in groups 0, A, and B, and this trend continued in groups C and D. On the other hand, MCP-1 levels of G93A rats were less in all groups compared to WT rats (Figure 3).

Trace element and mineral levels in the serum samples of $SOD1^{WT}$ and $SOD1^{G93A}$ rats

Fe levels increased in the $SOD1^{G93A}$ rats belonging to groups B, C, and D were decreased in group 0 compared to $SOD1^{WT}$ rats (Figure 4). Zn levels decreased in the $SOD1^{G93A}$ rats of the groups B, C, and D compared to the $SOD1^{WT}$ rats (Figure 4). Na and Mg levels significantly increased in the $SOD1^{G93A}$ rats compared to the $SOD1^{WT}$ rats belonging to groups C and D (Figure 4). Mg levels significantly decreased in the $SOD1^{G93A}$ rats compared to the $SOD1^{WT}$ in groups A and D; however, they increased dramatically in group C (Figure 4). However, K levels increased in the $SOD1^{G93A}$ of groups A, C, and D compared to the $SOD1^{WT}$ rats but significantly decreased in group B (Figure 4). Ca levels increased in the serum samples of $SOD1^{G93A}$ rats of groups 0 and C, were reduced in groups B and D compared to the $SOD1^{WT}$ rats (Figure 4).

Blood parameters of $SOD1^{WT}$ and $SOD1^{G93A}$ rats

% Lymphocyte and # lymphocyte values of $SOD1^{G93A}$ rats decreased in all groups compared to the $SOD1^{WT}$ rats; however, this decrease was significant in the groups C and D. % monocyte levels increased in the $SOD1^{G93A}$ rats compared to the $SOD1^{WT}$ rats belonging to the groups B, C and D. # monocyte and # granulocyte levels decreased in the $SOD1^{G93A}$ rats compared to the $SOD1^{WT}$ rats in all groups. % granulocyte levels increased in the $SOD1^{G93A}$ rats compared to the $SOD1^{WT}$ rats in all groups; however, this increase was significant in group C (Figure 5). WBC and PLT levels decreased in the $SOD1^{G93A}$ rats compared to $SOD1^{WT}$ of all groups. This decrease was significant in the C and D. HCT, and MCV levels decreased in the $SOD1^{G93A}$ rats compared to the $SOD1^{WT}$ rats in groups C and D. MCHC levels decreased in the $SOD1^{G93A}$ rats compared to the $SOD1^{WT}$ rats in groups 0, were increased in the groups C and D. RDW-SD decreased in all groups except A for $SOD1^{G93A}$ rats compared to the $SOD1^{WT}$ rats (Figure 5). RDW-CV and PCT levels decreased in the $SOD1^{G93A}$ rats in all groups except group A compared to $SOD1^{WT}$ (Figure 5). We have found that RBC, HGB, and MCH levels decreased in the $SOD1^{G93A}$ rats in groups 0, A, and D, and C increased in group D (Table 3).

Discussion

Administration of edaravone and riluzole can slow the progression of the disease at the early stages of disease; thus, rapid diagnosis is vital for ALS. On the other hand, symptoms of ALS are not highly specific and can mimic the symptoms of other neurological disorders; therefore, developing prognostic and diagnostic biomarkers for ALS is immediately needed for accurate and rapid diagnosis of the disease [11] [12].

Weight loss (WL) has been categorized as a clinical feature and predictive value for ALS and observed 56% to 62% in all cases [13], associated with morbidity and mortality respiratory and functional loss in ALS. Thus, bodyweight management enables the improvement of the prognosis of the patients [14]. Our data showed that $SOD1^{G93A}$ rats had significantly lost their weights than $SOD1^{WT}$ rats in groups C and D, indicating WL most probably started before the first symptoms showed up (Figure 1). WL can result from different factors, including hypermetabolic state, food intake, metabolic or hormonal levels, and physical activity status [15]. ROW is used to evaluate the toxicity of a substance and possible tissue damage in the organism [16]. Our data showed ROW of all $SOD1^{G93A}$ rat tissues significantly elevated compared to the $SOD1^{WT}$ in groups C and D except spleen (Table 1). Thus, we can propose that ALS disease progression in the early and late stages can induce tissue impair in the liver, kidney, lung, brain, testis, heart, and spinal cord tissues (Table 1).

Biomarkers in the blood and serum can be used for early and accurate diagnosis of the disease since riluzole and edaravone, the only two approved drugs, are only effective at the early stages of ALS [2]. Since ALS causes a hypermetabolic state leading to malnutrition, weight, and muscle loss in patients, various biomarkers can be used to evaluate the prognosis and progression of the ALS disease [17]. We have found that albumin, TP, and Ca levels slightly increased in the $SOD1^{G93A}$ rats compared to the $SOD1^{WT}$ ones in the groups B, C, and D groups meaning increases have started at the pre-symptomatic stages as well (Table 2). An increase in serum albumin and TP correlates with the body's inflammatory status, and albumin levels positively correlate with ALS progression and survival. Additionally, albumin has antioxidant effects on metabolism [18,19] On the other hand, Ca homeostasis plays a vital role in ALS pathogenesis and disease progression, especially in SOD1-linked ALS; for instance, Ca buffering and metabolism are impaired in the motor neurons, mitochondria, and CNS even at the pre-symptomatic stages according to the various studies. Increased Ca levels in ALS patients have been found, a however possible mechanism behind Ca homeostasis should be further investigated [20,21].

Creatinine has been characterized as an independent prognostic biomarker for ALS and elevated in the earlier phases of the disease as muscle mass destruction and decreased at the late stages of the disease as a hallmark of malnutrition. Additionally, reduced creatinine levels are associated with reduced survival time and poor prognosis in male and female patients [22]. Creatinine levels increased in the $SOD1^{G93A}$ rats of group 0 and decreased compared to the $SOD1^{WT}$ rays by aging and disease progression in groups A, B, C, and D (Figure 2). That indicated that creatinine could be used as a biomarker at the pre-symptomatic stages of ALS. Cholesterol is being discussed as another possible biomarker for ALS since neuron death occurs during the disease progression resulting in elevated levels of cholesterol in the CSF [23].

Interestingly increased oxidative stress in the SOD1^{G93A} mice results in the formation of oxidized-cholesterol products existing in the serum of the rats [24]. Elevated levels of cholesterol have been observed in ALS patients. Increased cholesterol levels are correlated with risk for ALS and poor survival, resulting from oxidized cholesterol products and their harmful effects on the metabolic pathways [25]. We have found that cholesterol levels in the mutated rats increased compared to the wild-type ones and this increase was higher in groups C and D (Figure 2).

ALKP is a liver enzyme responsible for breaking down proteins and transporting phosphate groups, and lower levels of ALKP are associated with malnutrition in humans [26]. We have shown that ALKP levels started to decrease in the SOD1^{G93A} rats compared to the wild-type ones in group B, and the decrease reached significant levels in groups C and D (Fig. 2). There are no studies that showed the impact of the ALKP as a biomarker in the pre-symptomatic and symptomatic stages of ALS; the first time in the literature, we hypotheses that ALKP could be used as a biomarker (Fig. 2). On the other hand, bilirubin is associated with the inflammatory biomarker in inflammation-linked diseases such as ALS, multiple sclerosis (MS), Alzheimer's disease, and diabetes [27]. Since we found that TBIL levels dramatically increased in the mutated rats compared to the wild-type ones at the symptomatic stages, that can be used as a possible biomarker for the ALS diagnosis (Fig. 2). Phosphate is vital for cell signaling, energy, and mineral metabolism [28]. We have found that serum phosphate levels increased in the SOD1^{G93A} rats of groups 0, A, B, and C compared to the SOD1^{WT} rats; however, they significantly decreased in group D (Fig. 2). No published data evaluate the impact of phosphate as a potential biomarker; thus, further studies can be conducted. On the other hand, BUN levels increased in the mutated rats compared to the wild type ones according to our data (Figure 2); however, the importance of the BUN as a potential biomarker has not been studied until now, and thus a study can be conducted with the human cohort in the future.

ALS is a hypermetabolic disease; however, the possible mechanisms contributing to the homeostasis of the energy metabolism is not known. Besides serum biochemistry biomarkers, we evaluated serum hormone and glucose levels in the SOD1^{WT} and SOD1^{G93A} rats (Fig. 2). Glucose metabolism is considered one of the possible targeting approaches to cure people with ALS since it is vital for OXPHOS, synthesis of neurotransmitters, and oxidative stress metabolism [29]. Glucagon induces catabolism of the glycogen storages of the body resulting in the glucose release into the blood. On the other hand, c-peptide can be measured instead of insulin since it is more stable and has a longer half-life.

Additionally, it is produced in equal amounts with insulin, and both are responsible for lowering blood glucose [30]. Interestingly we have found that serum glucagon levels increased in the SOD1^{G93A} rats compared to the SOD1^{WT} rats in groups B, C, and D (Fig. 3). C-peptide levels increased in the mutated rats compared to wild-type animals in groups 0, A, and B; however, they decreased in groups C and D (Figure 3). Despite increased glucagon and decreased c-peptide levels, glucose levels in the SOD1^{G93A} rats increased in groups A and B. Still, they significantly reduced in groups C and D (Figıre 3).

On the other hand, PYY is a gut hormone involving energy expenditure, appetite, and fat oxidation. Increased levels of PYY in the blood result in increased energy expenditure, elevated levels of fat oxidation, and decreased appetite [31]. However, we have found that PYY levels decreased in the mutated rats compared to the wild-type ones in the pre-symptomatic stages but increased in groups C and D (Figuıre 3). MCP-1 expression is induced by insulin and plays a role in diabetes because of impaired glucose metabolism. Increased levels of MCP-1 are associated with impairment in adipocyte function decreased glucose uptake into the cells [32]. We have found that MCP-1 levels increased in the $SOD1^{G93A}$ rats compared to $SOD1^{WT}$ rats in all groups, where MCP-1 levels decreased in the wild-type rats by aging (Fig. 3). All these data indicate that increased catabolic activity and impairment in glucose metabolism have started at the pre-symptomatic stages and worsened at the late stages of ALS. Thus, PYY, MCP-1, glucagon, c-peptide, and glucose levels can be evaluated as possible biomarkers for ALS prognosis and progression as one of the hallmarks of impaired glucose metabolism [29].

We have also evaluated blood biomarkers as potential biomarkers in the $SOD1^{WT}$ and $SOD1^{G93A}$ rats that have become a hot topic in a couple of years. RBC and MCH levels were suggested as possible early biomarkers of early stages of ALS by an *in-silico* study [33]. Additionally, decreased levels of RBC count have been reported as an early biomarker in ALS [34]. Additionally, increased HGB levels are associated with prognosis and decreased survival rate in ALS [35]. We have found that RBC, HGB, and MCH levels decreased in the $SOD1^{G93A}$ rats in groups 0, A, C, and D; however, they increased in group D (Table 3). We suggest that MCH, HGB, and RBC count can be considered possible biomarkers in ALS and give insight into disease progression.

Blood biomarkers indicating the impairment in the peripheral immune system and inflammation are vital for ALS prognosis and diagnosis since neuroinflammation contributes to ALS pathogenesis [36]. Increased levels of monocyte, granulocyte, and neutrophil and decreased lymphocyte counts have previously been reported in ALS patients[36–38]. We found that monocyte and granulocyte counts have started to increase in the mutated rats in the pre-symptomatic (B) and continued in the symptomatic stages (C and D) (Figure 4). In groups B, C, and D, lymphocyte levels decreased in the $SOD1^{G93A}$ rats (Figure 4). Therefore, granulocyte, lymphocyte, and monocyte levels can be used as potential biomarkers in the ALS prognosis and progression, indicating the impaired inflammation and immune response, which is one of the hallmarks of the disease [1,36]. In this study, we have reported that MCH, RDW-SD, MCV, RDW-CV, PCT, and HCT values significantly decreased in the $SOD1^{G93A}$ rats in comparison with $SOD1^{WT}$ rats at the early stage (C) and late-stage (D) of ALS first time in the literature (Fig. 4). We have found that WBC levels decreased in the mutated rats compared to the wild-type ones in all groups and PLT levels decreased in the mutated rats in the pre-symptomatic (B) and symptomatic groups (C and D) first time literature as well (Fig. 4). All the indicated parameters can be used as hallmarks of the impaired blood homeostasis and immune system since WBC, PLT, MCH, RDW-SD, HCT, MCV, PCT, and RDW-CV levels are impaired in the alteration of the indicated metabolisms [39]. Furthermore, these parameters can be evaluated in the human cohort to validate the accuracy of our data.

Serum mineral and trace element levels are vital for the various metabolisms such as antioxidant homeostasis, blood metabolism, neuron integrity, and nutrition balance, for instance, Fe plays a crucial role in the energy, blood, and oxidative stress metabolisms and elevated levels of Fe indicate higher levels of oxidative stress in the organism. Additionally, elevated levels of iron can disturb Ca, Cu, and Zn levels in the body. We have found that Zn, Fe, Ca, Na, Mg, and K levels were impaired in the $SOD1^{G93A}$ rats compared to the $SOD1^{WT}$ rats that may indicate impaired oxidative stress metabolism, neuronal homeostasis, and mineral metabolism [2,10,40–44] (Fig. 5). Thus, these minerals and trace elements can be evaluated as potential biomarkers for ALS prognosis and progression. In conclusion, first time in the literature, we have investigated possible biomarkers in the blood and serum by eliminating the effects of aging and indicated promising biomarkers could be used in the ALS disease prognosis and progression.

Declarations

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Duygu Aydemir: Conceptualization (supporting); investigation (lead); experimental procedures (lead); data curation (lead); formal analysis (lead); writing–original draft (lead); writing–review and editing (lead). **Selcuk Sürücü:** Experimental procedures (supporting), writing–review and editing (supporting) **Ayse Nazli Basak:** Conceptualization (supporting); writing–review and editing (supporting), sources (lead); supervision (lead) **Nuriye Nuray Ulusu:** Conceptualization (lead); data curation (supporting); sources (lead); investigation (supporting); writing–review and editing (supporting); funding acquisition (lead).

Consent to Participate

Not applicable.

Consent for Publication

All authors consent to publication.

Ethical approval

All experimental procedures and animal use were approved by the Ethics Committee of Koc University with the number 2019.HADYEK.006.

Data Availability

Data are not public since our study has conducted first time in the literature, however, the datasets used and analyzed during this study are available from the corresponding author upon reasonable request.

Code Availability

Not applicable.

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Tables

Table.1. Relative organ weight is represented by % body weight for each rat. All results were given as mean ± SD.

		Relative Organ weights (% Body weight)						
		Brain	Lung	Liver	Spleen	Heart	Kidney	Testis
Group 0	*WT*	0.828 ± 0.119	0.609 ± 0.047	4.752 ± 0.875	0.236 ± 0.021	0.356 ± 0.035	0.832 ± 0.071	1.291± 0.082
	G93A	0.923 ± 0.164	0.660 ± 0.040	4.057 ± 0.595	0.246 ± 0.018	0.367 ± 0.033	0.856 ± 0.034	1.226 ± 0.029
Group A	*WT*	0.607 ± 0.045	0.505 ± 0.037	4.196 ± 0.443	0.203 ± 0.030	0.338 ± 0.016	0.803 ± 0.093	1.028 ± 0.063
	G93A	0.578 ± 0.084	0.476 ± 0.072	4.292 ± 0.595	0.198 ± 0.020	0.329 ± 0.013	0.761 ± 0.078	1.073 ± 0.100
Group B	*WT*	0.502 ± 0.041	0.449 ± 0.078	3.903 ± 0.194	0.171 ± 0.022	0.317 ± 0.026	0.724 ± 0.057	0.854 ± 0.069
	G93A	0.473 ± 0.053	0.486 ± 0.056	3.671 ± 0.427	0.189 ± 0.059	0.284 ± 0.031	0.688 ± 0.225	0.824 ± 0.054
Group C	*WT*	0.432 ± 0,061	0.526 ± 0.103	3.528 ± 0.195	0.156 ± 0.010	0.278 ± 0.014	0.657 ± 0.049	0.793 ± 0.111
	G93A	0.561 ± 0.075[b]	0.601 ± 0.088	3.977 ± 0.316[b]	0.164 ± 0.015	0.333 ± 0.021[c]	0.755 ± 0.049[c]	0.876 ± 0.128
Group D	*WT*	0.429 ± 0.042	0.518 ± 0.096	3.355 ± 0.273	0.163 ± 0.030	0.297 ± 0.021	0.632 ± 0.038	0.795 ± 0.101[b]
	G93A	0.657 ± 0.119[c]	0.660 ± 0,125[a]	3.886 ± 0.445[a]	0.164 ± 0.022	0.341 ± 0.026[c]	0.872 ± 0.084[c]	1.06 ± 0.217

[a] represents significantly different from $SOD1^{WT}$ group ($p \leq 0.05$), [b] represents significantly different from $SOD1^{WT}$ group ($p \leq 0.001$), [c] represents significantly different from control $SOD1^{WT}$ group ($p \leq 0.0001$)

Table.2. Serum levels of albumin, ALT, amylase, calcium, globulin and TP in the $SOD1^{WT}$ and $SOD1^{G93A}$ rats were given as mean ± SD of n=16 animals for each group.

		Serum biochemistry parameters					
		Albumin (g/dL)	ALT (U/L)	Amylase (U/L)	Calcium (mg/dL)	Globulin (g/dL)	TP (g/dL)
Group 0	*WT*	3.11 ± 0.76	53.50 ± 15.33	1327 ± 256.8	10.39 ± 0.42	2.657 ± 0.67	5.743 ± 1.43
	G93A	2.80 ± 0.08	57.00 ± 6.30	1249 ± 298.9	9.957 ± 0.37	2.443 ± 0.20	5.214 ± 0.25
Group A	*WT*	2.80 ± 0.35	76.20 ± 24.16	1653 ± 390.4	10.72 ± 0.94	2.90 ± 0.45	5.700 ± 0.71
	G93A	2.61 ± 0.02	69.83 ± 10.03	1624 ± 284.7	10.06 ± 0.11	2.483 ± 0.18	5.100 ± 0.43
Group B	*WT*	2.71 ± 0.24	77.33 ± 12.33	1556 ± 129.4	10.08 ± 0.23	2.90 ± 0.12	5.617 ± 0.34
	G93A	3.17 ± 1.30	74.17 ± 19.76	1516 ± 302.1	10.23 ± 0.47	2.933 ± 0.47	5.667± 0.25
Group C	*WT*	2.80 ± 0.23	65.60 ± 13.83	1555 ± 136.6	9.980 ± 0.32	2.880 ± 0.30	5.680 ± 0.47
	G93A	2.98 ± 0.22	65.11 ± 8.22	1467 ± 213.1	10.40 ± 0.56	3,170 ± 0.23	6.060 ± 0.27
Group D	*WT*	2.86 ± 0.05	58.67 ± 19.35	1559 ± 227.2	10.00 ± 0.43	2.800 ± 0.43	5.583 ± 0.31
	G93A	3.02 ± 0.25	59.20 ± 13.75	1366 ± 387.4	10.35 ± 0.57	2.810 ± 0.35	5.820 ± 0.46

Table.3. Blood parameters in the $SOD1^{WT}$ and $SOD1^{G93A}$ rats were given as mean ± SD of n=16 animals for each group.

		BLOOD parameters				
		RBC (10^{-6}/µl)	HGB (g/dL)	MCH (pg)	MPV (fL)	PDW (%)
Group 0	*WT*	7.024 ± 1.26	13.58 ± 3.03	19.10 ± 1.21	7.280 ± 0.28	8.320 ± 0.81
	G93A	6.528 ± 1.15	13.28 ± 2.73	16.77 ± 5.02	7.967 ± 0.70	9.083 ± 1.38
Group A	*WT*	7.640 ± 1.62	14.66 ± 3.77	18.99 ± 1.07	8.286 ± 1.48	8.743 ± 1.01
	G93A	7.475 ± 0.80	14.18 ± 2.03	17.64 ± 2.90	8.960 ± 1.89	8.620 ± 0.94
Group B	*WT*	9.156 ± 1.86	17.63 ± 3.51	19.11 ± 0.94	8.600 ± 1.42	8.829 ± 0.87
	G93A	9.109 ± 1.24	16.77 ± 2.96	18.61 ± 1.06	8.500 ± 1.62	8.914 ± 1.69
Group C	*WT*	8.926 ± 0.35	16.14 ± 0.83	18.02 ± 0.57	10.32 ± 2.89	10.42 ± 2.59
	G93A	8.416 ± 0.78	15.22 ± 2.21	17.96 ± 1.09	9.120 ± 1.11	8.720 ± 1.04
Group D	*WT*	7.803 ± 1.38	14.48 ± 3.03	29.53 ± 1.99	9.700 ± 2.67	9.967 ± 2.62
	G93A	8.151 ± 1.25	15.15 ± 3.05	31.69 ± 2.07	9.225 ± 1.58	8.788 ± 0.90

Figures

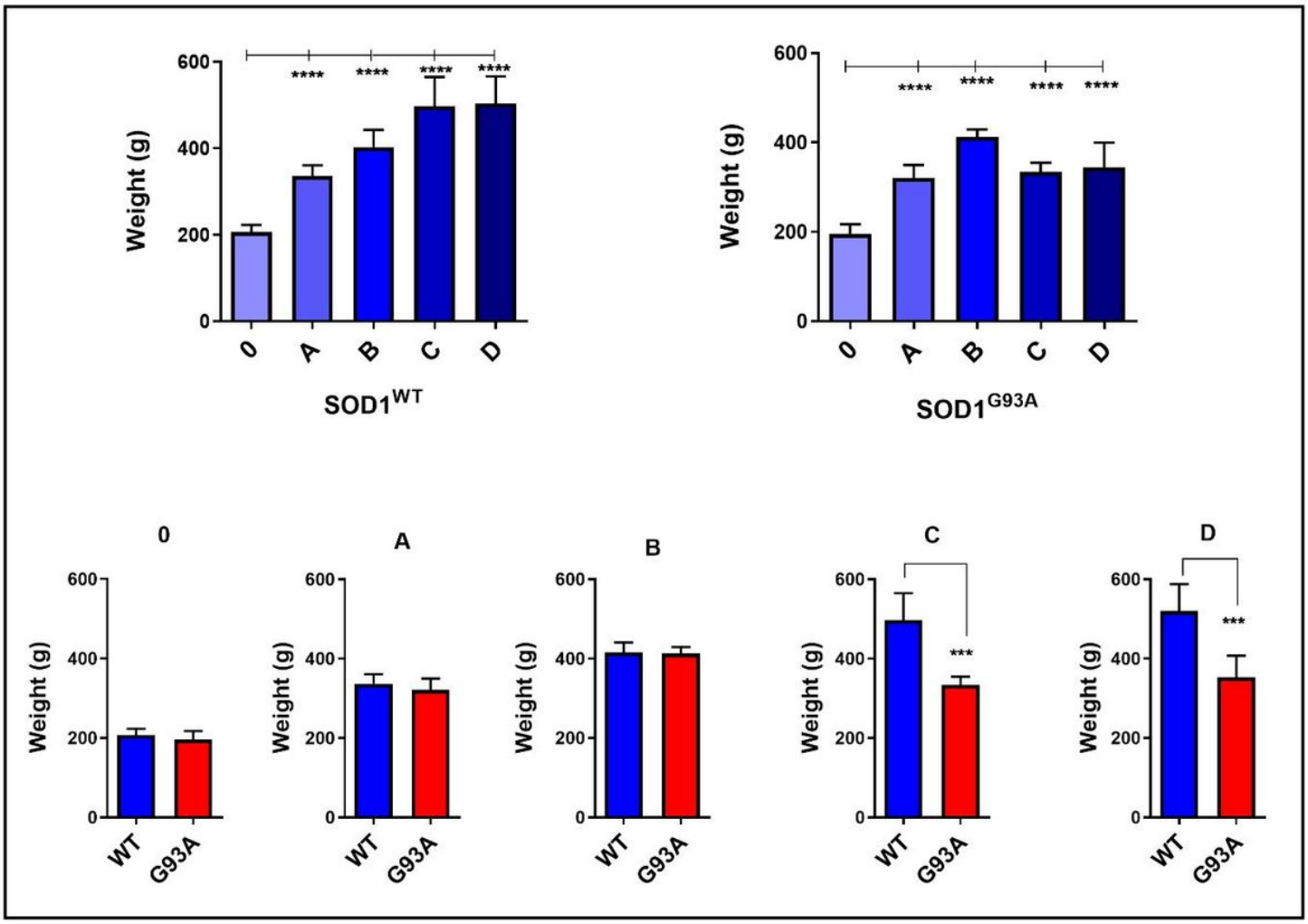

Figure 1

Body weights of the $SOD1^{G93A}$ and $SOD1^{WT}$ rats. All results were given as mean ± SD of n=16 animals for each group. Notes: * ($p \leq 0.05$), **($p \leq 0.001$) and *** ($p \leq 0.0001$)

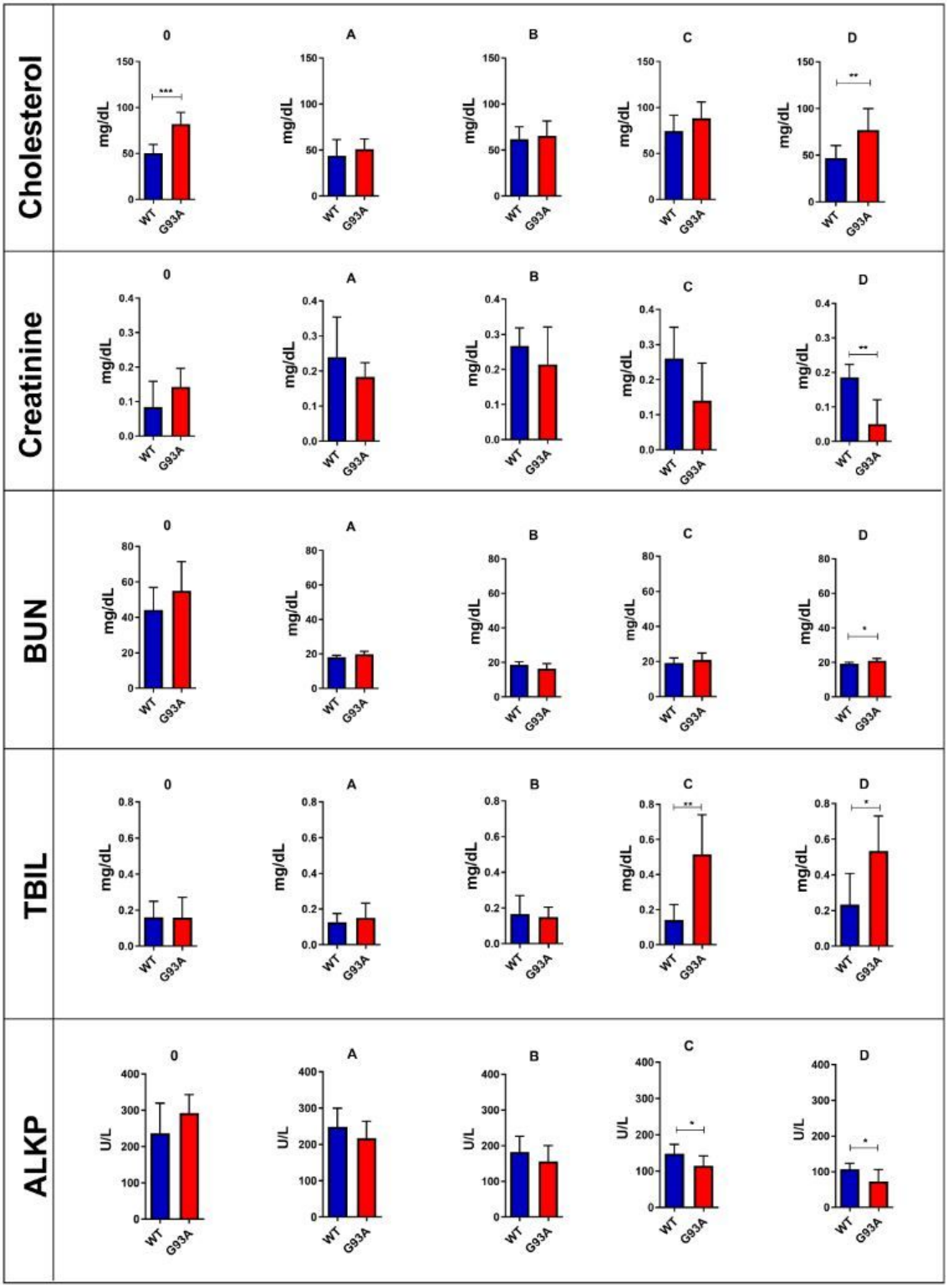

Figure 2

Serum biochemical parameters of the $SOD1^{G93A}$ and $SOD1^{WT}$ rats. All results were given as mean ± SD of n=16 animals for each group. Notes: * ($p \leq 0.05$), **($p \leq 0.001$) and *** ($p \leq 0.0001$)

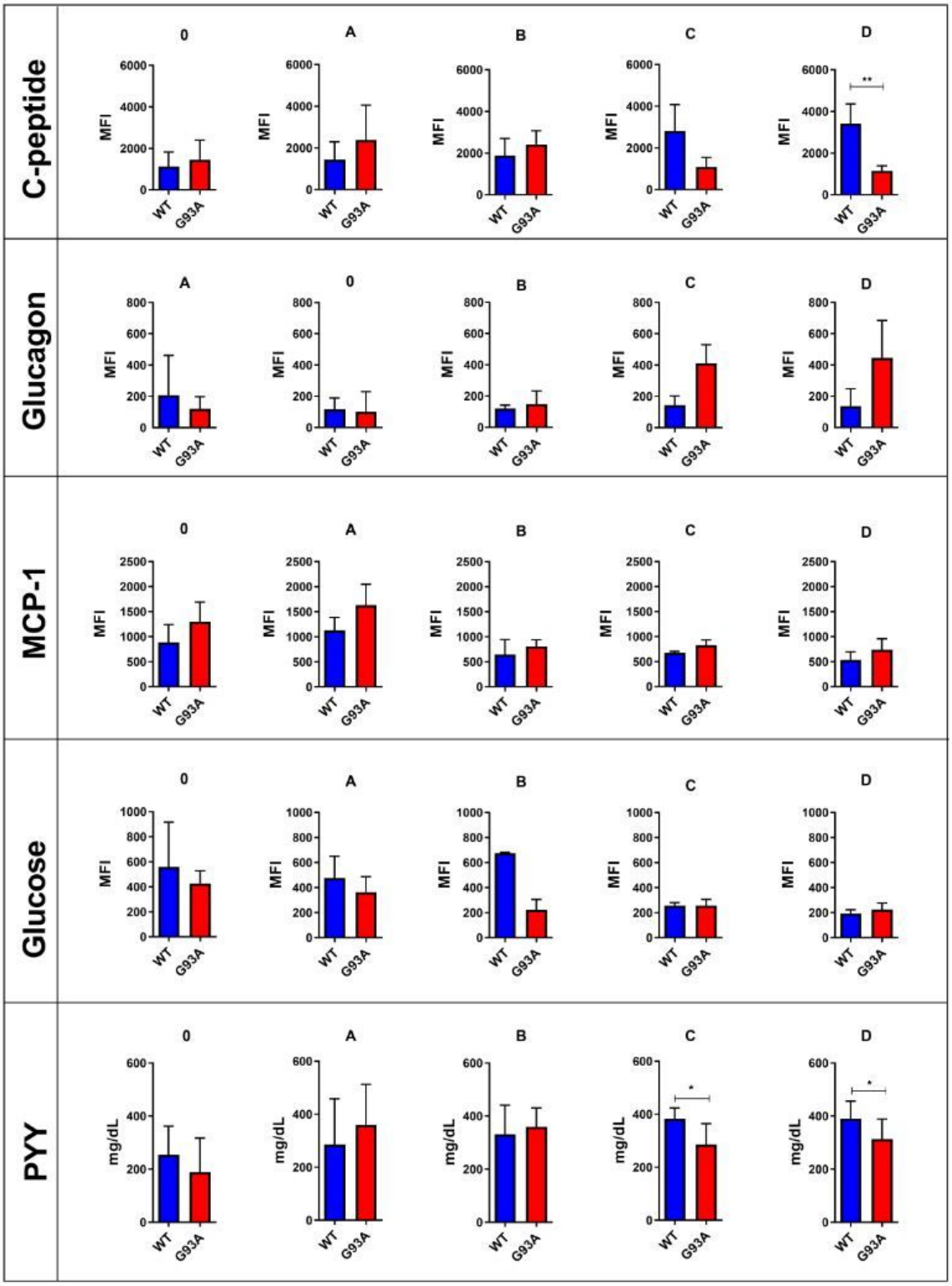

Figure 3

Serum hormone parameters of the SOD1^{G93A} and SOD1^{WT} rats. All results were given as mean ± SD of n=16 animals for each group. Notes: * ($p \leq 0.05$), **($p \leq 0.001$) and *** ($p \leq 0.0001$)

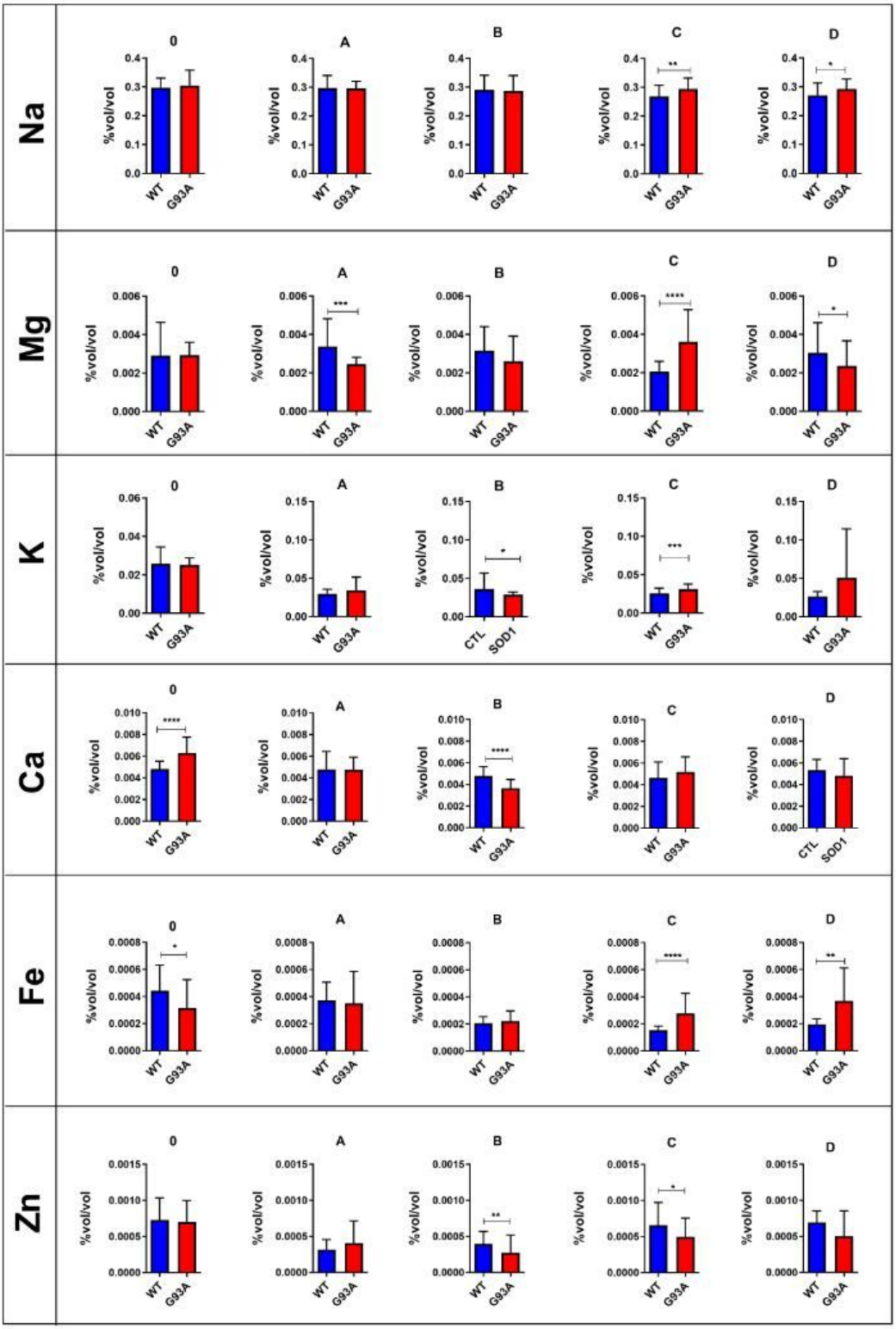

Figure 4

Serum mineral and trace element levels of the $SOD1^{G93A}$ and $SOD1^{WT}$ rats. All results were given as mean ± SD of n=16 animals for each group. Notes: * ($p \leq 0.05$), **($p \leq 0.001$) and *** ($p \leq 0.0001$)

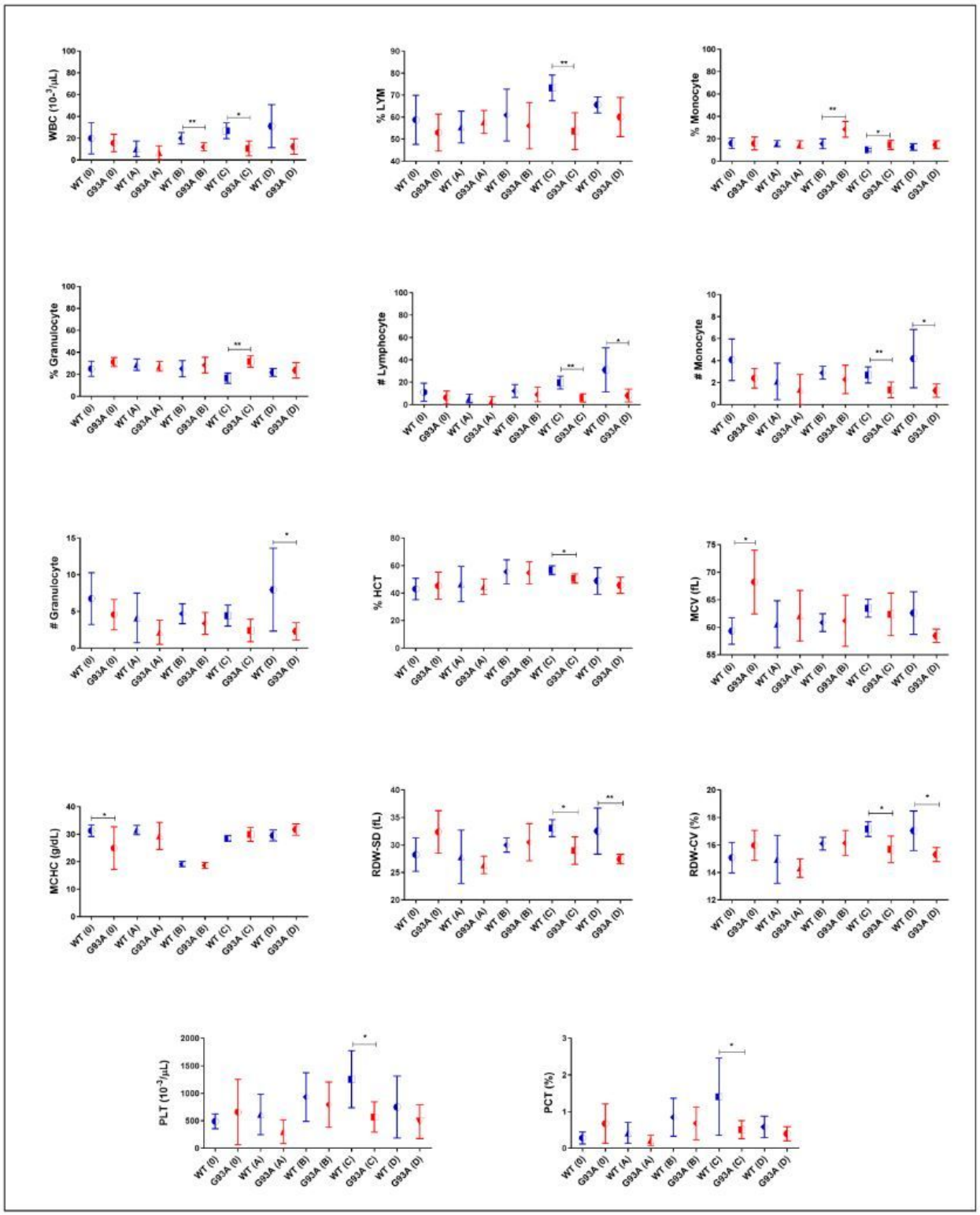

Figure 5

Blood parameters of the SOD1^{G93A} and SOD1^{WT} rats. All results were given as mean ± SD of n=16 animals for each group. Notes: * ($p \leq 0.05$), **($p \leq 0.001$) and *** ($p \leq 0.0001$)

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