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Determination of adenosine by CRISPR-Cas12a system based on duplexed aptamer and molecular beacon reporter linked to gold nanoparticles

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Abstract

Adenosine as a potential tumor marker is of great value for clinical disease diagnosis. Since the CRISPR-cas12a system is only capable of recognizing nucleic acid targets we expanded the CRISPR-cas12a system to determine small molecules by designing a duplexed aptamer (DA) converting g-RNA recognition of adenosine to recognition of aptamer complementary DNA strands (ACD). To further improve the sensitivity of determination, we designed a molecule beacon (MB)/gold nanoparticle (AuNP)-based reporter, which has higher sensitivity than traditional ssDNA reporter. In addition, the AuNP-based reporter enables more efficient and fast determination. The determination of adenosine under 488-nm excitation can be realized within 7 min, which is more than 4 times faster than traditional ssDNA reporter. The linear determination range of the assay to adenosine was 0.5–100 μM with the determination limit of 15.67 nM. The assay was applied to recovery determination of adenosine in serum samples with satisfactory results. The recoveries were between 91 and 106% and the RSD values of different concertation were below 4.8%. This sensitive, highly selective, and stable sensing system is expected to play a role in the clinical determination of adenosine and other biomolecules.

Keywords Adenosine · CRISPR-cas12a · Duplexed aptamer · Molecule beacon · Gold nanoparticle · Fluorescence spectrometry

Introduction

Adenosine, as a purine nucleoside, plays a huge role in regulating tumor growth, which is a response factor that can be produced by immune cells in the fight against tumor cells [1–4]. Adenosine concentrations in the microenvironment of tumor cells have been reported to be 10 to 20 times higher than that in the microenvironment of healthy humans [5, 6]. There have been many studies on the anti-tumor effect of adenosine, which confirmed that inhibition of adenosine overexpression can effectively inhibit tumor growth and spread [7]. Therefore, as a potential tumor marker, rapid and sensitive determination of adenosine is of great significance.

Aptamers are single-stranded nucleotides that can specifically recognize and bind to targets. Because of its simple synthesis, high selectivity, good chemical stability, and low cost, aptamer has become a common recognition component in biosensors [8, 9]. At present, most of the determination of adenosine uses adenosine aptamer. Aptamer undergoes structural transformation after binding to the target molecule [10, 11]. Based on this feature, aptamer complementary DNA strand (ACD) combined with aptamer was designed to convert a single aptamer into duplexed aptamer (DA). ACD plays the role of blocker in DA, which can block aptamers. When target molecules exist, the structure of aptamers will be transformed after specific recognition of target molecules, and ACD will be dissociated and become a new determination signal. This

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DA-based signal conversion has higher activity and wider application than a single aptamer [12].

Clustered regularly interspaced short palindromic repeats (CRSIPR) and CRISPR-associated proteins (cas) are collectively known as the CRISPR-cas system [13]. Due to its high sensitivity, low determination limit, and good stability, CRISPR-cas12a system has been widely used in clinical determination of nucleic acid substances. For example, the well-known SHERLOCK system and DETECTOR system are widely used in commercial determination of HPV virus [14]. In the latest report, the CRISPR-cas12a system has been preliminarily developed into an efficient nucleic acid test strip for COVID-19 virus determination [15]. The CRISPR-cas12a system works on the principle that when g-RNA recognizes and binds to a single strand of target DNA, the cas12a protein is activated to perform trans-cleave, which can achieve non-selective cleave of the surrounding single strand DNA (ssDNA) other than the target DNA. Therefore, ssDNA (Reporter) with a luminescent group at one end and a fluorescence quenching group at the other end is designed [16, 17]. When the trans cleavage ability of cas12a protein is activated, Reporter is cleaved to emit fluorescence for determination.

In some of the latest reports, the CRISPR-cas12a system can recognize not only ssDNA, but also RNA or other non-nucleic acid material. For example, Luo et al. found that the CRISPR-Cas12a system can specifically recognize microRNAs and performed fluorescence determination [18]. The traditional CRISPR-cas12a system uses straight-strand ssDNA as reporter, but its slow cutting rate limits its application [19, 20]. The molecular beacon (MB), due to its bulges, seems to be more easily cleaved by activated cas proteins for more sensitive and rapid determination. The maximum emission spectrum of FAM luminescent group is well matched with the maximum absorption spectrum of gold nanoparticles (AuNPs). This allows a fluorescence resonance energy transfer (FRET) process between AuNPs and MB to quench the fluorescence of FAM groups. Furthermore, the MB attached to AuNPs formed a hairpin structure and increased the concertation of MB per unit volume, which could be cleaved more efficiently by cas protein [21, 22].

Here, we designed a novel CRISPR-cas12a determination system for adenosine determination (Scheme 1). The aptamer chain in DA can effectively recognize adenosine molecules, and g-RNA was designed to bind ACD. Once adenosine molecules are present, duplexed aptamer will undergo structural transformation so that ACD is freed and combined with gRNA to stimulate trans cleavage activity of cas protein. The reporter was designed as AuNPs/MBs. One end of the MBs was connected with FAM luminescent group, and the other end was connected with sulfhydryl group to covalently connect with AuNPs. When cas12a protein was not activated with trans cleavage activity, the fluorescence of MBs was quenched by AuNPs. Once the trans cleavage ability of cas12a protein was activated, the MBs on the AuNPs would be cut off, and the fluorescence of the FAM group would be restored. Based on this, rapid, sensitive, and stable determination of adenosine can be performed.
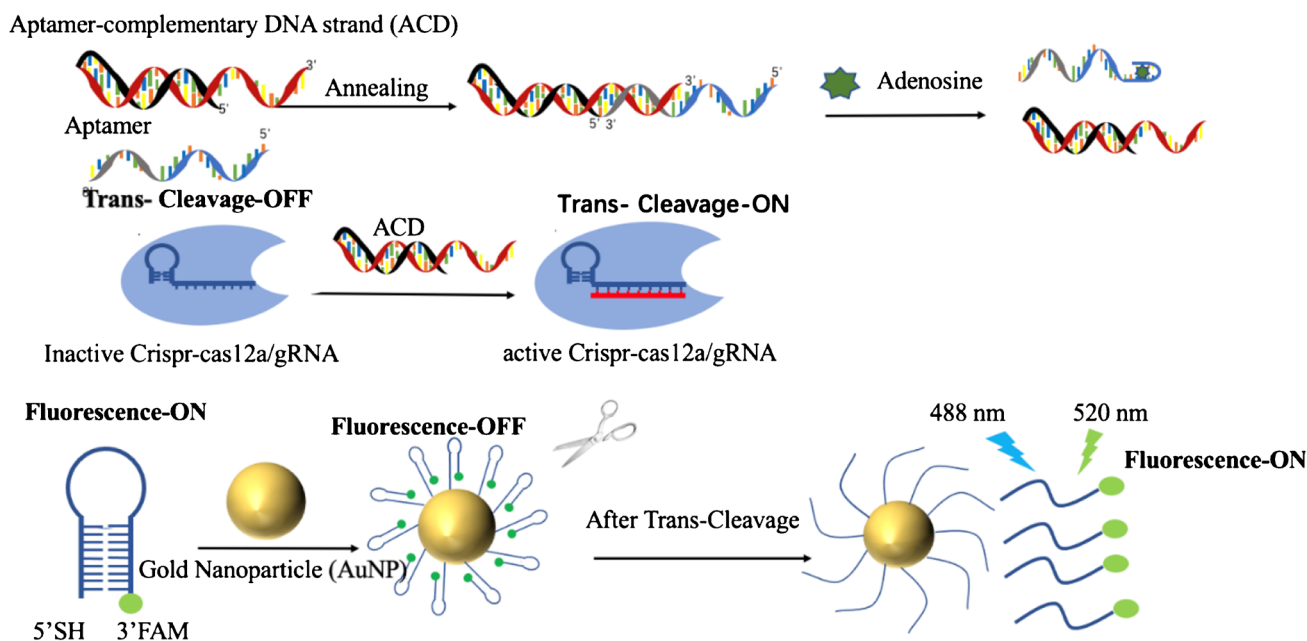
Scheme 1 Mechanism of CRISPR-cas12a system based on DA and MB/AuNPs for adenosine determination

Experimental section

Materials and reagents

EnGen Lba Cas12a (cpf1), NEbuffer2.1, and TEbuffer were all purchased from New England Biolabs (Ipswitch, MA). All DNA sequences as well as g-RNA sequences were synthesized by Sangon Biotech (Shanghai, China) and the sequences are shown in Table S1. The storage lifetime of cpf1 is 6 months under −20 °C, the g-RNA sequences should be stored at −80 °C no more than 1 month, and all the DNA sequences can be stored at −20 °C for 3 months. DEPC water was also obtained from Sangon Biotech. $HAuCl_4 \cdot 4H_2O$ (10 mg/mL) and adenosine (99.9%) were provided by Macklin biotech (Shanghai, China). Agarose, 1*TAE solution, Gelstain dye, loading buffer (6%), and marker (20–500 bp) were all purchased from Sangon Biotech (Shanghai, China). All the chemical reagents used in this work are analytical grade.

Experimental apparatus

All fluorescence measurements were performed on a fluorometer F-7000 (Hitachi, Japan). Transmission electron microscope (TEM) testing of the particle morphology of AuNPs and AuNP/MB was performed by FeL-Tecnai G2 F20 (USA). The AuNPs and AuNP/MB particle sizes were characterized using a liquid Zeta particle size analyzer Malvern-Zetananosizer S90 (UK). The maximum ultraviolet absorption of AuNPs and MB/AuNPs was tested by UV-visible spectrum UV1780 (Shimadzu, Japan). Elemental analysis of AuNP/MB was performed by Tescan Mira4 and energy spectrum Xplore 30 EDS. Electrophoresis experiments were carried out on gel electrical system (Bio-Rad Laboratories, Electrophoresis, Hercules, CA) (80 V, 60 min). Gel imaging was performed on ZF-388 Full Automatic Gel Imaging System (Shanghai, China).

The synthesis of AuNPs/MB

The synthesis of AuNPs is based on the sodium citrate reduction method of $HAuCl_4$ that we used previously [23]. In simple terms, 1 mL of $HAuCl_4 \cdot 4H_2O$ (10 mg/mL) was added into 99 mL of deionized water. The mixture was heated and stirred until boiling. Then a solution of 2% (w/V) sodium citrate was quickly added, and the solution immediately turned black and then slowly turned fuchsia. The mixture was heated and refluxed for 15 min, then cooled to room temperature. The prepared AuNPs are stored in a refrigerator at 4 °C until ready for use.

The method of ligating sulfhydryl modified MB to AuNPs is an improvement of the method previously reported in the literature [24]. Typically, 3 μL of 100 μM of MB was added to 5 mM Tris (2-chloroethyl) phosphate (TECP) and reduced for one hour. Then 100 μL of the prepared AuNPs was added and the mixture was frozen in the refrigerator at −4 °C overnight. After returning to normal temperature, it was centrifuged three times in a refrigerated centrifuge (4 °C, 12000 rpm, 30 min). After removing the supernatant, the obtained AuNP/MB were redispersed in 100 μL deionized water.

Synthesis of duplexed aptamer

The aptamer and ACD were all configured with TEBuffer. According to literature reports [25], aptamer and ACD were added at a concentration of 4:1 to NEbuffer2.1. The mixture was then heated to 95 °C for 5 min, and then slowly cooled and annealed for about 4h to reach room temperature to obtain duplexed aptamer.

CRISPR-Cas12a system for adenosine determination

The adenosine solution was prepared with DEPC water, and different concentrations of adenosine solution were added to the DA mixture, while the concentration of aptamer was maintained at 300 nM. The mixed solution of adenosine and DA was reacted in the water bath at 37 °C for 30 min. CRISPR-cas12a was prepared with special diluent to make its concentration reach 1 μM. g-RNA was prepared with DEPC water into 1.25 μM solution. Cas12a and g-RNA were mixed and added into NEbuffer2.1. The crispr-cas12a/g-RNA mixture was incubated in a water bath at 37 °C for 30 min. The entire assay system was configured with 40 nM cas12a, 50 nM g-RNA, 120 nM aptamer, 30 nM ACD, and 10 μL Au-MB reporter. The entire determination system was added to the cuvette and determined by the fluorometer F-7000 with an excitation wavelength of 488 nm.

Results and discussion

Activation of DA-based CRISPR-cas12a system by adenosine

The activation effect of adenosine on DA-based CRISPR-cas12a system was verified by agarose gel electrophoresis. We selected seven holes to add different contents for the experiment. The added contents and experimental results are shown in Fig. 1a. The annealed duplexed aptamer was added in hole 1, and there are three bands, which are unpaired aptamer, ACD, and combined DA. When MB is added to well 2, a bright band can be
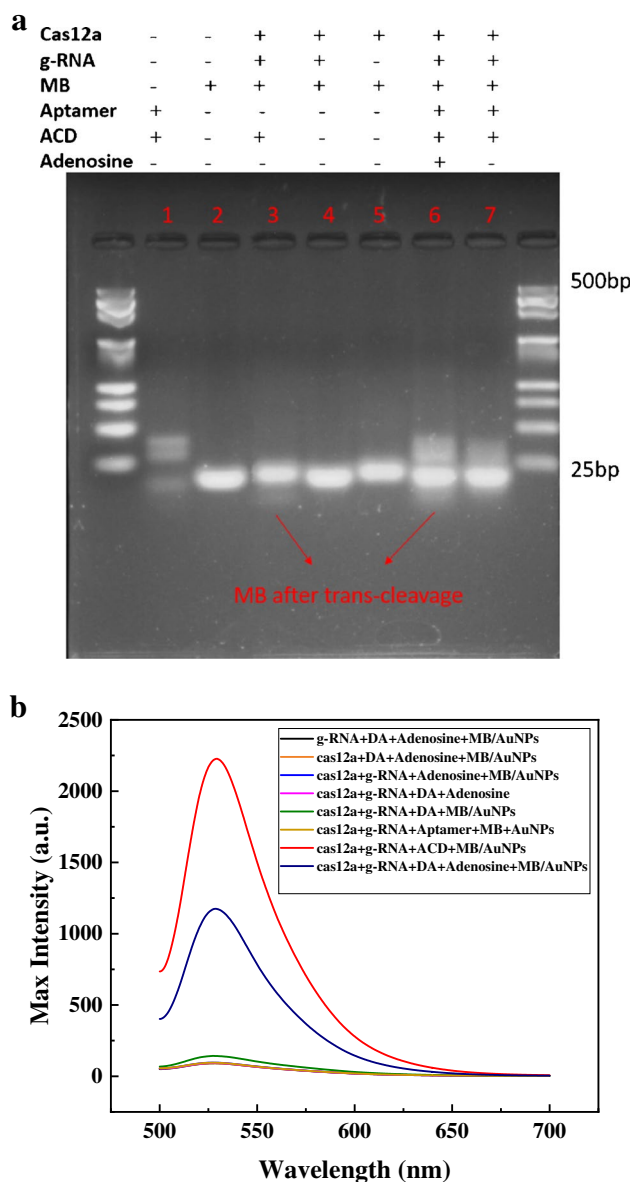
Fig. 1 **a** Image of CRISPR-cas12a system based on DA and MB/AuNPs by agarose gel electrophoresis; **b** Fluorescence spectra of adenosine determination under 488-nm excitation by CRISPR-cas12a system based on DA and MB/AuNPs

observed. Using ACD to activate the trans cleavage function of cas12a in hole 3, it can be found that a new band is generated under the bright band, which is the product of MB after cleavage. Similarly, in the experimental group, well 6 also saw the emergence of a new cleaved band under the MB band, which proved that adenosine can well activate the DA-based CRISPR-cas12a system. To further verify that adenosine can stimulate the trans-cleavage activity of the DA and MB/AuNP-based CRISPR-cas12a system, fluorescence measurements of different fractions were performed (Fig. 1b). The measured results were in consistent with the results of gel electrophoresis. Cas12a

activated by ACD alone showed the strongest cleavage ability, and the experimental group supplemented with adenosine and DA also produced strong fluorescence. None of the other controls produced fluorescence. In the blank group, because a certain amount of ACD and aptamer could not be completely combined, weak fluorescence was also produced.

Structure analysis of AuNP/MB

TEM images (Fig. S1a and S1b) show that the morphology of AuNPs before and after MB modification does not change significantly. However, as shown in the EDS scan (Fig. S2), AuNP/MB contained DNA components such as C, O, P, and S. This is initial proof that the MB was successfully linked to the AuNPs. Then, the particle size of the AuNPs modified with MB was analyzed by dynamic light scattering (DLS). As shown in Fig. S3, the diameter of the AuNPs synthesized was about 16 nm, while that of the AuNPs modified with MB was about 33 nm. The particle size of AuNPs increased significantly after MB modification, which further indicated that MB was successfully connected to AuNPs. Then, UV-VIS measurements were performed on AuNPs before and after MB modification (Fig. S4). It was found that the absorption spectra of AuNPs overlapped well with the emission spectra of the FAM group, allowing the FRET process to occur and effectively quench the fluorescence of the FAM group. The absorption spectrum of AuNPs modified with MB has a certain degree of red shift, and a new absorption peak (generated by MB) appears at 250 nm, which also proved that the MB is connected to the AuNPs.

Cleavage activity of AuNP/MB

In order to verify the efficient and rapid cleavage ability of AuNP/MB, we used traditional reporter ssDNA as a comparison, and measured their real-time fluorescence (Fig. 2). The 600 nM ssDNA and the same concentration of AuNP/MB were added to the DA-based CRISPR-cas12a system for determination. As can be seen, within the time range of 800 s, the fluorescence response of the traditional reporter grows slowly and cannot reach the maximum fluorescence value within 800s. AuNP/MB, however, reacted violently when added to the CRISPR-cas12a system, reaching the maximum fluorescence response within 7 min. This proves that our AuNP/MB reporter amplification method can effectively increase CRISPR-cas12a cleavage activity.

Linear determination range of adenosine by CRISPR-cas12 system based on DA and AuNP/MB

After optimizing the experimental conditions, the CRISPR-cas12a system based on DA and AuNP/MB was applied to quantitatively determine adenosine. Different concentrations

Fig. 2 Comparison of ssDNA determination efficiency with traditional SSDNA determination efficiency
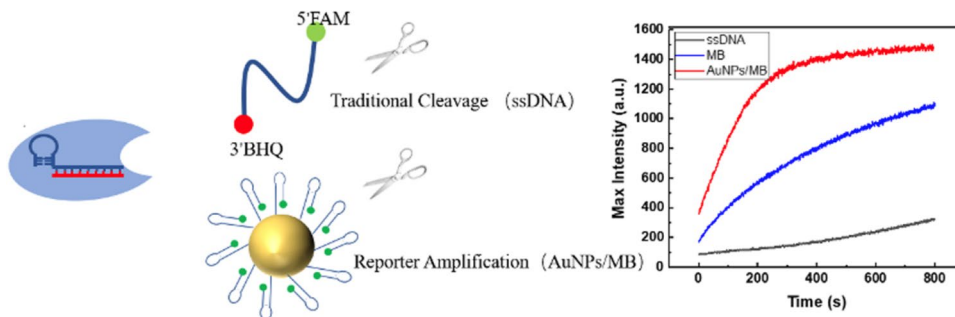
of adenosine solutions were added to DA and incubated, then added to CRISPR-cas12a system for 7 min, and then the maximum fluorescence value was measured. As can be seen from Fig. 3a, when the concentration of adenosine increases from 500 nM to 100 µM, the maximum fluorescence value also increases linearly. According to the regression plot between the maximum fluorescence value and the concentration of adenosine, the linear equation (Fig. 3b) is MI (a.u.) $=10.3\pm0.334C$ (µM) $+102\pm7.45$ ($R^2=0.993$). According to the formula LOD$=3S/k$ (S=standard deviation of the blank $n=10$), the determination limit of the system for adenosine is 15.67nM, and its linear range is 0.5–100µM. This proves that our sensing system is sensitive and fast for adenosine determination. The determination performance of our method for adenosine was compared with other reported methods. As shown in Table 1, it can be seen, our method has advantages in limit of determination and determination speed.

Selectivity and reproducibility of the assay

Since the contents of serum are rather complex, therefore, we selected some substances in the serum or have a structure similar to adenosine as potential interferences (glucose, caudate, uric acid, glutamate, guanine, cytidine, thymidine, and uridine) to test the selectivity of the assay. In this experiment, 100 µM concentration of adenosine was selected as the standard and 10mM concentration of interfering substance

was selected. As can be seen from Fig. S5, the responses of the assay to these substances are similar to that of the blank control, which is mainly due to the high selectivity of the aptamer for adenosine. After mixing all interferences with adenosine and then determined, the fluorescence response was found to be similar to the determination of adenosine alone, which means that our CRISPR-cas12a system based on DA and AuNP/MB has high selectivity for adenosine in complex environment and can be further applied to the determination of adenosine in serum.

To study the reproducibility of the assay, 1 µM and 100 µM of adenosine were measured independently for 5 times. The relative standard deviation (RSD) of the testing results was 2.6% and 4.0%, respectively, proving the assay has good reproducibility for both high and low concentrations of adenosine.

Measurement of adenosine content in serum

The CRISPR-cas12a system based on DA and AuNP/MB was finally applied to measure adenosine content in healthy human serum (male, 24 years old, stored at −20 °C). The original adenosine concentration in the serum found by the assay was 0.881µM ± 0.094µM ($n=3$). Then, a standard addition method was applied to verify the accuracy of the assay for the determination of adenosine in serum. Three concentrations of adenosine, 1 µM, 10 µM, and 100 µM of adenosine, were added to the serum samples and then

Fig. 3 a Fluorescence spectra of changes in adenosine concentration under 488-nm excitation (0.5, 1, 2, 5, 10, 25, 50, 75, and 100µM); **b** Linear regression equation
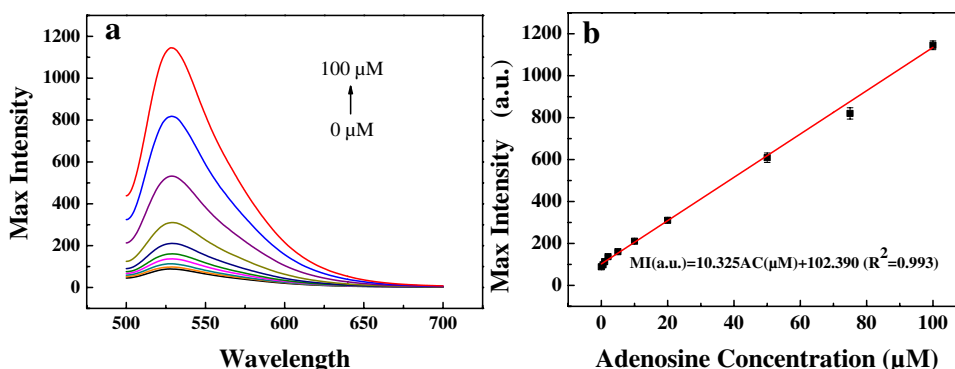
Table 1 Comparison with other methods for determination adenosine

Method	Determination range (μM)	LOD (nM)	Determination time (min)	Ref.
Fluorescence	30–680	6000	240	[26]
Colorimetric	5–60	39.00	20	[27]
SERS	0.005–0.5	5.000	/	[28]
Fluorescence	1–100	420.0	40	[29]
Light diffraction measurement	0.0001–1000	0.09	/	[30]
This work	0.5–100	15.67	7	

Table 2 Recovery in actual serum samples

Serum No.	Added (μM)	Found (μM)	Recovery (%)	RSD (%)
1	1.00	1.06	106	4.8
2	10.0	9.9	99	2.8
3	100	91	91	1.4

determined. The calculated recoveries were between 91 and 106% (Table 2). This proves the reliability of our assay for determination of adenosine in serum samples.

Conclusion

This work designed a DA- and AuNP/MB-based reporter amplification CRISPR-cas12a system for the determination of adenosine in serum. The assay converts recognition of adenosine to recognition of ACD. The reporter amplification method based on AuNP/MB greatly shortened the determination time and improved the sensitivity of the CRISPR-cas12a system. Adenosine in serum was determined by standard addition method with reliable results, which proved the feasibility of this sensing system in clinical determination. This assay paved a new way for the CRISPR-cas12a-based system to determination of not only nucleic acid, but also other substances, which may find wide applications. However, the procedure of the assay is still a little complex that needs to be simplified.

Declarations

Ethical approval All experiments were in accordance with the guidelines of the National Institute of Health, China, and approved by the Institutional Ethical Committee (IEC) of Central South University. We also obtained informed consent for any experimentation with human serum samples.

Conflict of interest The authors declare no competing interests.

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