

Journal of Nephrologist

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Serum neutrophil gelatinase-associated lipocalin and oxidized neutrophil proteins in patients with nephropathy caused by acute alcohol poisoning

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ARTICLE INFO

Article type:
Original Article

Article history:
Received: 12 February 2020
Accepted: 7 January 2021
Published online: 13 February 2021

Keywords:
Toxic nephropathy, Acute alcohol poisoning, Kidney damage, Neutrophils, Advanced oxidation protein products, Neutrophil gelatinase-associated lipocalin (NGAL)

ABSTRACT

Introduction: Alcohol use has been identified as a major risk factor for disease burden and premature mortality.

Objectives: We studied the serum neutrophil gelatinase-associated lipocalin (NGAL) and advanced oxidation protein product (AOPP) concentrations in neutrophils to assess the possibility of their using for the early detection of kidney damage in patients with acute alcohol poisoning (AAP). The impact of eGFR (estimated glomerular filtration rate) on the NGAL and AOPP levels was also studied.

Patients and Methods: The study included 89 patients with AAP. Healthy individuals and patients with chronic kidney disease (CKD) served as comparison groups. Participants were represented by men, aged between 20 and 40 years. Results of laboratory tests of kidney function were also taken into account. Serum NGAL level was measured using ELISA kit. AOPP was determined using the method of Witko-Sarsat et al.

Results: We detected a significant increase in serum NGAL and AOPP level both in toxic nephropathy with a clinical picture of acute kidney injury (AKI) and in the "preclinical stage" of kidney damage. Hence a single trend in the changes of these indicators existed in patients with AAP. At the same time, our study revealed opposite trends in patients with CKD. There was no significant increase in serum NGAL in patients with CKD.

Conclusion: We propose to consider an increased eGFR together with an increased serum NGAL concentration in patients with AAP as the stage, preceding the nephropathy or AKI, even in the absence of clinical and laboratory signs of impaired renal function.

Implication for health policy/practice/research/medical education:

This study has implication for practical medicine, public health and scientific community because it considers the issue of the possibility of using the serum neutrophil gelatinase-associated lipocalin (NGAL) and oxidized neutrophil proteins for the early detection of kidney damage in patients with acute alcohol poisoning, until the moment when it can be diagnosed with using only serum creatinine. Additionally, in this study we compared these indexes at toxic nephropathy and early stages of chronic kidney disease, having revealed opposite trends in their changes.

Please cite this paper as: Demidchik LA, Klyuyev DA, Muravlyova LM, Borisovich Molotov-Luchanskiy V, Yemelevna Bakirova R, Ponamareva OA, Kolesnikova YA, Baymagambetova DD, Kiziltunc A. Serum neutrophil gelatinase-associated lipocalin and oxidized neutrophil proteins in patients with nephropathy caused by acute alcohol poisoning. J Nephrologist. 2021;10(3):e27. DOI: 10.34172/jnp.2021.27.

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Introduction

Alcohol use has been identified as a major risk factor for disease burden and premature mortality and as a global significant problem (1). Alcohol poisoning is constantly recorded in many countries around the world. Kazakhstan ranks 34th place in terms of alcohol consumption from 188 countries in the world (10.96 L of alcohol per capita) and first among the countries of Central Asia (2). The number of alcohol poisoning in Kazakhstan in 2013 was 86.6 per 100 000 thousand population and the number of alcohol poisoning deaths was 5.6 per 100 000 population (2).

One of the pathological syndromes or complications in acute alcohol poisoning (AAP) is toxic nephropathy which defeats the glomerular apparatus and kidney tissue (3). The changes are due to the direct toxic effect of high ethanol and acetaldehyde concentrations on the renal tissue. The common name “nephropathy” means a group of secondary-caused kidney diseases, ultimately leading to the development of nephrosclerosis that is the morphological basis of chronic renal failure (CRF).

The course of nephropathy is prolonged in time, irreversible and progressive. In the preclinical stage there is an accumulation of structural changes in the kidney, which could be revealed, until some time, only by puncture biopsy. The first clinical stage is characterized by proteinuria, passing into the stage of nephrotic syndrome and, as the outcome, the stage of CRF (4). The term “preclinical kidney disease” has become widely known since 2006 (5,6). It began to be used for persons with normal glomerular filtration rate (GFR; according to serum creatinine), but with elevated serum cystatin C level. It was proposed to use cystatin C detection to identify patients at high risk of chronic kidney disease (CKD) (5,7). For the early diagnosis of diabetic nephropathy and detection of renal damage it has also been proposed to use neutrophil gelatinase-associated lipocalin (NGAL), the level of which in plasma or urine gradually increases during the development of renal dysfunctions (8-10). NGAL is a proven and preferred marker of acute kidney injury (AKI) in various clinical settings (11-13). However, similar studies and assessment of the capabilities of serum NGAL in the diagnosis of toxic nephropathy have not been conducted. The study of oxidized neutrophil proteins in patients with AAP, in particular advanced oxidation protein products (AOPP) is also of interest. There is no doubt that the presence of markers opens up the possibility of detection the “preclinical kidney disease”, as well as timely treatment and prevention of the disease progression to CRF (corresponding to CKD 3-5 stages). Currently, the limitations of serum creatinine for the early detection of kidney damage are widely known, because

serum creatinine rises only when the function of about 50 percent of nephrons is lost (14,15).

The toxicological departments in our country most often use the classification of toxic nephropathy and its diagnostic criteria proposed by Luzhnikov et al back in 1989 (16). At the same time, despite the subsequent appearance of improved classifications, the decrease in eGFR (Estimated glomerular filtration rate), progressing with the nephropathy severity remains a common diagnostic criterion for them. However, as mentioned before, the experience of recent years, in particular with diabetic nephropathy, shows the “insensitivity” of serum creatinine to the early stages of kidney damage and its inability to detect the preclinical stage. Late diagnosis significantly reduces the effectiveness of treatment and, as known, AKI is a strong risk factor for CKD.

Objectives

The purpose of the study was to assess the possibility of using the serum NGAL and oxidized neutrophil proteins for the early detection of kidney damage in patients with AAP. Additionally, the impact of the alcohol poisoning severity and eGFR on the level of NGAL and oxidized neutrophil proteins was studied.

Patients and Methods

Study protocol

This prospective cross-sectional study was conducted on the basis of the biochemical laboratory of Karaganda Medical University together with the toxicological department of the Regional Medical Center (from January 2018 to May 2019). The diagnosis of alcohol poisoning and determination of the severity of intoxication were carried out according to the protocol “The toxic effect of alcohol (adults and children)” recommended by the Expert Council of the Ministry of Health and Social Development of the Republic of Kazakhstan (30.10.2015). The diagnosis confirmation was based on the thorough medical history, an objective examination, laboratory tests and determination of blood alcohol concentration (BAC). In addition to objective data and laboratory tests (AST/ALT ratio, serum GGT activity) the CAGE questionnaire was used to confirm AAP and exclude chronic alcohol intoxication.

The study included 89 patients with AAP, 25 healthy donors (control group) and 25 patients with CKD (CKD stage 1, n=15 and CKD stage 2, n=10). The control group and the group of patients with CKD served as comparison groups. In the study, the influence of alcohol poisoning severity (moderate degree, n=42; severe degree, n=47) was also taken into account. All participants in the study were mostly represented by men

(90%) aged between 20 and 40 years (mean age 31.8; SD: 5.7). Exclusion criteria from the study were: chronic alcohol intoxication, alcoholic or viral hepatitis, the presence of acute infectious and inflammatory processes of other organs during the study period, as well as acute or chronic pyelonephritis of infectious etiology, acute or chronic glomerulonephritis, diabetes and/or diabetic nephropathy, obesity. In addition, persons younger than 18 years or older than 40 years were excluded from the study. The control group was represented by individuals without clinical and laboratory signs of impaired renal function, without signs of AAP (confirmed by the absence of alcohol in the blood) or chronic alcohol exposure. CKD was diagnosed according to the criteria recommended by KDIGO 2012. A prerequisite for the diagnosis of CKD stage 1 and stage 2 with normal or elevated eGFR, as well as in patients with its initial decrease ($60 \leq \text{eGFR} < 90 \text{ mL/min/1.73 m}^2$) was the presence of signs of kidney damage (detection of markers of kidney damage, in particular increased albuminuria/proteinuria, persisting for at least three months). In our study, 82% of patients with CKD were in remission.

Blood sampling was carried out early in the morning on the second day of hospitalization, since it is believed that this time is enough for a response of serum creatinine in the case of AKI (17). Blood was stabilized by heparin. All blood tests were conducted within two hours after the blood collection. Serum NGAL level was measured using a commercially available ELISA kit (Affymetrix eBioscience, Vienna, Austria); the unit of measure was ng/mL. The protocol of Fedorova and Levin was used to obtain neutrophils from whole blood and to prepare cell-lysate (18). Cell count was standardized up to 1 million in 1 mL of medium. The count of neutrophils was enabled by using a Mindray BC-3200 Hematology Analyzer.

The suspension of neutrophils (10^6 cells/mL) was lysed by freeze-thawing (19). AOPP level in neutrophil lysate was determined using the method of Witko-Sarsat et al (20). The main laboratory indicators of kidney functions were also evaluated. eGFR was calculated according to serum creatinine concentration, using the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) formula (unit in mL/min/1.73 m²). Patients' BAC averaged 2.2 permille (‰, BAC by mass) with total range 0.6–4.7‰.

Ethical issues

The study was approved by the ethics committee of Karaganda Medical University and was conducted in accordance with the Helsinki Declaration. Informed consent was obtained from all the patients before their inclusion in the study. During the presence of patients in the toxicological department all of them received standard

therapy, corresponding to the poisoning severity and developed complications.

Statistical analysis

The Statistica program, version 12 was used to analyze the received data. One-way ANOVA for independent variables was used (21) to determine significant differences between the groups. The choice of this statistical method was due to the normal distribution of data (Shapiro-Wilk normality test, $P > 0.05$) and homogeneity of variances (Levene's test, $P = 0.591$) (22). The differences were considered reliable at significance level $P < 0.05$. The post-hoc Bonferroni test was used to identify pairs of samples, differing from each other in means. Correlation analysis was done using Pearson parametric correlation. Parametric data are presented as mean \pm SD.

Results

Analysis of serum NGAL level

In our study we found an inverse correlation between the serum NGAL concentration and eGFR in patients with CKD 1, 2 ($r = -0.5$, $P < 0.05$), which corresponds to the results of another study (10). However, there was no significant correlation between serum NGAL level and eGFR or serum creatinine concentration in patients with AAP ($P > 0.05$). Then, to study the impact of eGFR on the serum NGAL concentration in patients with AAP we ranked conditionally eGFR indexes into three groups: from 90 to 120 mL/min/1.73 m² – “normal” eGFR, above 120 mL/min/1.73 m² – “increased” eGFR (from 121 to 140) and less than 90 mL/min/1.73 m² – “reduced” eGFR (from 50 to 89). The effect of gender was excluded because the study was dominated by men. We found that eGFR did not affect the serum NGAL level ($F = 2.21$, $P = 0.12$) and its increase was observed both at “reduced” and “increased” eGFR only relative to the control group values ($P < 0.001$). Serum NGAL level depended on eGFR in patients with AAP and control group has been shown in Table 1.

Next, to analyze the effect of an increase in serum NGAL concentration, observed both at “reduced” and “increased” eGFR, we added the results of laboratory tests of kidney function to the study. We divided the patients with AAP into the following groups: group I ($n = 25$) – with “normal” eGFR without clinical and laboratory signs of impaired renal function; group II ($n = 20$) – with “increased” eGFR without clinical and laboratory signs of impaired renal function; group III ($n = 17$) – with “normal” or “increased” eGFR and minor shifts in urine analysis: moderate leukocyturia (from 4 to 14 cells in the field of view) and minor proteinuria (0.03–0.1 g/L). And finally, group IV ($n = 27$) – patients with toxic nephropathy, caused by AAP (it corresponds to the clinical picture of

Table 1. Serum NGAL level depending on eGFR in patients with AAP and control group

	Mean (ng/mL)	Standard deviation	-95% CI	+95% CI
Control group	98.17	85.13	62.22	134.12
“Normal” eGFR 90-120	266.17	158.08	153.09	379.25
“Increased” eGFR 121-140	508.46*	390.58*	347.23*	669.68*
“Reduced” eGFR 50- 89	571.03*	399.67*	302.53*	839.53*

* Reliability of differences with the control group, $P < 0.001$.

eGFR was calculated according to serum creatinine, unit in mL/min/1.73 m².

AKI), confirmed by proteinuria, hematuria, leukocyturia, cylindruria, increased serum creatinine, as well as reduced eGFR and daily diuresis. The control group and the group of patients with CKD stage 1 (eGFR mean 101.0; SD: 7.9) and stage 2 (eGFR mean 66.3; SD: 13.9) were used as comparison groups.

Serum NGAL level in the studied groups is presented in Table 2. Thus, in group I with “normal” eGFR without clinical and laboratory signs of impaired renal function, the level of NGAL increased slightly, however without a statistically significant difference with the control group. The level of NGAL in groups II, III and IV increased by 5–6 times relative to the control group values, was significantly higher than in group I and exceeded the values of CKD stage 1 group. However, there were no significant differences in NGAL level between groups II, III and IV. The maximum values, reaching 1177 ng/mL were found in groups III and IV. Serum NGAL level in patients with CKD stage 1 and stage 2 did not differ from the control group values and from each other (Table 2).

Alcohol poisoning severity, according to our data, did not have a statistically significant effect on the serum NGAL concentration ($F = 0.81$, $P = 0.37$). Therefore, serum NGAL level was much higher than the control group values both in moderate degree ($m = 527.02$; CI 95%: 334.06-719.98) and severe degree of alcohol poisoning ($m = 425.25$; CI 95%: 285.31-565.19), ($P < 0.005$). Additionally, a correlation analysis showed

that serum NGAL level did not correlate with BAC, while the eGFR correlated with BAC ($r = 0.3$, $P < 0.05$) while the higher BAC, the higher eGFR.

Analysis of AOPP level in neutrophils

We also ranked conditionally eGFR indexes into three groups to study the impact of eGFR on the AOPP level in patients with AAP. We found that eGFR did not affect the AOPP level ($F = 0.067$, $P = 0.936$). An upward trend in AOPP concentration was revealed both at “increased” and “normal” eGFR compared with the control values, however, statistically significant increase was detected only in patients with “reduced” eGFR (mean 0.042; SD: 0.023) relative to the control group values (mean 0.021; SD: 0.013) ($P = 0.042$). There was no significant correlation between the AOPP level and eGFR or serum creatinine concentration, according to our data. Then, we added the results of laboratory tests of kidney function to study AOPP level in patients with AAP (Table 3). We detected the increased AOPP level in groups II, III and IV compared with the control group values ($P < 0.03$). In patients with CKD stage 1 and stage 2, AOPP level was significantly higher than the control group values and exceeded the values of group I ($P < 0.03$). We did not reveal a significant correlation between the AOPP level and serum NGAL or BAC. One-way ANOVA did not confirm the effect of alcohol poisoning severity on the AOPP level ($F = 2.86$, $P = 0.09$).

Table 2. Serum NGAL level in the studied groups, depending on the eGFR and results of laboratory tests of kidney function

	Mean (ng/mL)	Standard deviation	-95% CI	+95% CI
Control group	98.17	85.13	62.22	134.12
Group I	219.64	178.42	111.83	327.46
Group II	540.07 ^{a,b,c}	341.59 ^{a,b,c}	333.65 ^{a,b,c}	746.49 ^{a,b,c}
Group III	647.37 ^{a,b,c}	452.27 ^{a,b,c}	85.80 ^{a,b,c}	1208.94 ^{a,b,c}
Group IV	569.42 ^{a,b,c}	402.11 ^{a,b,c}	346.74 ^{a,b,c}	792.10 ^{a,b,c}
CKD stage 1	162.32	99.61	85.75	238.88
CKD stage 2	276.36	124.86	160.89	391.84

^a Reliability of differences with CKD stage 1, $P < 0.03$;

^b Reliability of differences with the control, $P < 0.001$;

^c Reliability of differences with group I, $P < 0.05$

Group I: “normal” eGFR without clinical and laboratory signs of impaired renal function;

Group II: “increased” eGFR without clinical and laboratory signs of impaired renal function;

Group III: “normal” or “increased” eGFR and minor shifts in urine analysis;

Group IV: patients with toxic nephropathy, caused by AAP.

Table 3. AOPP level in neutrophils in the studied groups, depending on the eGFR and results of laboratory tests of kidney function

	Mean (ng/mL)	Standard deviation	-95% CI	+95% CI
Control group	0.017	0.006	0.014	0.020
Group I	0.028	0.017	0.019	0.038
Group II	0.040 ^a	0.028 ^a	0.026 ^a	0.053 ^a
Group III	0.042 ^a	0.020 ^a	0.029 ^a	0.055 ^a
Group IV	0.045 ^a	0.027 ^a	0.035 ^a	0.054 ^a
CKD stage 1	0.054 ^{a,b}	0.015 ^{a,b}	0.045 ^{a,b}	0.063 ^{a,b}
CKD stage 2	0.057 ^{a,b}	0.026 ^{a,b}	0.037 ^{a,b}	0.077 ^{a,b}

^a Reliability of differences with the control, $P < 0.03$.

^c Reliability of differences with group I, $P < 0.03$.

Group I: "normal" eGFR without clinical and laboratory signs of impaired renal function.

Group II: "increased" eGFR without clinical and laboratory signs of impaired renal function.

Group III: "normal" or "increased" eGFR and minor shifts in urine analysis.

Group IV: patients with toxic nephropathy, caused by AAP.

Discussion

A slight increase in serum NGAL level, detected in patients with AAP in the group with "normal" eGFR (estimated according to serum creatinine) without clinical and laboratory signs of impaired renal function is possible, in our opinion, due to the direct effect of ethanol on the liver. In addition, all patients had leukocytosis with a high percentage of segmented neutrophils. It was previously reported that the liver and neutrophils are sources of NGAL in the blood (11). In our study, we did not find a correlation between BAC and serum NGAL level; also, alcohol poisoning severity did not affect the NGAL values. The correlation between eGFR and BAC is due to the effect of ethanol. The mechanisms of eGFR increasing in the case of AAP have been described in detail and are associated mainly with hypertonic dehydration and an increase in the osmolarity of blood plasma (23). It deserves attention, that the same mechanism underlies the increase in eGFR in the case of diabetic nephropathy due to hyperglycemia.

Firstly, the results of our study showed a multiple increase in serum NGAL concentration and AOPP level in patients with toxic nephropathy with a clinical picture of AKI. Secondly, our study demonstrated a multiple increase in serum NGAL concentration and AOPP level in patients with "increased" eGFR even in the absence of clinical and laboratory signs of impaired renal function, as well as in patients with "normal" or "increased" eGFR and minor abnormalities in urine analysis. In our opinion, both these groups can be considered as patients with a "preclinical stage" of kidney damage and a high risk of nephropathy or AKI development. Moreover, NGAL values in the aforesaid patients' groups exceeded not only the control values, but also the values of patients with "normal" eGFR, without clinical and laboratory signs of impaired renal function. It deserves attention that changes in AOPP level in neutrophils in the aforesaid groups corresponded

to changes in serum NGAL level, which is a single trend for them. This situation is similar to the other, observed in patients with diabetes mellitus whose microalbuminuria is often accompanied by increased eGFR and is considered as the initial stage of diabetic nephropathy. It was also shown that the initial stage of kidney damage in the case of hypertension is also manifested by an increase in eGFR, while at later stages – by decline (24). Thereby, eGFR reflects early, intermediate and later stages of kidney damage, but changes in eGFR are not equivalent. The decrease in eGFR in combination with the diagnostic criteria of toxic nephropathy, proposed by Luzhnikov et al reveal only the late stage of kidney damage when the function of a significant part of nephrons is lost. Our study showed the possibility of using the serum NGAL in patients with AAP to detect the "preclinical stage" of reduced renal function, until the moment when it can be diagnosed with using only serum creatinine. We propose to consider an increase in eGFR together with an increase in serum NGAL concentration in patients with AAP as a stage, preceding the nephropathy or AKI, even in the absence of clinical and laboratory signs of impaired renal function. Obviously, the changes in the kidneys are reversible exactly at the "preclinical stage" in the case of elimination of etiological factor and appropriate therapy. While in diagnosed nephropathy, even though the etiological factor is eliminated, the renal function worsens progressively and the stage of CRF develops as an outcome (4). However, the main point why and due to what mechanisms the changes in the kidneys, that have arisen initially, continue to progress inevitably even with the elimination of etiological factor, has not yet found an exhaustive explanation.

Thus, there was a single trend in the changes in serum NGAL and AOPP level in neutrophils both in toxic nephropathy with a clinical picture of AKI and in the "preclinical stage" of kidney damage, described above. At

the same time, in patients with CKD stage 1 and stage 2 our study revealed opposite trends in the changes of these indicators. NGAL level in patients with CKD did not differ from the control group values and was significantly lower than in patients with toxic nephropathy or in patients with the “preclinical stage” of kidney damage. In contrast, AOPP level in patients with CKD significantly exceeded the control values and had a clear tendency to increase compared to the patients with toxic nephropathy or patients with the “preclinical stage” of kidney damage. It was shown earlier that plasma AOPP level increased in patients with alcohol intoxication together with the degree of severity and reached a maximum in the case of toxic nephropathy (25). Thus, increased AOPP level in neutrophils may be due to their capture from the blood. Currently, it is believed that AOPP is formed mainly under the influence of chlorine-containing oxidants and are mediators between neutrophils and monocytes, potentiating a “respiratory burst” in monocytes, thus are mediators of inflammation. This fact, undoubtedly, contributes to the progression of renal damage and promotes the transition from reversible changes to irreversible. It deserves attention that there was no significant increase in serum NGAL concentration in patients with CKD stage 1 and stage 2. However, previously it was reported that patients with progressive CKD have increased serum NGAL in comparison with patients without progression. It is suggested that serum creatinine or eGFR are markers of the number of functioning nephrons while serum or urine NGAL is indicator of current kidney damage activity (26). Thus, a significant increase in serum NGAL, which is a marker of tubular lesions can be observed due to acute tubular necrosis and allows us to predict the severity of renal damage.

Conclusion

We propose to consider an increase in eGFR together with an increase in serum NGAL concentration in patients with AAP as the stage, preceding the nephropathy or AKI, even in the absence of clinical and laboratory signs of impaired renal function. We detected a single trend in the changes in serum NGAL and AOPP level in neutrophils both in toxic nephropathy with a clinical picture of AKI and in the “preclinical stage” of kidney damage. At the same time, in patients with CKD stage 1 and stage 2 our study revealed opposite trends in the changes of these indicators. It deserves attention that there was no significant increase in serum NGAL concentration in patients with CKD stage 1 and stage 2.

Limitations of the study

In our research we studied the possibility of serum NGAL and oxidized neutrophil proteins in the early detection of

kidney damage in patients with AAP, we did not consider the patients with chronic alcoholism. Additionally, all indicators were determined once. It is of great interest to study these indicators in dynamics.

Authors' contribution

The final manuscript has been read and approved by all the authors. All authors have made a substantial contribution to the conception, design, gathering, analysis and interpretation of data, writing and intellectual content of the article. LAD participated in the design, conduct of the study (data collection), statistical analysis and interpretation of data and preparing the manuscript. DAK participated in describing the methodology of the study and amending the manuscript draft of the article. LYM participated in describing the methodology of the study, statistical analysis and amending the manuscript draft of the article. VBML prepared the draft of the proposal and participated in conduct of the study (selection of patients in the study according to inclusion and exclusion criteria, diagnosis). RYB prepared the draft of the proposal and participated in conduct of the study (selection of patients in the study according to inclusion and exclusion criteria, diagnosis). OAP participated in conduct of the study (data collection, enter data to software) and preparing the manuscript draft. YAK participated in the design, conduct of the study (data collection) and statistical analysis. DDB participated in conduct of the study (data collection, enter data to software) and preparing the manuscript draft. AK participated in the design of the study and amending the manuscript draft of the article.

Conflicts of interest

The authors report no conflict of interest

Ethical considerations

Ethical issues including plagiarism, double publication, and redundancy have been completely observed by the authors.

Funding /Support

This study was supported by an interior grant from the Karaganda Medical University, devoted to the study of extracellular vesicles and neutrophil reactivity in AKI (001/KFNI3). The authors declare that all views, expressed in the article are their own and not an official position of the institution, that supported this study. This research did not receive funding sources from any third party and any commercial organizations or pharmaceutical companies.

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