RESEARCH REPORT

The development and evaluation of the efficacy of ovine-derived experimental antivenom immunoserum against *Macrovipera lebetina obtusa* (MLO) venom

Arsen Kishmiryan, Gevorg Ghukasyan, Lusine Ghulikyan, Anna Darbinyan, Lilia Parseghyan, Armen Voskanyan, Naira M Ayvazyan*

L. A. Orbeli Institute of Physiology of the National Academy of Sciences of the Republic of Armenia, Laboratory of Toxinology and Molecular Systematics, 0028, Orbeli 22, Yerevan, Armenia

*Correspondence to: Naira Ayvazyan, Email: taipan@ysu.am, nairaayvazyan@physiol.sci.am, Tel: +374 91 587344, Fax: +374 10 272247

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ABSTRACT

Here we describe the processing and development of animal-derived monovalent antibody serum against *Macrovipera lebetina obtusa* venom by purification and concentration of the immunoglobulins using caprylic acid. We demonstrate that this new viper venom antiserum is pre-clinically effective in neutralizing lethal toxicity and hemorrhagicity of the venom of the Armenian Levantine viper – a significant public health problem in Armenia and a wide region from south-east parts of Europe to south-west Asia. The developed product shows a high capacity to inhibit metalloproteinases and phospholipase activity of venom included in the study in comparison to current specific antivenoms, and following additional experimental approvals, it will be possible to derive the monovalent antivenom satisfying international standards, which will be much cheaper and accessible compared with the current market rivals.

KEYWORDS: snake venom, vipers, ovine antivenom, *Macrovipera lebetina obtusa*

INTRODUCTION

Snakebite is a significant public health problems, resulting in thousands of deaths each year as well as leaving the survivors with life-long and life-changing disabilities (Kasturiratne et al, 2008; Gutiérrez, 2018). World Health Organization has recently classified snakebites as a neglected tropical disease with prevalence in Asia, Sub-Saharan Africa, Latin America, and Oceania (WHO, 2019). The only scientifically proven treatment for snakebites is the injection of specific antivenoms (WHO, 2018), which represent immunoglobulins derived from the serum of immunized animals (complete immunoglobulin molecules or Fab/ fragments) (Gutiérrez et al, 2011).

Viperidae snakebites produce notable morbidity and mortality and have a significant impact on health care. These snakes are widespread throughout Eurasia, especially Southeast (the Caucasus including Armenia, Turkey, Iran, etc.). Between 1999 and 2008, several genus-level name changes have occurred, most notably the transfer of some species of Vipera and *Macrovipera* to the genera Daboia and Montivipera (usefully summarized in WHO, 2010, and Reptile database, 2010, and references therein).

In Armenia, the majority of snakebites are due to *Macrovipera lebetina obtusa* (MLO), which is a subtype of viper family with venom containing the proteins belonging to few main families: Zn$^{2+}$- metalloproteinases (PIII and PI), phospholipase A2 (PLA2), serine proteinases, L-amino acid oxidase, disintegrins (short and dimeric), cysteine-rich secretory proteins, Bradykinin-potentiating Peptides and C-type Natriuretic Peptides (Sanz et al, 2008; Siigur et al, 2019; Pla et al, 2020). According to the data provided by the Ministry of Health of Armenia (MOHA), during the timeframe of 2015-2019, there were recorded 89-146 cases
per year of snakebites with several mortalities. Despite this devastating impact on public health, MOHA does not purchase and provide medical institutions in Armenia with antivenoms. For a period of several years, antivenom of quite high quality produced by the Institute of Immunology of Zagreb was available in Yerevan (Kurtović et al, 2014). Regrettably, the Institute stopped production of the antivenom in 2014. Currently, the available antivenom is imported by a private organization (“Armen Farm”) from Uzbekistan, and, according to its itinerary, it is trivalent antivenom against Vipera lebetina, Echis carinatus, Naja oxiana snakebites. Vipera lebetina (now Macrovipera) snake group involves 5 subspecies (M. l. cernovi, M. l. lebetina, M. l. obtusa, M. l. transmediterranea, M. l. turanica), which differ in their venom composition (Aaspõllu and Siigur, 2018; Sanz et al, 2008). Taking into account the fact that in Uzbekistan, there is only one subtype of this viper (M. l. turanica), also frequent dissatisfaction of clinicians with the effect of antitoxin, there is a huge concern regarding the quality and effectiveness of currently available antivenom. This product is not listed in the World Health Organization List of Antivenoms database (apps.who.int/bloodproducts/snakeantivenoms/database/default.htm), where against Macrovipera lebetina venom could be found only three polyvalent products: Anti-viperin/Institut Pasteur d’Algerie/Algeria; Gamma-Vip/Institut Pasteur de Tunis/Tunisia; Polyvalent Snake Antivenom/Razi Vaccine & Serum Research Institute/Iran. Only Iranian antivenom is a product of immunization by the M. l. obtusa and it is not a market-available product. On the other hand, this subspecies of Levant blunt-nosed viper has a widest geographical distribution: from Turkey, through Syria, Lebanon, Iraq, north Jordan, Armenia, Azerbaijan, Dagestan, Iran, southern Afghanistan, Pakistan (Kashmir), and north India. Unfortunately, the epidemiological situation with snakebites in these countries patchy and scarce, but it is no doubt that, although the incidence is not such high comparing to the tropical and sub-tropical world, severe envenomations often require antivenom because of the strong inflammatory and necrotizing properties of this venom (Ladnova et al, 2018; Pla et al, 2020). Considering these facts, the implementation of the production of Armenian antivenom and the development of antivenom production guidelines will have a significant impact on public health.

This article discusses the production of ovine antibody serum (ABS) development against MLO snake. The methods presented herein corresponds to effective protocols of antivenom production against Vipera ammodytes (European Pharmacopoeia (Ph.Eur.01/2008:0145); Lang Balija et al, 2005; Căpitănescu et al, 2008) and satisfies WHO current instructions of antivenom production (Figure 1) (Bolton et al, 2014; WHO, 2018).

**MATERIALS AND METHODS**

**Chemicals and snake venom**

Sodium alginate and polyethylene glycol 4000 were from Medisar LLC (Armenia); caprylic acid from Carl Roth (Germany); other solutions from Sigma–Aldrich (USA). *Macrovipera lebetina obtusa* (MLO) venom was pooled from 20 adult individuals between 90 and 120cm in length and older than 3 years of both sexes, collected during the day in spring and autumn in different regions of the Republic of Armenia. Venom was vacuum-dried at room temperature and stored at -5°C until used.

**Animals**

Sheep for antibody serum production were chosen according to their availability, the ease and expenses of their care and nutrition. The animals were medically examined against various infections and diseases. The animal-derived experimental venom antibody serum was raised by immunizing two 1.5-2 year-old male sheeps (40kg) in accordance with the WHO Guidelines for the Production, Control, and Regulation of Snake Antivenom Immunoglobulins (WHO, 2018). The animals were kept in constant light (07.00-19.00) and temperature (25±2°C), and provided with food and water according to their needs.

Determinations of the neutralization of lethal toxicity and hemorrhagic activity were performed with non-pure bred male mice (18-22gm) and rats (180-220gm), bred at the Orbeli Institute of Physiology. All animal experimentation was carried out in accordance with Council Directive 2010/63/EU of the European Parliament, the Council of 22 September 2010 on the protection of animals used for scientific purposes, and approved by the Committee of
Ethics of Yerevan State Medical University (YSMU) (Yerevan, Armenia).

Immunization
The immunization of the animals was done with subcutaneous injections of multiple and increasing dosages of venom of *Macrovipera lebetina obtusa* snake. Immunization included two stages (Table 1). During the first stage, the animals were injected with MLO venom; to decrease the chance of tissue damage with venom and adjuvant, the injections made in two different anatomical regions on the animal’s neck in areas close to axillary lymph nodes. The corresponding dosages of venom dissolved into adjuvant, sodium alginate. The use of adjuvant increases the antibody formation, due to the fact, that adjuvant makes venom depots and slows venom absorption into the systemic circulation, giving the immune system the opportunity to constantly produce antibodies (León et al, 2011). The solutions envisaged for immunization were prepared immediately before the injection. In the room temperature sodium alginate dissolved into the 0.9% (w/v) NaCl until reaching 1.25% (mass/volume) for 15min. Thereafter, MLO venom dissolved into the 1.5ml of the solution (Table 1).

During the second stage of immunization, the MLO venom without adjuvant was injected into an animal’s neck multiple times. The venom dissolved in 0.9% (w/v) NaCl and injected according to Table 1. After the last injection, the 2-week break was taken to allow the organism to produce enough quantity of antibodies. Thereafter, 1-2 times a week 350ml of blood was collected via the jugular vein into a sterile, pyrogen-free 0.4l bags. The collected blood was rotated for 20min to accelerate clotting, then centrifuged for 15min at 3000rpm (BECKMAN, GS-6R centrifuge), the blood cells were separated and the serum kept in the freeze (maximum 2 weeks) until the serum kept in the freeze (maximum 2 weeks) until the antibodies separated and purified. To conserve the adequate titer of antibodies in animals, subsequently, they were immunized regularly according to the second stage of immunization. Immunizations were carried out every four months for a period of two years and blood was collected during three weeks after each immunization.

**The separation and purification of antibodies using caprylic acid**
The serum containing IgG antibodies were subsequently processed by caprylic acid to separate these antibodies. During this stage, every 100ml serum slowly mixed with 5.27ml caprylic acid (5%, w/v, analytical grade, ≥99,5%) with a magnetic stirrer in room temperature (22-25°C). The mixing was continued for one hour and maintaining solution pH in 5.2-5.4 range using 4N NaOH. Subsequently, this solution centrifuged for 15min at 3000rpm (BECKMAN, GS-6R centrifuge) and the sediment was separated. To separate this sediment from the remnants of caprylic acid it was dialyzed for 36hr using 1.35l 0.9%, w/v, NaCl. During this time the NaCl solution changed every 9hr. Dialysis subsequent yield purified IgG antibodies which were condensed 3 times by placing the dialysis bag into a Berzelius flask that contains 300gm polyethylene glycol 4000 (this step could be repeated few times). The final condensed solution containing IgG antibodies was divided into Eppendorf vials and refrigerated (-4°C) until subsequent experiments. The validation of the purified antibodies from sheep sera was done by the Immunofixation capillary electrophoresis (Helena Bioscience Europe, UK). Each experimental antiserum used in the study was normalized by protein concentration (by Lowry and Bradford in parallel) and

| Table 1. Timetable of the immunization scheme of sheeps (2 phases). |
|-----------------|-----------------|-----------------|-----------------|
| Week number     | Tube number     | Volume of venom solution injected (mL) | Quantity of venom injected (mg) |
| 1               | 1               | 0.1             | 1               |
| 2               | 2               | 0.2             | 2               |
| 3               | 3               | 0.3             | 3               |
| 4               | 4               | 0.4             | 4               |
| 5               | 5               | 0.5             | 5               |
| 6               | 6               | 0.6             | 6               |
| 7               | 7               | 1               | 10              |
| 8               | 8               | 1.5             | 15              |
| 9               | 9               | 0.1             | 1               |
| 10              | 10              | 0.3             | 3               |
| 11              | 11              | 1               | 10              |
| 12              | 12              | 1.8             | 18              |
| 13              | 13              | 1               | 20              |
| 14              | 14              | 1.8             | 36              |
contain 53-60mg IgG/ml at 1.5ml/ampoule. The IgG yield from this downstream processing is 16 to 21ml of concentrate antibody serum per 100ml of ovine blood.

**Immunodiffusion Assay**

Double diffusion test according to Ouchterlony and Nilsson (1958) used to study antigenic relationships between the different antigens. Holes 5mm in diameter were blown in horizontal gels containing 1.2%, w/v, agarose in 1x PBS. Protein fractions of several types of viper venom and cobra venom (20µl) were placed in the peripheral wells and ABS in the central well. The diffusion was let to proceed for 24hr at 37°C. The gel was then cleaned with saline solution and dried. The precipitin lines were visualized with Coomassie Brilliant Blue staining.

**Animal survival assay**

To evaluate the effectiveness of ABS, SLD<sub>50</sub> dosage of MLO venom lethality was neutralized with antibody serum. The LD<sub>50</sub> of MLO venom (18.4±1.4µg/mouse, i.v.) was used according to previous studies (Kurtović et al, 2014). The experiments were undertaken on the white male mice (18-20gm) grown in the vivarium of the Orbeli Institute of Physiology. Experimental animals were divided into 5 groups of four mice (n=4). Mice of control group i.v. injected with exclusively venom solution, the rest of groups injected with SL'D<sub>50</sub> dosage of venom pre-incubated with different dilutions of ABS (Dilution Factor (DF): 8, 4, 2, and without dilution). Each mouse in the control group was injected with a SL'D<sub>50</sub> dosage of venom (92µg/mouse) of venom dissolved in 0.5ml of 0.9%, w/v, NaCl. In the case of the rest groups, the same dosage dissolved into 250µl 0.9%, w/v, NaCl, thereafter mixed with the above-mentioned dilutions of ABS in 1:1 ratio, and incubated for 30min in 37°C. Centrifugation (5min, 3000xg) and separation of the sediment were undertaken and each mouse was injected with 0.5ml of this solution. During a 24hr period after injections mice were observed to record the lethal cases, as it is recommended by WHO guidelines for the intravenous test (WHO, 1981; WHO, 2018). The Spearman–Karber method (Ph.Eur.01/2008:0145; WHO, 1981; Saganuwan A, 2016) used to calculate the median effective dose LD<sub>50</sub> which constitutes the amount of undiluted antiserum (in ml) efficient for neutralizing the lethality of used venom dose in 50% of animals. Using LD<sub>50</sub> value, Protective efficacy (R) was calculated and represents the amount of LD<sub>50</sub> possible to be neutralized with 1ml of undiluted antivenom.

**Assay of hemorrhagic activity and its neutralization**

The hemorrhagic activity was evaluated according to Theakston and Reid (1983), with experimental details described by T. Kurtovic et al (2014). Briefly, each rat on the dorsal side received 100µl of saline containing venom (i.d.) prepared in doses ranging from 1.28 to 50µg. After 24h their skin was removed and from its inner surface, the perpendicular diameters of the hemorrhagic lesions were measured. ABS (diluted 2-, 5- or 10-fold) was incubated with an equal volume of the venom solution (1mg/ml) at 37°C for 30min. Aliquots of 100µl were i.d. administered to a group of four rats. As a negative control, saline instead of ABS was used. The hemorrhagic lesions on the inner surface of the removed skin were observed 24h later, their perpendicular diameters measured and corresponding surfaces calculated, from which the average value for each dose was obtained. Results are expressed as NHA (%) = [(PV – P<sub>vav</sub>)/PV] x 100±SE, where NHA is a Neutralization of Hemorrhagic Activity (%), PV represents the mean value of lesion surfaces induced by the venom alone and P<sub>vav</sub> the mean value of lesion surfaces induced by the venom mixed with ABS.

**Assays of the in vitro inhibition of the enzymatic activity of svPLA<sub>2</sub> and svMPs (express-tests).**

For the in vitro assays of the MLO venom PLA<sub>2</sub> and Zn-MPs enzymatic activities and their rapid inhibition by the experimental ABS antiserum, two simple express-tests were developed in our Institute based on the methods of the coagulation of the egg yolk (Nieuwenhuizen et al, 1974) and caseinolitic activity inhibition (Vejan et al, 2017) with certain modifications (Voskanyan et al, 2017). The methods are briefly described below.

The yolks of fresh chicken eggs were mixed at 1/1 ratio with a PBS (pH 7.4) resulting in Yolk Buffer Solution (YBS). The MLO venom was added to the YBS at 1:2500 ratios. In the case of the rest groups, the same dosage of venom, thereafter mixed with the undiluted and two times diluted ABS in 1:1 ratio, and incubated for 2hr at room temperature. After incubation, the whole 0.5ml of this mixture was added to 2ml of YBS. The MLO/ABS/YBS solution was incubated at 38°C for 1hr. Then, the tubes were placed in boiling water for 15min. The YBS without venom or venom with inhibited phospholipase A<sub>2</sub> activity coagulates into a light yellow solid clot. The mixture pre-incubated with venom remains liquid due to the emergence of free fatty acids by the phospholipase A<sub>2</sub> activity.

For the determining of the caseinolitic activity the natural cow’s milk was incubated with the MLO venom in a ratio of 1: 2500 (venom/milk). Intact MLO venom curdles milk during the first 10min of the incubation. The venom in which Zn-MPs enzymatic activity inhibited by ABS does not curdle the milk at all (observation time: 60min).

**RESULTS**

The venom of *M. l. obtusa* exerted both lethal and hemorrhagic activities. The LD<sub>50</sub> and MHD values of the venom, measured in mice and rats, respectively, have been summarized previously (Kurtovic et al, 2014). We evaluated the purity (Figure 2) and effectiveness of the developed experimental antivenom to neutralize the lethal dosage of MLO venom, the data is shown in Table 2. The mice in the control group died immediately after injection of SDL<sub>50</sub> dosage of the venom, whereas in the groups were ABS used was, the percentage of mortality decreased inversely to the dilution of the antibody serum. Particularly, the experimental antivenom diluted 2x displayed complete neutralization of venom lethal toxicity. TheLD<sub>50</sub> of this ABS compiles 0.05257ml/mouse or 2.6285ml/kg. This data allowed calculation of the ABS Protective efficacy (R), which equals 76 (Table 2).

The results of immunodiffusion experiments with experimental ABS also showed some cross-reactivity (Figure...
Figure 2. The IgG yield after first stage of immunization (A) by Immunofixation capillary electrophoresis data (IgG – 73.9% of the overall blood serum protein content after precipitation); IgG yield after second stage of immunization (B,C); Immunofixation capillary electrophoresis gel film (D) - the phenotyping of IgG, heavy chains and/or light chains (kappa and lambda).

Table 2. Effective dose fifty ($ED_{50}$) and Protective efficacy (R) calculation for antibody serum (ABS).

| Injection volume (ml/mouse) | Antivenom volume (ml/mouse) | Number of mice | Percentage of deaths (%) |
|-----------------------------|-----------------------------|----------------|--------------------------|
|                            |                             | Died | Survived | Total |                |
| MLOv+ABS                    | 0.5                         | 0    | 4        | 4     | 0               |
| *ABS ( DF=2)                | 0.5                         | 0    | 4        | 4     | 0               |
| *ABS ( DF=4)                | 0.5                         | 2    | 2        | 4     | 50              |
| *ABS ( DF=8)                | 0.5                         | 3    | 1        | 4     | 75              |
| MLOv                        | 0.5                         | 4    | 0        | 4     | 100             |

The $ED_{50}$ was calculated according to Spearman and Karber (Saganuwan, 2016):

$$log_{10}ED_{50} = log_{10}X_{100} - \frac{log_{10}DF}{n}(\Sigma t-n/2) = -1.27925$$

$$ED_{50} = 0.05257 \text{ ml/mouse}$$

**The $ED_{50}$ is the effective volume of ABS that will protect 50% of the mouse population when injected with $LD_{50}$s.**

$$R = \frac{(T_{v} - 1)}{ED_{50}}$$

$$= (5-1)/0.05247$$

$$= 76$$

**$T_{v}$** is the amount of $LD_{50}$ injected in one mouse.
It is evident that when anti-MLO IgG experimented against crude MLO venom, a wide pattern shown in Figure 3 (top well) was obtained pointing coalescence of antigens. While the same ABS was tested against the venom of *Montivipera raddei* one precipitin line was formed. The comparatively weak immunoprecipitation has been observed also for the venoms of *Montivipera latifi* and *Daboia russelii*, but not for the *Naja oxiana* venom (data not presented).

The potential of ABS to neutralize hemorrhagicity of MLO venom tested in a dose of 50µg was evaluated in rats. As expected, the ABS exhibited the most effective protective power against the hemorrhagic activity of the venom when undiluted. It also undoubtedly showed neutralization ability, although to a lesser degree, towards MLO venom (Figure 4) after dilutions. Namely, complete neutralization of 50µg of the MLO venom was obtained with a double-volume of antibody serum. Achievement of the 72% of the effect against the equal dose of MLO venom required its application in a 1:1 volumes ratio of ABS vs MLO venom, and 56% - if the volume of ABS was half of the venom volume in the venom-antivenom mixture.

Rapid *in vitro* express tests clearly show the capability of the presented ABS product to neutralize two main enzymatic activities of the MLO venom, namely, the PLA2 and Zn-MPs effects (Figures 5 and 6). Being simple and qualitative, these tests are well suited to preliminary test of the experimental antivenom potency of the product. Interestingly, the lyophilization of the ABS and consequent experiments with the dry IgG antibodies dissolved in the same volume of physiological solution demonstrated the identical results both for the assay of lethal toxicity neutralization and the neutralization of the hemorrhagic activity of MLO venom. The experiments with dry ABS let us measure the active weight of IgGs for both approaches: the 1ml of ABS contained 53mg of dry protein (from which the 94.8% (91-95%) is IgG, Figure 2) was enough to neutralize 76 LD₅₀ doses of MLO venom, while more than 100mg of ABS should be applied to neutralize the hemorrhagic activity of venom completely. These data are in a good accordance with the experimental results of Archundia et al (2011) and Alagon et al (2019) for the pentavalent antiserum and of Pla et al (2020) for the cross-reactive Russian new anti-*Vipera berus berus* product efficacy.

**DISCUSSION**

Horses are the preferred species for sourcing antivenom immunoglobulins for the industrial production of antivenoms. However, it has been suggested that ovine antivenoms are safer products for humans (Landon and Smith, 2003; Gerardo et al, 2017). Nevertheless, until now no clinical evidence has shown significant differences of adverse reactions caused by both types of antivenoms (Abubakar et al, 2010; Gutiérrez et al, 2018). The WHO guide for standard protocols of snake antivenoms purification does not restrict manufacturers in the source and method for antivenom production (WHO, 2018). The main rule for the preferable method for this should be the one that renders the highest purification with the fewest number of steps. In contrast to pepsin digestion, usually performed for the equine immunoglobulin F(ab')₂, fragments production,
enzymatic digestion with papain is used to produce sheep Fab fragment-based antivenoms. The León et al (2000) papain digestion method does not reduce the potency of plasma, but because of its pharmacokinetic properties, Fab fragments are rapidly eliminated from the body by renal filtration after intravenous administration (Gutiérrez et al, 2018), which leads of antigenemia and envenomation (Seifert and Boyer, 2001; Bush et al, 2015) and hence, to the need of more doses of antivenom. All these studies appear to support the use of protocols to produce the antivenom using sheep whole IgG. This is also because of the ease/price/yield ratio for our country (Hamza et al, 2016). Thus this experimental product could become a prototype for the monovalent anti-Macrovipera lebetina obtusa antivenom, as its manufacturing is needed not only in Armenia, but also in the neighboring countries in our region.

Figure 4. The hemorrhagic activity neutralization assay in rats performed with AV antivenom. Antivenom (undiluted and diluted 1:2 or 1:3) was pre-incubated with 50μg of Ma. l. obtusa (MLO) and presented as Neutralization of hemorrhagic activity (%).

Figure 5. The ability of experimental AV to inhibit the svPLA2 activity. C. control (egg yolk with PBS after boiling; 3min in 98°C in waterbath); 1 and 2. egg yolks with MLO pre-incubated with undiluted and two times diluted antivenom (1:1 ratio); 3. egg yolks with intact MLO.
It is generally understood that the increased polyvalency in antivenoms entails diminished potency against a venom, although only a few studies have addressed this question directly (Raweerith and Ratanabanangkoon, 2005, for some Asian elapids; Dos-Santos et al, 2010, for some American vipers). In the case of the genus *Vipera*, there are several recent publications concerning the para-specificity and cross-reactivity of some polyvalent antivenoms raised against *Vipera* and *Macrovipera* venoms (Archundia et al, 2011, Pla et al, 2020), including our previous investigation concerning this question (Kurtovic et al, 2014). Based on all these works and our own observations, we suggest that in many cases it may be preferable to have the monovalent antivenom, which has cross-reactivity with the venom of other vipers of the region rather than polyvalent, which can lead to adverse reactions in as many as 60% of the clinical cases.

Overall, our data indicate that the experimental antibody serum developed against MLO venom described in this article displays high effectiveness and targets the components of viper venom that compile the toxic potential of the venom. However, further pre-clinical investigations are needed before clinical trials of these antivenoms. Considering the lack of easy availability and accessibility of antivenoms in Armenia such studies are of considerable importance.

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COMPETING INTERESTS

None declared.

LIST OF ABBREVIATION

ABS: antibody serum  
PBS: Phosphate buffered saline

MLO: *Macrovipera lebetina obtusa*  
MOHA: Ministry of Health of the Republic of Armenia  
PLA₂: Phospholipase A2  
YSMU: Yerevan State Medical University after M. Heratsi  
Zn-MPs: Zn²⁺-dependent metalloproteinases

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