nature portfolio

Peer Review File

A Pre-Diagnostic Tool for Familial Mediterranean Fever a Rare Genetic Disorder

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Overall Comments:
The authors have fabricated a label-free, plasmonic chip-based biosensing platform utilizing gold nanoparticles and optofluidic technology for Familial Mediterranean Fever (FMF) patient diagnostics. This approach incorporates visible light spectroscopy and post-processing algorithms to test clinical samples all run via a Raspberry Pi. Additionally, the authors show a working prototype that could be utilized in a clinical setting as a FMF preliminary laboratory test. Such a device would allow doctors to get results within hours, as opposed to weeks or even months for genetic testing, allowing patients to immediately begin treatment. Though this work has great potential and is arguably viewed as top 50%, meaning very significant, it lacks crucial scientific depth including specificity tests, sample validation, statistical analysis of diagnostic results and literature support for methodology and limit of detection/quantification calculations. This manuscript has serious deficiency including no structural data on gold nanoparticle analysis, characterization of plamonic sensor surface, data processing, etc. The manuscript should not be published in any moderately high impact journal. This reviewer has listed some additional comments for authors to consider for the future submission.

Major Comments:
1. Overall, the authors claim they are using an LSPR-based method to detect pyrin protein for FMF diagnostics. Most LSPR based sensors measure the absorption, scattering, or extinction spectra of a nanoparticle or plasmonic sensor and utilize the obtained LSPR dipole peak position as their data. However, here the authors are measuring the LSPR response of their sensor by employing a “spectral integral method, which cumulatively evaluates spectral variations” (line 317-318). It is stated that “transmission resonance is integrated within a spectral window and the sum of all spectral changes within this region is used to determine the overall spectral change” (line 318-320). However, the only citation given is for monitoring spectral changes at multiple wavelengths, not their integration. Is there literature support for using this method? The problem being that other factors, such as chemical damping, can reduce dipole peak intensity and cause peak broadening which can greatly impacting the peak integration over a set range that has nothing to do with analyte detection or the concentration of analyte present. This needs to be addressed in detail and discussed further in terms of how the authors are definitively linking peak integrations to the amount of analyte present.
2. The authors state that (3-aminopropyl)triethoxysilane was used to functionalize the glass substrates. Why was this compound used instead of (3-mercaptopropyl)triethoxysilane? This is significant as the amino group only affords an electrostatic attraction, while the sulfur of the mercapto group would instead form a much stronger covalent bond with the Au nanoparticles.
3. Additionally, it is stated that the Au nanoparticles were evaluated using several methods, including SEM, however, no images are provided. It is common practice to include a spectral figure of the as synthesized nanoparticles as well as an SEM image of the fully functionalized glass coverslip in the manuscript. Nanoparticle attachment needs to have a density and distribution that is reproducible. Any batch-to-batch variations would lead to varying

reaction concentrations and heterogeneity in the resulting functionalized glass coverslips. This could result in sensor inconsistencies and inaccurate results. The authors also state that "random particle distribution resulting from the particle synthesis-based fabrication method" (line 453-454) does occur, so without such documentation, the manuscript is incomplete.

4. Could the authors please explain the amount of sample that is used and the number of repeat measurements taken. It is stated that the clinical samples were diluted with PBS buffer but it is not clearly stated how much total sample was used for testing over the 20 minute flow cycle and how many replicate tests were done for each. The calibration curve does mention that "5 independent experiments were conducted" (line 347) but it is unclear if that was also the number of replicate tests per sample for patient samples. For analytical chemistry, n=6 is typically used. The authors should expand their measurements to at least 6 if they have not already done so.

5. When discussing the detection of purin protein with their biosensing platform, the authors state they obtained an LOD of 0.3 ng/mL. They also state that this is "highly sensitive" (line 39). LSPR-based sensing mechanisms used to quantify proteins are seen to have an LOD in the aM to zM range. 0.3 ng/mL might be considered sensitive but it would not be considered highly sensitive. Additionally, the determination of that LOD seems be solely based on the fact that less than 0.3 ng/mL could not be readily distinguished by the biosensing platform due to significant overlap. This is not how LOD is typically calculated for sensors. In most publications, the LOD is derived by using a Z value of the blank (Z= mean + 3σ, σ = standard deviation of blank). This is typically determined from six separate measurements using six different sensors and substituting the Z value into the "Y" in the calibration curve best fit line equation allowing for the LOD concentration ("X") to be obtained. The limit of quantification (LOQ) should also be determined and discussed with LOD for a sensor such as this.

6. With any newly developed sensor fabrication, specificity testing is vital to conduct prior to pubication. However, there is no specificity testing presented in this work despite the authors claim that this approach "ensures high selectivity and specificity" (line 534). Contrastingly, the author mentions that whole blood samples could "lead to unspecific binding" (line 204) and that "clinical samples contain a high number of other proteins that may interfere with the capturing of pyrin protein (line 378-379). The author states that diluting clinical samples with PBS buffer and subtracting out the integral values for the control channel is their "correction procedure" to "account for nonspecific binding" (line 398). This, however, is not the typical method for conducting specificity tests for sensors that prove the spectral changes observed are a direct result of target analyte detection and nothing else. For this reason, other protein biomarkers for FMF found in blood should be tested along with other circulating proteins. The proteins used for specificity testing should be tested individually and as a mixture do determine if nonspecific binding is occurring.

7. When patient samples are analyzed by a new sensing construct, validation of the samples is required. The manuscript does not include validation of their findings via an FDA approved technique. For example, validation testing for proteins can be accomplished using electrochemiluminescence, ELIZA or western blot. The samples tested should be analyzed by one of these validation methods and the results compared to the data obtained from the plasmonic biosensor. A correlation graph should be made to determine the Pearson's r value and included in the manuscript.

8. On Figure 4B and C the authors present their plasmonic biosensor results, however, no

statistical significance is shown. A statistical analysis of diagnostic results is typically done with a program, such as GraphPad Prism, to show how well a sensor can differentiate patient or sample groups. Both a Mann Whitney t-test and an area under the curve (AUC) of the receiver operating characteristic (ROC) graph can be conducted to determine p values and star significance of the data. Without this type of statistical analysis of the plasmonic biosensor results, they are meaningless.

Minor Comments:
1. The total number of citations for this manuscript is 33 indicating that it is severely under-cited. The authors should address this.
2. The “Plasmonic Biosensor” used in the schematic illustration on Figure 6 does not match the construct used in previous figures in this paper where the Au nanoparticle is shown in between the glass and the bound A/G-IgG-Pyrin.
3. The authors did not mention the shelf-life stability of the plasmonic chip. If this is something that would be used in a clinical setting, this information would be of great importance.
4. The calibration curve provided in Figure 3D does not include error bars for the spectral integral measurements shown. The inset for the figure does include error bars. Line 347 states that 5 independent experiments for each concentration were conducted. Was that only done for the concentrations in the inset? If every concentration was conducted 5 times, there should be error bars shown for each concentration on the figure.

Reviewer #2 (Remarks to the Author):

The manuscript titled "A Pre-Diagnostic Tool for a Rare Disease: Familial Mediterranean Fever (FMF)" has undergone a thorough review. This study presents an innovative approach to FMF diagnosis by measuring pyrin levels in patient samples, a protein affected by variations in the MEFV gene.

Research on the pathogenesis of Familial Mediterranean Fever (FMF) has led to the discovery of numerous inflammatory pathways within the innate immune system, including PAMPs, DAMPs, inflammasomes, pyroptosis, and NETosis. These investigations have significantly advanced our understanding of the pathogenesis and treatment of several important diseases, such as atherosclerosis and diabetes. Despite these notable breakthroughs, many aspects of FMF's pathogenesis and diagnosis remain mysterious.

FMF diagnosis primarily relies on compatible symptoms and a positive response to colchicine treatment. However, a definitive diagnosis necessitates the confirmation of specific pathogenic variations in the MEFV gene. Nonetheless, genetic testing is only informative for approximately 50% of patients. Another significant challenge arises from the presence of gene variants of unknown significance (VUS) and their potential contribution to the disease's pathogenesis. Functional studies have provided some insights into these unanswered questions, but undoubtedly, we require practical techniques like the one explored in this study to address these challenges effectively.

I believe the manuscript is well-written, and its subject matter remains highly relevant in the present context. The research is original and of high quality, holding substantial potential for future citations. The authors have meticulously outlined their methodologies, providing ample information for the replication of experiments. Furthermore, it's noteworthy that their method may find applicability in addressing other conditions characterized by variations in protein quantity.

However, I do have a few notable concerns about the study, such as the utilization of the technique on a limited group of homogeneous subjects, among others, which I will elaborate on below.

Major Points:
1. Line 30 and in other instances, the use of "variant" instead of "mutation" is the preferred terminology for expressing genetic differences between individuals.
2. Lines 31 and 71, "The MEFV gene encodes a protein called pyrin, and mutations in this gene lead to abnormally increased pyrin synthesis." It should be clarified that MEFV variants alter the structure and interactions of pyrin, potentially leading to increased pyrin production due to positive feedback loops involving IL-1 beta on pyrin synthesis.
3. The nature of the patient sample used in experiments is unclear. It's crucial to specify whether it was full blood, serum, cell lysate, or another source, as this information is critical given that free pyrin is not present in blood and pyrin is preferentially expressed in myeloid lineage cells.
4. In Figure 4B, the y-axis unit should be indicated, and absolute numerical values should be provided on the bars.
5. Investigate if there is a correlation between serum inflammatory markers (C-reactive protein & serum amyloid A) and corrected SI of pyrin obtained from the technique.
6. Specify the MEFV variants of the patients and investigate if there were any differences between patients based on the variants they harbored.
7. Line 510: Provide this test's numerical values of sensitivity and specificity.
8. Discuss the potential limitations of this technique's specificity for FMF diagnosis, considering that various factors, including IL-1β, TNF-α, IFN-γ, infections, and trauma, stimulate pyrin expression. I strongly suggest authors to consider testing the technique in individuals with asymptomatic carriers, patients with different variants (single heterozygous, complex heterozygous, homozygous), and other rheumatic diseases, infections, and trauma.
9. Suggest that the primary utility of this technique may lie in revealing the pathogenicity of variants of unknown significance and distinguishing between individuals with a single heterozygous variant who are sick or carriers. Include these points in the introduction and discussion sections.
Minor Points:
1. Remove "(FMF)" from the title.
2. Remove the word "disease" after "FMF."
3. Consider moving or removing the sentence, "Children are particularly affected by FMF, and the disease process can be distressing for them," affecting the text's flow.
4. Consider using "presumptive diagnosis" or “preliminary diagnosis” instead of "pre-diagnosis" for better terminology.
5. Remove the phrase "FMF was first documented in a case report in 1908, and it was clearly described in 1945. Moreover," as it is unrelated to the primary subject and disrupts the

manuscript's flow.
6. On Line 62, replace "pain" with "restriction."
7. Remove the phrase "and serositis" from Line 62.
8. On Line 70, change "Interleukin-1" to "interleukin-1β."
9. On Lines 80-83 and 507, note that per EULAR FMF treatment recommendations, a clinical diagnosis of FMF is sufficient to initiate colchicine treatment; genetic diagnosis is not necessary for treatment initiation.
10. On Lines 101-111, consider moving this paragraph to the methods section.
11. Provide information about institutional or commercial sources of the used pyrin and anti-pyrin antibody materials.

REVIEWERS' COMMENTS

REVIEWER #1

The authors have fabricated a label-free, plasmonic chip-based biosensing platform utilizing gold nanoparticles and optofluidic technology for Familial Mediterranean Fever (FMF) patient diagnostics. This approach incorporates visible light spectroscopy and post-processing algorithms to test clinical samples all run via a Raspberry Pi. Additionally, the authors show a working prototype that could be utilized in a clinical setting as a FMF preliminary laboratory test. Such a device would allow doctors to get results within hours, as opposed to weeks or even months for genetic testing, allowing patients to immediately begin treatment.

We sincerely appreciate the reviewer's positive comments regarding the practical usability of our technology for detecting a rare disease. His/her feedback is encouraging and reinforces the significance of our work.

Though this work has great potential and is arguably viewed as top 50%, meaning very significant, it lacks crucial scientific depth including specificity tests, sample validation, statistical analysis of diagnostic results and literature support for methodology and limit of detection/quantification calculations. This manuscript has serious deficiency including no structural data on gold nanoparticle analysis, characterization of plasmonic sensor surface, data processing, etc. The manuscript should not be published in any moderately high impact journal.

We wish to extend our gratitude to the reviewer for his/her comprehensive evaluation of our manuscript. By addressing his/her concerns, we have significantly enhanced the quality of our work. The reviewer's valuable input has been invaluable in refining our work.

Particularly, we agree with the reviewer's comments regarding the plasmonic chip fabrication. Our manuscript primarily focuses on the technology for diagnosing a rare disease, which is why we did not provide extensive details on particle synthesis for plasmonic chip fabrication. We have already collected a significant amount of the data requested by the reviewer, and here we incorporated these details, either as new figures or within the Supporting Information section. The reviewer's feedback has guided us in this direction, and we believe these additions enriched our manuscript.

This reviewer has listed some additional comments for authors to consider for the future submission.

MAJOR COMMENTS

1. *Overall, the authors claim they are using an LSPR-based method to detect pyrin protein for FMF diagnostics. Most LSPR based sensors measure the absorption, scattering, or extinction spectra of a nanoparticle or plasmonic sensor and utilize the obtained LSPR dipole peak position as their data. However, here the authors are measuring the LSPR response of their sensor by employing a "spectral integral method, which cumulatively evaluates spectral variations" (line 317-318). It is stated that "transmission resonance is integrated within a spectral window and the sum of all spectral changes within this region is used to determine the overall spectral change" (line 318-320). However, the only citation given is for monitoring spectral changes at multiple wavelengths, not their integration. Is there literature support for using this method?*

Following the reviewer's observation, it is worth noting that a predominant approach in LSPR-based biosensors, as seen in much of the existing literature, involves detecting the presence of analytes by monitoring the shift in the peak position of the plasmonic resonance determined from absorption, scattering, or extinction spectra. In this work, we employed a spectral integral method, wherein we calculated the integral of the transmission curve within a well-defined spectral window.

In our original manuscript ("Limit-of-Detection of the Biosensor Platform" Section), we introduced the spectral integral method in the following sentence:"

"*One potential approach is to simultaneously monitor spectral changes at multiple wavelengths instead of a single one.*[Ref1] *To accurately determine the presence of analytes at low concentrations, we employed a spectral integral method, which cumulatively evaluates spectral variations. In this method, the transmission resonance is integrated within a spectral window, and the sum of all spectral changes within this region is used to determine the overall spectral change.*"

In this text, Ref1 is associated with the article below, in which we, for the first time, introduced the spectral integral method to the literature.

Ref1: Cetin, A. E. et al. Plasmonic Nanohole Arrays on a Robust Hybrid Substrate for Highly Sensitive Label-Free Biosensing. ACS Photonics 2, 1167–1174 (2015).

We acknowledge the reviewer's feedback, and we recognize that our previous statement may have given the impression that we did not cite any literature employing the spectral integral method. To address this concern, we have corrected the text in "Limit-of-Detection of the Biosensor Platform" Section as follows:

"*One potential approach is to simultaneously monitor spectral changes at multiple wavelengths instead of a single one.*[Ref1] *This simultaneous monitoring could be achieved through their integration, accurately determining the presence of analytes at low concentrations. Here, the spectral integral method cumulatively evaluates spectral variations, where the transmission resonance is integrated within a spectral window, and the sum of all spectral changes within this region is used to determine the overall spectral change.*"

We applied this method to various applications where the monitoring of spectral variations at extremely low analyte concentrations was necessary. To underscore our method's versatility, we have included the following text to "Limit-of-Detection of the Biosensor Platform" Section to emphasize its suitability for plasmonic biosensor applications, particularly in challenging refractive index conditions.

"*Utilizing this method, we achieved a remarkable refractive index sensitivity of* 2×10^{-5} *RIU (refractive index unit) through the employment of periodic gold nanohole arrays.*[Ref1] *Moreover, this technique facilitates the monitoring of minute mass changes in cancer cells, with a mass accumulation rate on the order of picogram per hour.*[Ref2] *Additionally, our method extends its applicability to nucleic acid sequencing via an integrated sensing-by-binding approach.*[Ref3]"

Ref2: Cetin, A. E., Topkaya, S. N., Yalcin-Ozuysal, O. & Khademhosseini, A. Refractive Index Sensing for Measuring Single Cell Growth. ACS Nano (2021) doi:10.1021/acsnano.1c04031.

Ref3: Cetin, A. E. et al. Plasmonic Sensor Could Enable Label-Free DNA Sequencing. ACS Sensors 3, (2018).

The problem being that other factors, such as chemical damping, can reduce dipole peak intensity and cause peak broadening which can greatly impacting the peak integration over a set range that has nothing to do with analyte detection or the concentration of analyte present. This needs to be addressed in detail and discussed further in terms of how the authors are definitively linking peak integrations to the amount of analyte present.

We agree with the reviewer that an increase in the refractive index not only shifts the spectral position of the dipole but also broadens its linewidth. For example, the linewidth of the transmission resonance, which is ~51 nm, increases to ~76 nm after immersing the plasmonic chip into a PBS solution. However, for FMF diagnosis, we are comparing the transmission resonance obtained after the attachment of pyrin protein in the patient sample with that associated with pyrin antibody, which does not cause a dramatic shift as in the case of air-PBS interchange. For example, the total spectral shift from the antibody to 100 µg/mL pyrin protein results in only a ~1.6 nm linewidth broadening. Considering the much smaller spectral shifts in the presence of smaller amount of pyrin protein in the patient samples, broadening is expected to be much lower, resulting in a negligible impact on the spectral integral calculations.

To address this fact, we added the following text to the modified manuscript in "Limit-of-Detection of the Biosensor Platform" Section:

"*As an additional note, the increase in the refractive index not only shifts the spectral position of the dipole but also broadens its linewidth. For example, the linewidth of the transmission resonance, ~51 nm, increases to ~76 nm after immersing the plasmonic chip in a PBS solution. However, for FMF diagnosis, we are comparing the transmission resonance obtained after the attachment of pyrin protein in the patient sample with that associated with pyrin antibody, which does not cause a dramatic shift as in the case of the air-PBS interchange. For example, the total spectral shift from the antibody to 100 µg/mL pyrin protein results in only a ~1.6 nm linewidth broadening. Considering the much smaller spectral shifts in the presence of a smaller amount of pyrin protein in the patient samples, broadening is expected to be much lower, resulting in a negligible impact on the spectral integral calculations.*"

2. *The authors state that (3-aminopropyl)triethoxysilane was used to functionalize the glass substrates. Why was this compound used instead of (3-mercaptopropyl)triethoxysilane? This is significant as the amino group only affords an electrostatic attraction, while the sulfur of the mercapto group would instead form a much stronger covalent bond with the Au nanoparticles.*

We would like to thank the reviewer for highlighting this important issue. Within the scope of the study, 3-aminopropyltriethoxy silane (APTES) was utilized as the binding molecule to attach gold nanoparticles to the glass surface. The APTES structure forms Si-O bonds through a sol-gel reaction with the –OH groups on the substrate surface, creating an amino functional surface structure. Similar to APTES, (3-mercaptopropyl)trimethoxysilane (MPTES) is an organosilane with three alkoxy groups that can react with hydroxyl groups on the substrate surface to form Si–O bonds, leaving terminal functional thiol groups available for immobilization. Both molecules are commonly used for surface binding of AuNPs [doi:10.3390/nano9071034]. However, there is no quantitative evidence to determine which functionality is superior for incorporating AuNPs in the immobilization process [doi:10.3390/nano9071034]. It has been established that the weak interaction is due to electrostatic bonding, while the strong interaction is a result of covalent bonding. Both bonding agents effectively bind and coat the Au groups onto the surface [doi:10.3390/nano9071034].

Despite this, there are four reasons why the APTES molecule was preferred in this study:

(i) The interaction of the APTES structure with Au nanoparticles is weaker than the interaction of 3-mercaptopropyl groups with Au nanoparticles. Therefore, it does not disrupt the crystal structure of Au nanoparticles (doi: 10.1002/adma.202211624). This weak interaction also leads to a lower degree of passivation compared to thiols (doi: 10.1039/C4CP01963F). Therefore, since the Au surface is less passivated, subsequent modifications can be easily carried out.

(ii) Amino groups on the APTES molecule, which adheres to the glass surface via sol-gel chemistry, bind to Au nanoparticles through electron transfers and electrostatic interactions. In contrast, HS- groups on MPTES have two unshared electron pairs on the S atom, resulting in a stronger interaction with Au nanoparticles. This may lead to irregular and multilayer bonding, as well as single-row Au accumulation on the surface, which is undesirable for optical sensors. (doi: 10.1021/ar200260p and 10.1021/acs.jpclett.2c01616)

(iii) Our study targets a sensor system with disposable measurement units, allowing field measurements with instant results. To achieve widespread and cost-effective measurements, the disposable sample unit was designed to be low-cost. The chemical cost of 3-mercaptopropyltriethoxy silane is higher than that of APTES (500 mL of 98% APTES costs 288 euros, whereas 500 g of 95% MPTES costs 580 euros). Cost is therefore a crucial factor in choosing APTES.

www.sigmaaldrich.com/TR/en/product/aldrich/741442,
www.sigmaaldrich.com/TR/en/product/aldrich/175617)

(iv) Additionally, literature indicates that Au films deposited on the glass surface using APTES are faster than those derived with MPTES (doi: 10.1002/adfm.201002021). This further supports the preference for APTES.

In conclusion, our study investigates the use of an amine functional silane, specifically APTES, allowing for the conjugation of various amines to gold surfaces.

To clarify the rationale behind the selection of APTES for nanoparticle synthesis, we have included the following text in the "Plasmonic Chip" Section of the modified manuscript.

"*In our pre-diagnostic technology, plasmonic chips were created by synthesizing gold nanoparticles. 3-aminopropyltriethoxy silane (APTES) served as the binding molecule to affix gold nanoparticles to the glass surface. The APTES structure forms Si-O bonds through a sol-gel reaction with the -OH groups on the substrate surface, resulting in an amino-functional surface structure. Although an alternative molecule forming a much stronger covalent bond with the Au nanoparticles, e.g., (3-mercaptopropyl)trimethoxysilane (MPTES), could have been chosen as the binding molecule, we preferred APTES for our fabrication methodology. MPTES is an organosilane with three alkoxy groups capable of reacting with hydroxyl groups on the substrate surface to form Si–O bonds, leaving terminal functional thiol groups available for immobilization. Both APTES and MPTES are commonly used for surface binding of AuNPs.*[Ref1] *However,*

there is no quantitative evidence to determine which functionality is superior for incorporating AuNPs in the immobilization process.[Ref1] In this context, the weak interaction is attributed to electrostatic bonding, while the strong interaction results from covalent bonding. Both bonding agents effectively bind and coat the Au groups onto the surface.[Ref1] Despite these general considerations, four specific reasons justify the preference for APTES in our study.

(i) The interaction of the APTES structure with gold nanoparticles is weaker than the interaction of 3-mercaptopropyl groups with gold nanoparticles. This characteristic ensures that it does not disrupt the crystal structure of gold nanoparticles, maintaining their integrity.[Ref1] Additionally, the weak interaction results in a lower degree of passivation compared to thiols, making the gold surface less passivated.[Ref3] Consequently, subsequent modifications could be easily carried out. (ii) Amino groups on the APTES molecule, adhering to the glass surface via sol-gel chemistry, bind to gold nanoparticles through electron transfers and electrostatic interactions. In contrast, HS- groups on MPTES possess two unshared electron pairs on the S atom, leading to a stronger interaction with gold nanoparticles.[Ref4,Ref5] This stronger interaction could potentially result in irregular and multilayer bonding, as well as single-row gold accumulation on the surface, which is undesirable for optical sensors. (iii) In this study, our focus was on a pre-diagnostic technology employing disposable measurement units. Achieving cost-effective measurements with disposable chips necessitates a low-cost disposable sample unit, which is a requirement met with the use of APTES. (iv) As demonstrated in the literature, APTES allows for faster derivation of gold films, providing a practical advantage in the context of this study.[Ref6]"

Ref1: Rao, X. et al. A Comparison Study of Functional Groups (Amine vs. Thiol) for Immobilizing AuNPs on Zeolite Surface. Nanomaterials 9, 1034 (2019).

Ref2: Lyu, Y. et al. The Interaction of Amines with Gold Nanoparticles. Adv. Mater. (2023) doi:10.1002/adma.202211624.

Ref3: Olmos-Asar, J. A., Ludueña, M. & Mariscal, M. M. Monolayer protected gold nanoparticles: the effect of the headgroup–Au interaction. Phys. Chem. Chem. Phys. 16, 15979 (2014).

Ref4: Pensa, E. et al. The Chemistry of the Sulfur–Gold Interface: In Search of a Unified Model. Acc. Chem. Res. 45, 1183–1192 (2012).

Ref5: Pensa, E., Azofra, L. M., Salvarezza, R. C. & Carro, P. Effect of Ligands on the Stability of Gold Nanoclusters. J. Phys. Chem. Lett. 13, 6475–6480 (2022).

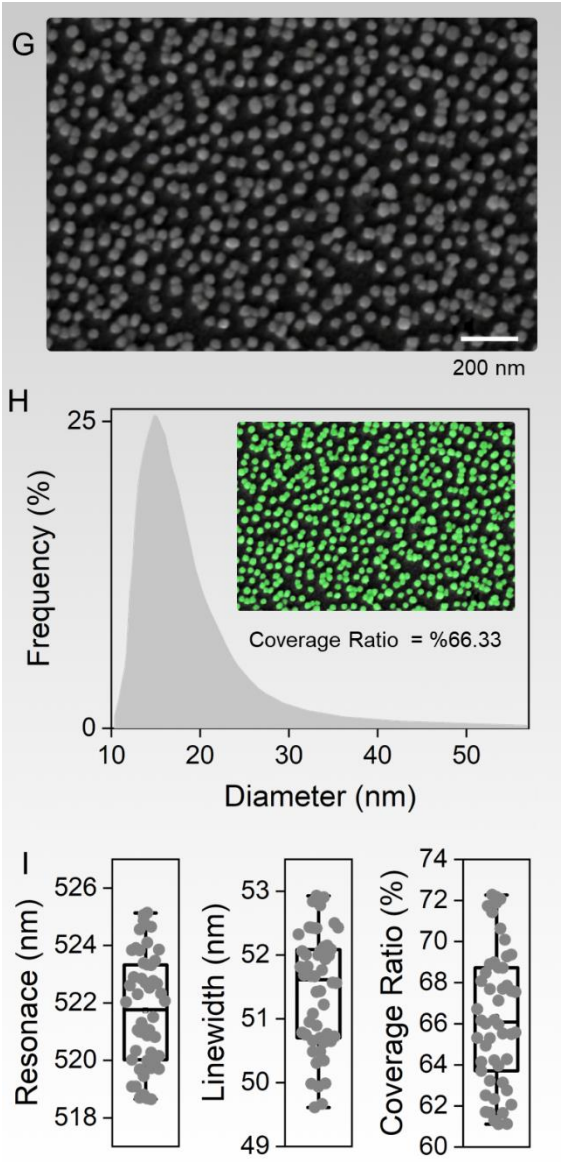
Ref6: Stec, H. M., Williams, R. J., Jones, T. S. & Hatton, R. A. Ultrathin Transparent Au Electrodes for Organic Photovoltaics Fabricated Using a Mixed Mono-Molecular Nucleation Layer. Adv. Funct. Mater. 21, 1709–1716 (2011).

3. *Additionally, it is stated that the Au nanoparticles were evaluated using several methods, including SEM, however, no images are provided. It is common practice to include a spectral figure of the as synthesized nanoparticles as well as an SEM image of the fully functionalized glass coverslip in the manuscript. Nanoparticle attachment needs to have a density and distribution that is reproducible. Any batch-to-batch variations would lead to varying reaction concentrations and heterogeneity in the resulting functionalized glass coverslips. This could result in sensor inconsistencies and inaccurate results. The authors also state that "random particle distribution resulting from the particle synthesis-based fabrication method" (line 453-454) does occur, so without such documentation, the manuscript is incomplete.*

We would like to thank the reviewer for bringing up this important issue. To address this crucial point, we performed an intensive analysis of the randomness in the synthesis of nanoparticles on the glass substrate. In the modified manuscript, we included a scanning electron microscopy (SEM) image and Zeta Sizer result to provide information on particle size. Additionally, we determined the ratio of the surface covered by the gold nanoparticles. Based on this information, we demonstrated how the spectral position and linewidth of the plasmonic resonance, supported by the gold nanoparticles, vary due to the randomness in the plasmonic features.

In the modified manuscript, we added the discussion to the "Plasmonic Chip" Section.

"*Figure 2G shows the scanning electron microscopy (SEM) images, revealing well-distributed random gold nanoparticles on the glass surface. The results of Zeta Sizer in Figure 2H shows the particle size distribution, demonstrating an average particle size of ~40 nm. The SEM image further shows that gold nanoparticles cover ~66% of the total surface, as indicated in Figure 2H-inset (green areas represent the gold-covered surface). Our fabrication methodology, based on nanoparticle synthesis, results in random antenna formations on the dielectric surface, which could lead to batch-to-batch variations in the position and linewidth of the plasmonic resonance supported by the nanostructures. Figure 2I compares the variations in the spectral position (left) and linewidth (middle) of the plasmonic resonance with the coverage ratio of the glass surface by the gold nanoparticles (right). Analyzing 50 plasmonic chips, we found that the surface coverage ratio centers at 66.32% with a standard deviation of 3.32%. These variations in the particle distribution lead to variations in the optical properties of the plasmonic chips, e.g., resonance location is centered at 521.74 with a standard deviation of 1.91 nm, and the linewidth is centered at 51.43 with a standard deviation of 0.91. As an important note, despite the batch-to-batch variations resulting in minor variations in the optical properties of the plasmonic chips, they could be critical when evaluating diagnostic conditions based on low biomarker concentrations in bodily fluids. As explained in the next section, batch-to-batch variations were eliminated by evaluating each test using its own plasmonic chip, where the position and linewidth of the transmission resonance were solely responsible for the calculations performed with the associated plasmonic chip.*"



We also added 3 new figures to Figure 2.

Figure 2. *(G) SEM image of the gold nanoparticles bounded on the glass surface. (H) Zeta Sizer result demonstrating particle distributions based on diameter. Figure inset shows the substrate surface covered with gold particle (highlighted with green), demonstrating a 66.33% coverage ratio. (I) Variations within the resonance wavelength (left) and the linewidth (middle) of the plasmonic resonances supported by 50 plasmonic chips with different surface coverage ratios (right).*

We also agree with the reviewer that the position of the spectral integral varying for each test conducted with a plasmonic chip is crucial. This variability arises due to inherent spectral position fluctuations in our fabrication technique based on nanoparticle synthesis. Nevertheless, by determining the position of the spectral integral window for each plasmonic chip, this methodology effectively mitigates the influence of these spectral variations when calculating the spectral integral values for various plasmonic chips. We added a note to the "Limit-of-Detection of the Biosensor Platform" Section to highlight that we can eliminate the effect of batch-to-batch spectral variations by interactively change the position of the spectral integral window:

"*It is essential to highlight that, for every test conducted with a plasmonic chip, the position of the spectral integral varies. This variation occurs because of the spectral position fluctuations inherent in our nanoparticle synthesis-based fabrication technique, as explained in the previous section. However, this*

methodology determining the position of the spectral integral window for each plasmonic chip, effectively eliminates the impact of these spectral variations when calculating the spectral integral values for different plasmonic chips."

4. *Could the authors please explain the amount of sample that is used and the number of repeat measurements taken? It is stated that the clinical samples were diluted with PBS buffer but it is not clearly stated how much total sample was used for testing over the 20 minute flow cycle and how many replicate tests were done for each. The calibration curve does mention that "5 independent experiments were conducted" (line 347) but it is unclear if that was also the number of replicate tests per sample for patient samples. For analytical chemistry, n=6 is typically used. The authors should expand their measurements to at least 6 if they have not already done so.*

We would like to thank the reviewer for highlighting this important issue. During the 20-minute sample testing, PBS solutions containing patient samples were flowed over the surface at a flow rate of 0.15 mL/min, requiring a total solution volume of 3 mL. To provide clarity regarding the sample volume used in each test, we added the following text to the manuscript (to "Clinical Sample Tests" Section).

"*Patient samples, diluted in PBS, were flowed over the plasmonic chip surface at a flow rate of 0.15 mL/min, which required a total of 3 mL of solution.*"

As for the second part of the reviewer's question; in response to the reviewer's suggestion, we repeated the biosensing experiments five more times. This repetition included both the calibration tests using commercial pyrin proteins and the testing of patient samples, which were the same patient samples used in this manuscript (the spare samples were stored at -80 °C). Furthermore, to address the reviewer's concern, we conducted these additional experiments using our stock proteins and patient samples.

We added the text below regarding our modification to "Clinical Sample Tests" Section.

"For high reliability in determining spectral integral ranges corresponding to healthy and patient responses, spectral tests were repeated ten times."

In the modified manuscript, we also added error bars, corresponding to double the standard deviation, to the control, healthy, and patient sample data in Figure 4B as shown below.

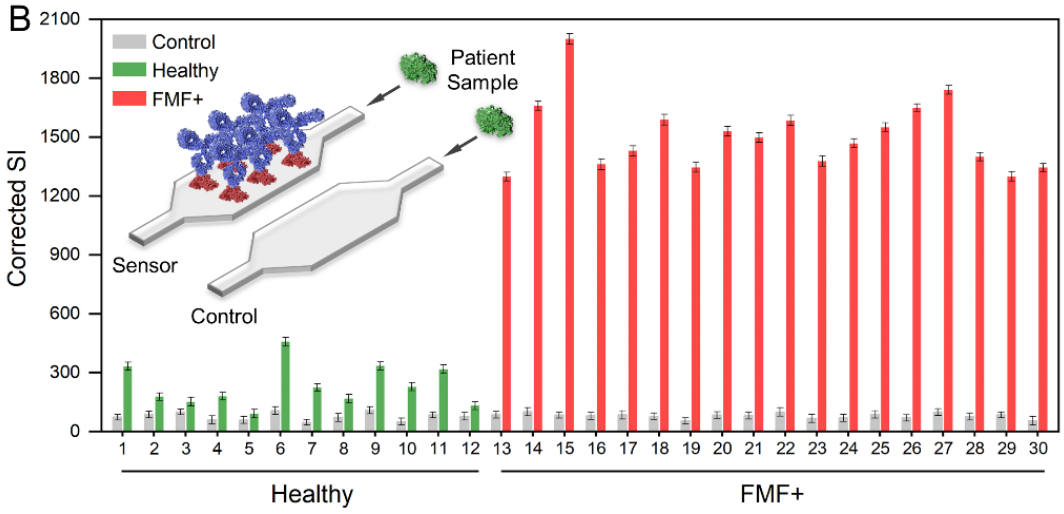


Figure 4B

5. *When discussing the detection of pyrin protein with their biosensing platform, the authors state they obtained an LOD of 0.3 ng/mL. They also state that this is "highly sensitive" (line 39). LSPR-based sensing mechanisms used to quantify proteins are seen to have an LOD in the aM to zM range. 0.3 ng/mL might be considered sensitive but it would not be considered highly sensitive.*

We acknowledge the reviewer's concerns regarding the sensitivity of our plasmonic substrate. Specifically, our system's limit of detection (LOD) is sufficient to detect the pyrin protein in clinical samples. Therefore, we have made the following modifications to our claims in the "Abstract" Section:

"*Our technology utilizes gold nanoparticles with a limit of detection of 0.24 ng/mL, which is sufficient for detecting the presence of pyrin protein in clinical samples using anti-pyrin antibodies.*"

Additionally, the determination of that LOD seems be solely based on the fact that less than 0.3 ng/mL could not be readily distinguished by the biosensing platform due to significant overlap. This is not how LOD is typically calculated for sensors. In most publications, the LOD is derived by using a Z value of the blank (Z= mean + 3σ, σ = standard deviation of blank). This is typically determined from six separate measurements using six different sensors and substituting the Z value into the "Y" in the calibration curve best fit line equation allowing for the LOD concentration ("X") to be obtained. The limit of quantification (LOQ) should also be determined and discussed with LOD for a sensor such as this.

In our manuscript, we initially used experimental data to estimate the system's LOD, although this approach may not provide the precise LOD information. In response to the reviewer's suggestion, we conducted a standard LOD calculation by applying a linear curve fit to the calibration data and determining the LOD based on the parameters derived from this linear regression. We have included the following text (in "Limit-of-Detection of the Biosensor Platform" Section) explaining the system LOD calculation, and added a reference regarding the calculation:

"*To more precisely determine the limit of detection (LOD) for our pre-diagnostic biosensor, we applied a linear regression model to the spectral integral vs. IgG concentration data, which yielded an R^2 value of 0.99857. Using the formula LOD = 3.3σ/s, where σ represents the standard deviation of the signal and s is the slope of the calibration curve, we determined the LOD to be 0.24 ng/mL.[Ref]*"

Regarding the limit of quantification of the system, we added the below sentence for the corresponding calculation to "Limit-of-Detection of the Biosensor Platform" Section:

"Furthermore, using the relation LOQ = 10σ/s, the limit of quantification of the system was calculated as 0.72 ng/mL.[Ref]"

Ref: Armbruster, D. A. & Pry, T. Limit of blank, limit of detection and limit of quantitation. Clin. Biochem. Rev. 29 Suppl 1, S49-52 (2008).

We also made modifications to Figure 3D-inset, particularly highlighting this linear regression.

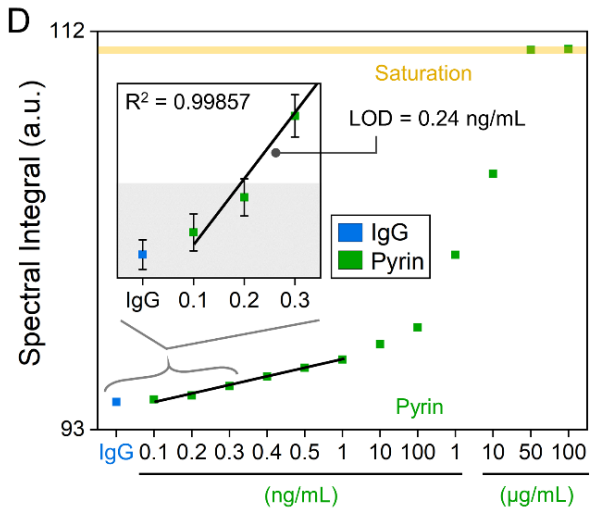


Figure 3. *(D) Spectral integral values calculated for different pyrin concentrations (green squares) compared to protein IgG (blue square). The yellow line highlights the spectral integral values associated with the surface saturation case for pyrin protein. For each concentration, the spectral integral values were calculated after a 15-minute pyrin injection. The figure inset zooms into a smaller pyrin concentration range. In the figure, squares correspond to the mean spectral values for ten independent experiments, and the error bars represent double the standard deviation. The black line corresponds to the linear regression model fitted to the calibration data to define system LOD. Gray area highlights the overlapping spectral integral datasets for different conditions.*

6. *With any newly developed sensor fabrication, specificity testing is vital to conduct prior to publication. However, there is no specificity testing presented in this work despite the authors claim that this approach "ensures high selectivity and specificity" (line 534). Contrastingly, the author mentions that whole blood samples could "lead to unspecific binding" (line 204) and that "clinical samples contain a high number of other proteins that may interfere with the capturing of pyrin protein (line 378-379). The author states that diluting clinical samples with PBS buffer and subtracting out the integral values for the control channel is their "correction procedure" to "account for nonspecific binding" (line 398). This, however, is not the typical method for conducting specificity tests for sensors that prove the spectral*

changes observed are a direct result of target analyte detection and nothing else. For this reason, other protein biomarkers for FMF found in blood should be tested along with other circulating proteins. The proteins used for specificity testing should be tested individually and as a mixture do determine if nonspecific binding is occurring.

We again would like to thank the reviewer for bringing attention to this critical issue, which holds significant importance for the technologies relying on sensing by binding methodologies. For FMF, no other protein or biomarker has been identified so far. The diagnosis of FMF is made by typical symptoms and nonspecific acute phase reactants (C-reactive protein, fibrinogen and erythrocyte sedimentation rate, serum amyloid A) that increase during the attack. Genetic tests are confirmed by detecting variations in the genes encoding pyrin in chromosome 16. Serum amyloid A, fibrinogen and c-reactive protein are acute phase proteins used to detect attacks in FMF patients and are non-specific inflammatory markers. Apart from this, resistin, calprotectin, pentraxin 3, CXC chemokine ligand 16, serum soluble fasLigand are not proteins specific to FMF and their levels increase or decrease during inflammation. Therefore, it has no diagnostic significance other than showing inflammation during an attack. Its level is found to be normal in periods when there are no attacks. Therefore, it will not be useful for evaluating the specificity of the method.

In order to address this need, we added the text below to the "Clinical Sample Tests" Section in the modified manuscript.

"*Typically, control channels should be covered with antibodies that lack affinity for the targeted analytes. In our case, the determination of FMF status relies on quantifying the amount of pyrin protein in clinical samples. However, no other protein or biomarker specific to FMF has been identified to date.*[Ref1-Ref4] *The diagnosis of FMF is based on typical symptoms and nonspecific acute phase reactants such as C-reactive protein, fibrinogen, erythrocyte sedimentation rate, and serum amyloid A, all of which increase during an attack. Genetic tests confirm FMF by detecting variations in the genes encoding pyrin on chromosome 16. While serum amyloid A, fibrinogen, and C-reactive protein are acute phase proteins used to detect attacks in FMF patients, they are non-specific inflammatory markers. In addition, proteins like resistin, calprotectin, pentraxin 3, CXC chemokine ligand 16, and serum soluble fasLigand are not specific to FMF. Their levels fluctuate during inflammation but lack diagnostic significance beyond indicating inflammation during an attack. Notably, their levels are normal during periods without attacks, rendering them unsuitable for assessing method specificity. Consequently, no other protein can serve as a control protein or antibody for control studies in our context.*"

We also added the corresponding references to strengthen our claims.

Ref1: Kılıçaslan, E. et al. Assessment of Serum Resistin and Plasma Calprotectin Levels as Biomarkers of Inflammation in Patients with Familial Mediterranean Fever Disease. Mediterr. J. Rheumatol. 33, 322 (2022).

Ref2: Bulut, M. et al. Higher Pentraxin-3 Levels are Associated With Inflammation in Familial Mediterranean Fever. J. Clin. Lab. Anal. 30, 978–981 (2016).

Ref3: Çakan, M. et al. Serum amyloid A as a biomarker in differentiating attacks of familial Mediterranean fever from acute febrile infections. Clin. Rheumatol. 39, 249–253 (2020).

Ref4: Ceri, M. et al. Serum soluble fas ligand levels in familial Mediterranean fever. Ren. Fail. 35, 835–837 (2013).

7. *When patient samples are analyzed by a new sensing construct, validation of the samples is required. The manuscript does not include validation of their findings via an FDA approved technique. For example, validation testing for proteins can be accomplished using electrochemiluminescence, ELIZA or western blot. The samples tested should be analyzed by one of these validation methods and the results compared to the data obtained from the plasmonic biosensor. A correlation graph should be made to determine the Pearson's r value and included in the manuscript.*

We express our sincere gratitude to the reviewer for highlighting this crucial concern, which significantly underscores the clinical applicability of our plasmonic methodology. To validate our methodology, we compared our findings with those obtained through ELISA (enzyme-linked immunosorbent assay). Specifically, we applied Pearson calculations separately to healthy and FMF+ samples. In this analysis, we employed an ELISA kit designed for the detection of pyrin from patient samples, ensuring a rigorous

evaluation of sensitivity and specificity in our assessments. Our calculations unveiled a substantial correlation between ELISA and our plasmonic biosensor, yielding Pearson's *r* values of 0.9561 and 0.9593 for healthy and FMF+ samples, respectively. The scale of Pearson's *r* ranges from -1 to 1, portraying both the strength and direction of a linear relationship. In our investigation, values approaching 1 denote an almost perfect positive linear relationship between the variables, accentuating the precision and reliability of our plasmonic technology in the analysis of clinical samples.

Beyond the statistical correlation, the clinical relevance of our findings is noteworthy. The robustness of the relationship, as indicated by Pearson's *r* values surpassing the widely acknowledged threshold of 0.7 for a strong correlation, underscores the potential of our plasmonic biosensor as a steadfast and trustworthy clinical tool. This puts our technique in the same league as the commonly used ELISA method, showing that it's reliable for regular use in dealing with FMF. The validation of a strong correlation with an FDA-approved technique further affirms the clinical applicability of our plasmonic biosensor, setting the stage for its seamless integration into the standard diagnostic repertoire for FMF and potentially other medical applications.

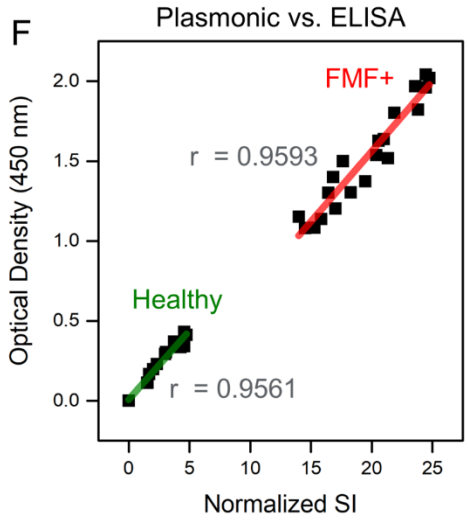
In the modified manuscript, we added a new section titled "Validation of the Plasmonic Technology with a Standard Technique" as below:

"*In this study, we conducted a thorough evaluation of clinical samples using an innovative plasmonic pre-diagnostic technology. To validate our methodology, we compared our findings with those obtained through ELISA (enzyme-linked immunosorbent assay). ELISA is a sophisticated laboratory technique designed for the precise detection and quantification of proteins or antibodies within biological fluids.*[Ref1] *Functioning on the principles of molecular recognition, ELISA operates akin to a lock-and-key system, where specific interactions occur between targeted molecules, namely antigens and antibodies. In the procedural sequence, the substances of interest are immobilized on a solid surface, thus initiating the assay. Stringent measures are then implemented to prevent non-specific interactions. ELISA (LifeSpan BioSciences Inc.) used as a reference standard, possesses versatility in analyzing clinical samples like serum, blood, plasma, or supernatants. Its sensitivity of 0.094 ng/mL and a broad working concentration range from 0.156 to 10 ng/mL make it a reliable tool in clinical diagnostics. See Supporting Information for the details of the ELISA test and Figure SI5 for the standard calibration curve of the ELISA kit performed with pyrin protein. Figure 4F illustrates the results of our parallel analyses using both ELISA and our plasmonic biosensor on samples from both healthy individuals and those diagnosed with FMF. In the figure, x-axis corresponds to the normalized SI values determined from our plasmonic methodology and y-axis corresponds to the optical density values determined at 450 nm using a microplate reader in ELISA. Green and red lines account for the linear regressions fitted to the experimental data (black squares) for the healthy and FMF+ clinical samples, respectively.*

In order to quantitatively measure the agreement between our methodology with a clinical tool, we calculated Pearson's correlation coefficient, a robust statistical method to measure the linear correlation between two data sets. Our analysis revealed a high correlation between ELISA and our plasmonic biosensor, with Pearson's r values of 0.9561 and 0.9593 for healthy (green line) and FMF+ (red line) samples, respectively. In this context, Pearson's r ranges from -1 to 1, signifying the strength and direction of a linear relationship. In our case, values near 1 indicate a nearly perfect positive linear relationship between the variables, emphasizing the precision and reliability of our plasmonic technology for clinical sample analyses. The significance of our findings extends beyond the high correlation, reaching into the realm of clinical relevance. The strength of the relationship, as indicated by Pearson's r values exceeding the widely accepted threshold of 0.7 for a strong correlation,[Ref2] *underscores the potential of our plasmonic biosensor as a robust and dependable clinical tool, comparable to the widely adopted ELISA method. Demonstrating a strong correlation with an FDA-approved technique (Food and Drug Administration) validates the clinical applicability of our plasmonic biosensor, paving the way for its integration into routine practice for FMF.*"

Ref1: Lequin, R. M. Enzyme Immunoassay (EIA)/Enzyme-Linked Immunosorbent Assay (ELISA). Clin. Chem. 51, 2415–2418 (2005).

Ref2: Ratner, B. The correlation coefficient: Its values range between +1/−1, or do they? J. Targeting, Meas. Anal. Mark. 17, 139–142 (2009).

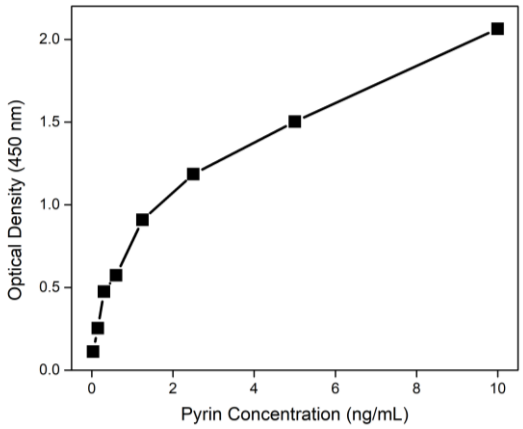


We also added a new figure (Figure 4F) to highlight the linear correlation between our methodology and ELISA.

Figure 4. *(F) Comparison between normalized SI values determined from the plasmonic pre-diagnostic technology and the optical density values determined from ELISA. In the figure, green (healthy) and red lines (FMF+) account for the linear regressions fitted to the experimental data (black squares).*

Furthermore, in the Supporting Information, we also added a section titled "Specifications of the ELISA Tests" to provide the details for the ELISA tests. We also added a new figure to the Supporting Information (Figure SI5), where we showed the standard calibration curve of the ELISA kit performed with purified pyrin protein.

Figure SI5. *ELISA Calibration curve for pyrin protein.*



8. *On Figure 4B and C the authors present their plasmonic biosensor results, however, no statistical significance is shown. A statistical analysis of diagnostic results is typically done with a program, such as GraphPad Prism, to show how well a sensor can differentiate patient or sample groups. Both a Mann Whitney t-test and an area under the curve (AUC) of the receiver operating characteristic (ROC) graph can be conducted to determine p values and star significance of the data. Without this type of statistical analysis of the plasmonic biosensor results, they are meaningless.*

We would like to thank the reviewer for raising this important issue. In response to the reviewer's suggestion, we conducted extensive statistical analyses to emphasize the accuracy of our sensing technology. We initially assessed the accuracy of our system's database, which was created using clinical samples. In this analysis, we compared the responses of healthy and FMF+ samples with the control group. These analyses demonstrated our ability to effectively distinguish the sensor response (in either FMF+ or FMF- format) from the control, underscoring our capability to determine the response of both healthy and patient samples accurately.

In the subsequent analysis, we compared the responses of healthy and FMF+ samples, demonstrating our accuracy in distinguishing between FMF+ and FMF- datasets. These comparisons were conducted using Welch's t-test, LDA, and ROC analyses.

To support these findings, we have incorporated associated discussions into both the main manuscript and the Supporting Information. Additionally, we have included new figures, such as Figure 4D, Figure 4E, and Supporting Information Figure SI3A and Figure SI3B.

Below, we outline all the modifications made to the main manuscript in "Clinical Sample Tests" Section:

"In order to demonstrate the robustness of the system's database in classifying healthy and FMF+ samples, we conducted Receiver Operating Characteristic (ROC) analysis following Linear Discriminant Analysis

(LDA) for healthy (green bars) and FMF+ (red bars) in comparison to control data sets (gray bars). LDA projects the two-dimensional spectral integral data onto a single axis that optimally separates these two data sets and establishes a classification threshold. Figure SI3A (Supplementary Information) displays the two-dimensional data corresponding to the spectral integral values before (x-axis) and after (y-axis) the pyrin infusion in both the control and sensor regions on the plasmonic chip. In the figure, the dashed black line represents the overlay of two orthogonal vectors, marking the threshold that separates the control group (gray squares) from the healthy (green squares) with FMF+ data (red squares), demonstrating a greater distinction for the FMF+ group. Subsequently, we conducted ROC curve analysis and calculated the Area Under the Curve (AUC), a metric that assesses the accuracy of classifying each clinical sample as either healthy or FMF+.[Ref] To provide context, a random classifier would yield an AUC of 0.5, whereas a perfect classifier would have an AUC of 1. Figure SI3B (Supplementary Information) shows the ROC curve conducted between control and healthy group (green curve), and between control and FMF+ group (red curve). ROC analyses demonstrate an excellent resolution between the healthy and FMF+ datasets from the control group, with AUC values calculated as 0.9792 for the healthy group and 1 for the FMF+ group (Figure SI3B-inset). To further distinguish clinical samples from the control group, we also conducted Welch's t-test at a significance level of α = 5%, where α ranges between 0 and 1, and p > 0.05 indicates no significant difference. Using spectral integral values for each dataset, we compared the FMF- and FMF+ subsets with the control group, with p-values corrected using the Bonferroni method. Welch's t-test demonstrates excellent separation from the control group for both clinical datasets, with the FMF+ subset showing a higher distinction compared to the healthy sample, e.g., p(Control vs. Healthy) = 5.68×10^{-5} and p(Control vs. FMF+) = 1.30×10^{-27}.

These statistical methods clearly demonstrate the perfect separation of clinical samples from the control group, ensuring the robustness of the system database. More importantly, classifying the clinical samples as healthy and FMF+ is critical to provide accurate test results. Using the normalized spectral integral data for healthy and FMF+ samples (Figure 4C), Welch's t-test demonstrates their perfect separation, with a p-value of 5.12×10^{-14}. Figure 4D shows the two-dimensional spectral integral data for clinical samples, where the LDA vector (black dashed line) accurately distinguishes between healthy (green squares) and FMF+ datasets (red squares). ROC analysis also demonstrates the perfect separation between healthy and FMF+ samples, yielding AUC = 1. These results confirm that the database created using the clinical samples, which exhibit distinctive spectral variations, is capable of accurately classifying the real samples as either healthy or patients."

Ref: Pencina, M. J., D'Agostino, R. B., D'Agostino, R. B. & Vasan, R. S. Evaluating the added predictive ability of a new marker: From area under the ROC curve to reclassification and beyond. Stat. Med. 27, 157–172 (2008).

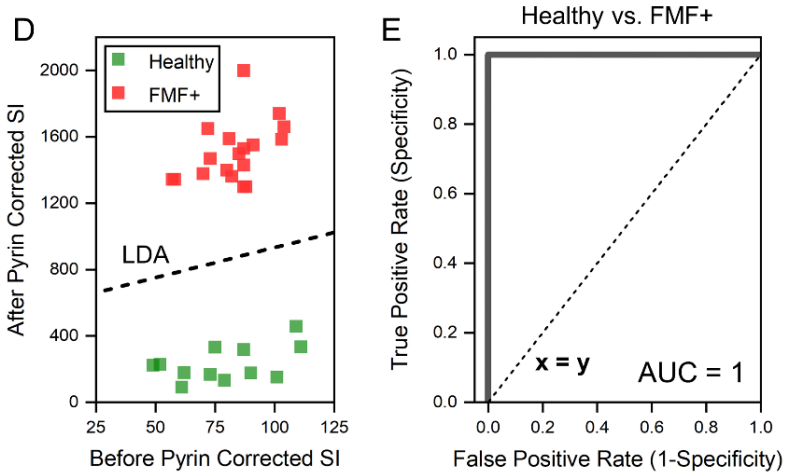


Figure 4. *(D) The two-dimensional spectral integral data determined before and after pyrin injection in the sensor region on the plasmonic chip, where an orthogonal LDA vector (black dashed line) separates the healthy (green squares) and FMF+ subsets (red squares). (E) ROC analysis conducted between healthy and FMF+ samples, demonstrating and AUC of 1.*

Below, we outline all the modifications made to the Supporting Information:

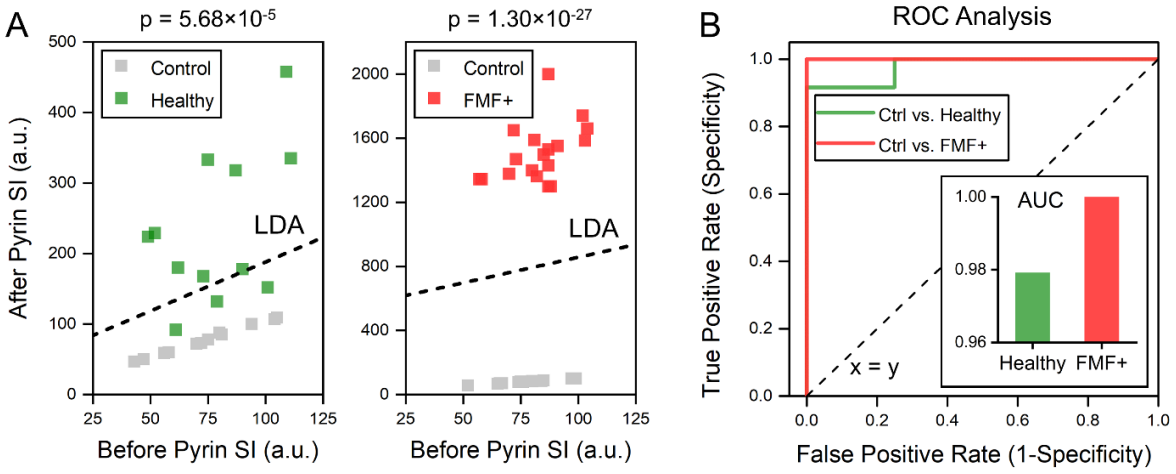


Figure SI3. *(A) The two-dimensional spectral integral data determined before and after pyrin injection in both the control and sensor regions on the plasmonic chip. Two orthogonal LDA vectors (black dashed lines) separating the healthy dataset (green squares) from the control (gray squares), and the FMF+ dataset (red squares) from the control. The figure also presents the calculated p-values using Welch's t-test to compare Control - Healthy and Control - FMF+, with the results Bonferroni corrected. (B) ROC analysis conducted between Control - Healthy (green line) and Control - FMF+ (red line). The figure inset displays the calculated AUC for the two different comparisons.*

MINOR COMMENTS:

1. *The total number of citations for this manuscript is 33 indicating that it is severely under-cited. The authors should address this.*

As the reviewer suggested, we added references related to FMF, plasmonic biosensors, the statistical methods used throughout the study, and our discussion regarding the applicability of our technology. The current number of references was increased to 58.

2. *The "Plasmonic Biosensor" used in the schematic illustration on Figure 6 does not match the construct used in previous figures in this paper where the Au nanoparticle is shown in between the glass and the bound A/G-IgG-Pyrin.*

We are grateful to the reviewer for bringing this issue to our attention. Figure 6 is pertinent to the overall operational concept of the pre-diagnostic platform, and features the plasma-on-a-chip enclosed within a rectangular gold-colored outline. As per the reviewer's suggestion, we revised Figure 6 in the modified manuscript.

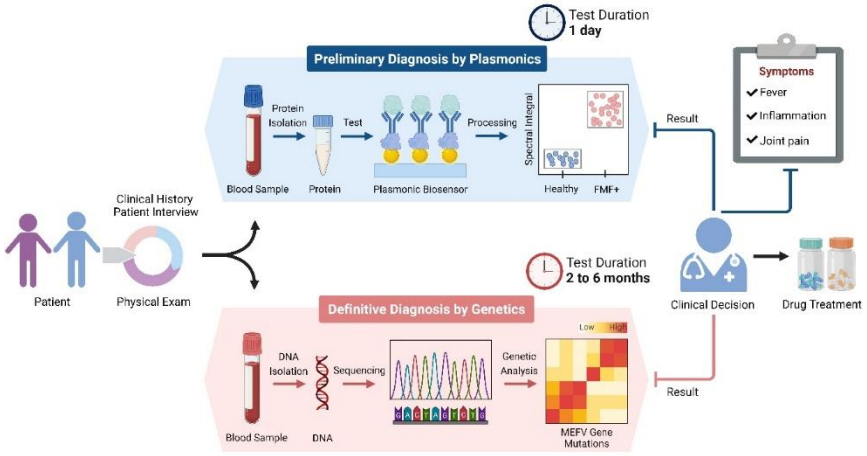


Figure 6

3. *The authors did not mention the shelf-life stability of the plasmonic chip. If this is something that would be used in a clinical setting, this information would be of great importance.*

We would like to express our gratitude to the reviewer for raising this critical issue, particularly relevant to the commercial clinical applications. In response to these concerns, we conducted a thorough analysis of the label-free binding response of plasmonic chips over a 24-month period. Our findings reveal that, during this duration, both the plasmonic and sensing properties exhibit negligible variations, underscoring the robust shelf-life stability of our plasmonic chips, which were fabricated using our nanofabrication methods.

Additionally, we included a dedicated paragraph in the main text to "Limit-of-Detection of the Biosensor Platform" Section to discuss this issue, and Figure SI4 to the Supporting Information for further clarity:

"*As an additional point to consider, while our technology, based on realizing the plasmonic chips through particle synthesis, offers cost-effectiveness, another crucial factor for the commercial applications of diagnostic platforms based on sensing-by-binding methodology is shelf-life stability. As previously noted, we monitored the plasmonic chip's response within a PBS environment. Initially, the resonance position of the plasmonic chip, represented by a mean value of ~674.5 nm (averaged across 10 chip responses), experienced a shift to ~693.5 nm (an average of 10 chip responses) following the attachment of protein A/G, pyrin antibody, and pyrin protein. Furthermore, the linewidth of the plasmonic resonance transitioned from ~76 nm to ~79 nm upon introduction of this protein complex. Figure SI4 in the Supplementary Information depicts the spectral position of the plasmonic resonance and its linewidth stability over a 24-month period, revealing minimal variations in these two parameters, each remaining below 0.5 nm. This analysis underscores the robust shelf-life stability afforded by our fabrication method, which is an essential attribute for potential commercial applications in the field of diagnostics.*"

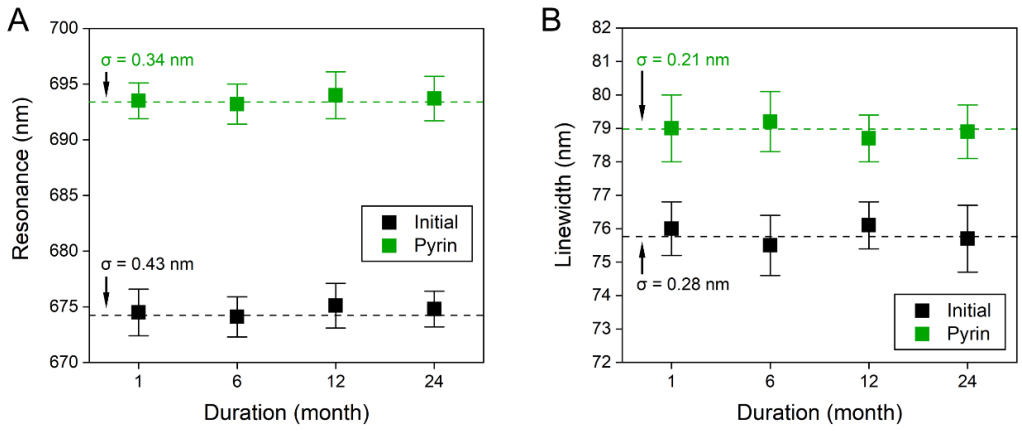


Figure SI4. *Variations in (A) spectral position and (B) linewidth of the plasmonic chip over a 24-month period. Black squares: The initial chip response under PBS. Green squares: The chip response after the attachment of Protein A/G, pyrin antibody, and pyrin protein. In the figure, squares correspond to the mean spectral values for ten independent experiments, and the error bars represent double the standard deviation. In figures, the black dashed lines represent the mean values, and σ refers to the standard deviation of the dataset containing the spectral positions and linewidths, which were averaged across 10 different chip responses, for each time point within the 24-month period.*

4. *The calibration curve provided in Figure 3D does not include error bars for the spectral integral measurements shown. The inset for the figure does include error bars. Line 347 states that 5 independent experiments for each concentration were conducted. Was that only done for the concentrations in the inset? If every concentration was conducted 5 times, there should be error bars shown for each concentration on the figure.*

We would like to express our gratitude to the reviewer for bringing this important issue to our attention. Repetition tests were conducted for all pyrin concentrations. In this figure, the spectral integral values were displayed in a range of approximately 30 (a.u.), with a standard deviation of around 0.1, which is very small compared to the mean values. Therefore, we were unable to visually represent the error bars in the zoomed-out figure.

To clarify this matter, in the caption for Figure 3D-inset, we have included the following note:

“The repetition tests were conducted for all pyrin concentrations, and the error bars are displayed only in the zoomed-in figure.”

In response to one of the reviewer's previous comments, we have increased the total number of repetition tests for each pyrin concentration to ten.

REVIEWER #2

The manuscript titled "A Pre-Diagnostic Tool for a Rare Disease: Familial Mediterranean Fever (FMF)" has undergone a thorough review. This study presents an innovative approach to FMF diagnosis by measuring pyrin levels in patient samples, a protein affected by variations in the MEFV gene.

Research on the pathogenesis of Familial Mediterranean Fever (FMF) has led to the discovery of numerous inflammatory pathways within the innate immune system, including PAMPs, DAMPs, inflammasomes, pyroptosis, and NETosis. These investigations have significantly advanced our understanding of the pathogenesis and treatment of several important diseases, such as atherosclerosis and diabetes. Despite these notable breakthroughs, many aspects of FMF's pathogenesis and diagnosis remain mysterious.

FMF diagnosis primarily relies on compatible symptoms and a positive response to colchicine treatment. However, a definitive diagnosis necessitates the confirmation of specific pathogenic variations in the MEFV gene. Nonetheless, genetic testing is only informative for approximately 50% of patients. Another significant challenge arises from the presence of gene variants of unknown significance (VUS) and their potential contribution to the disease's pathogenesis. Functional studies have provided some insights into these unanswered questions, but undoubtedly, we require practical techniques like the one explored in this study to address these challenges effectively.

I believe the manuscript is well-written, and its subject matter remains highly relevant in the present context. The research is original and of high quality, holding substantial potential for future citations. The authors have meticulously outlined their methodologies, providing ample information for the replication of experiments. Furthermore, it's noteworthy that their method may find applicability in addressing other conditions characterized by variations in protein quantity.

First and foremost, we extend our heartfelt gratitude to the reviewer for providing a comprehensive overview of FMF from a medical perspective. We also appreciate the reviewer for emphasizing the potential utility of our technology as a clinical tool. It's worth noting that we are actively engaged in advancing the development of this technology.

However, I do have a few notable concerns about the study, such as the utilization of the technique on a limited group of homogeneous subjects, among others, which I will elaborate on below.

We wish to express our sincere appreciation to the reviewer for bringing the following issues to our attention. We have diligently addressed all the points raised, and as a result of incorporating the reviewer's comments, we believe that the quality of our manuscript has significantly improved.

MAJOR POINTS:

1. *Line 30 and in other instances, the use of "variant" instead of "mutation" is the preferred terminology for expressing genetic differences between individuals.*

We would like to express our gratitude to the reviewer for his/her valuable suggestion.

The words “mutation” and “variant” are often used interchangeably. Typically, variants with specific pathogenic characteristics are also referred to as mutations. Changes in the genetic sequence are generally called variants, regardless of their pathogenic potential. The valuable contribution of the jury is highly appropriate.

As the reviewer suggested, to enhance the scientific accuracy of our manuscript and improves the overall readability, we replaced “mutation” by “variant” throughout the manuscript.

2. *Lines 31 and 71, "The MEFV gene encodes a protein called pyrin, and mutations in this gene lead to abnormally increased pyrin synthesis." It should be clarified that MEFV variants alter the structure and*

interactions of pyrin, potentially leading to increased pyrin production due to positive feedback loops involving IL-1 beta on pyrin synthesis.

Once again, we would like to thank the reviewer for assisting us in clarifying our statement regarding the impact of mutations in the MEFV gene on pyrin production.

The MEFV gene, responsible for this disease, encodes the pyrin protein, which serves as an innate immune sensor, triggering inflammation and facilitating the production of inflammatory mediators. Pyrin is predominantly expressed in neutrophils, eosinophils, monocytes, dendritic cells, and fibroblasts. It plays a pivotal role in regulating apoptosis, inflammation, and cytokine production. Variants in the MEFV gene lead to alterations in the protein's structure, resulting in uncontrolled interleukin-1 (IL-1) secretion and an increased inflammatory response. Furthermore, a positive feedback loop involving IL-1 beta leads to an upsurge in pyrin expression and synthesis.

To emphasize this, we replaced our previous statement in "Abstract" Section with the following:

"*MEFV variants induce structural and interaction changes in pyrin, which could potentially result in heightened pyrin production. This is driven by positive feedback loops involving IL-1 beta in the regulation of pyrin synthesis.*"

3. *The nature of the patient sample used in experiments is unclear. It's crucial to specify whether it was full blood, serum, cell lysate, or another source, as this information is critical given that free pyrin is not present in blood and pyrin is preferentially expressed in myeloid lineage cells.*

We would like to thank the reviewer for raising this issue. In order to specify the origin of the patient samples used in the label-free biosensing experiments, we have added the following text to the "Preparing Healthy and Patient Samples" Section.

"*Pyrin is primarily expressed in neutrophils, eosinophils, monocytes, dendritic cells, and fibroblasts, and it plays a crucial role in regulating apoptosis, inflammation, and cytokine production. To isolate pyrin, cell lysates were prepared from whole blood samples obtained from patients. These whole blood samples were centrifuged with erythrocyte lysis buffer to separate leukocytes, which were subsequently homogenized using a vortex. The resulting leukocyte pellets were then mixed with a solution containing protease inhibitor, Octylphenyl Polyethylene Glycol, and PBS. Afterward, the tubes were centrifuged to collect the supernatants, which contained cytoplasmic proteins.*"

4. *In Figure 4B, the y-axis unit should be indicated, and absolute numerical values should be provided on the bars.*

We would like to express our gratitude to the reviewer for bringing up this important issue. As demonstrated below, we have provided the values for the y-axis on the graph.

As stated in the manuscript:

"*For the sensor channel, the spectral integral values were corrected by dividing the spectral integral values obtained after the final PBS wash (representing the spectral integral value for the pyrin protein) by the ones determined for the anti-pyrin antibody.*"

Consequently, corrected SI values are unitless. Throughout the manuscript, all corrected and normalized SI values are unitless since they were derived through the division of two SI values. To clarify this matter further, we have included a note in the caption of Figure 4 as below:

"*As an additional note, both corrected and normalized SI values were calculated by dividing two SI values, making them unitless parameters.*"

5. *Investigate if there is a correlation between serum inflammatory markers (C-reactive protein & serum amyloid A) and corrected SI of pyrin obtained from the technique.*

We thank the reviewer for bringing this critical issue. Our article primarily aimed to establish a method for identifying patients during non-attack periods. The hypothesis is rooted in the observation that patients experiencing no attacks still exhibit elevated pyrin levels. Enrollment of patients into the study followed clinical and laboratory evaluations that confirmed the absence of FMF attacks. During non-attack periods, blood samples were collected from these patients, and those whose inflammatory markers, such as C-

reactive protein, fell within the normal range were included in the research. Consequently, the levels of inflammatory markers, specifically C-reactive protein, measured within the study were expected to be within the normal range.

Examining whether pyrin levels increase during an attack and their correlation with inflammatory markers could potentially be a separate research topic for future studies. To address this issue, we have included the relevant text in "Discussion" Section.

"*As an additional note, the main objective of this article was to develop a method for detecting patients during non-attack periods. The hypothesis is grounded in the observation that patients without attacks also exhibit elevated pyrin levels. Patients were enrolled after clinical and laboratory assessments confirmed the absence of FMF attacks. Blood samples were collected from these patients during non-attack periods, and individuals with inflammatory markers (such as C-reactive protein) within the normal range were included in the study. As a result, the levels of inflammatory markers (specifically C-reactive protein) measured were within the normal range.*"

6. *Specify the MEFV variants of the patients and investigate if there were any differences between patients based on the variants they harbored.*

We would like to express our gratitude to the reviewer for pointing out this important issue. It's worth noting that every patient included in the study carried at least one M694V variant. Specifically, 14 patients were homozygous for M694V, while an additional 6 had different exon 10 variants, creating compound heterozygous variations in conjunction with M694V. These specific details are now incorporated into the "Collecting Healthy and Patient Samples" Section. We also added the detailed information regarding the clinical samples as a new table in Supporting Information (Table S3).

"*All patients included in the study had at least one M694V variant. 14 of these are M694V homozygous, 6 are other exon 10 variants that form compound heterozygous variation with M694V. Table S3 in the Supporting Information shows the MEFV Variations in the patient samples.*"

MEFV Variations in the Patient Samples

Case Number	Age	Age at Diagnosis	Following Time (months)	MEFV Variations
DNU	14	5	44	M694V-hom
EC	17	16	4	M694V-het/V726A-het
TO	15	8.5	72	M694V-hom/R202Q-het
GS	11	9	30	M694V-het/M680I-het
EU	11	4	84	M694V-hom/R202Qhet
AS	14	6	84	M694V-hom
SS	16	3.5	144	M694V-hom
TE	8	4	36	M694V-het/M680I-het
YP	13	12.5	6	M694V-het;V726Ahet
AM	9.5	7	25	M694V-het/V726A-het
MNS	8	7	10	M694V-hom
AD	15.5	6	96	M694V-hom
GO	16.5	6	102	M694V-hom
HD	10	2.5	84	M694V-hom
AD	13.5	12	26	M694V-hom
BY	13.5	12	28	M694V-hom/A744S-het
MES	15	8	84	M694V-het/V726A-het/ R202Q-het
EAB	4	2.5	18	M694V-hom
GN	7	3	48	M694V-hom
ZB	15	8	84	M694V-hom

Table S3: MEFV Variations in the Patient Samples.

We also added the note below to the "Discussion" Section to highlight our future works planned on other MEFV variations.

"*Furthermore, as previously mentioned, all patients involved in this study exhibited the M694V variant in the MEFV gene. It's important to note that the investigation into the connection between other MEFV variants and pyrin will be the focus of a forthcoming study, which has been planned based on the findings of this research.*"

7. *Line 510: Provide this test's numerical values of sensitivity and specificity.*

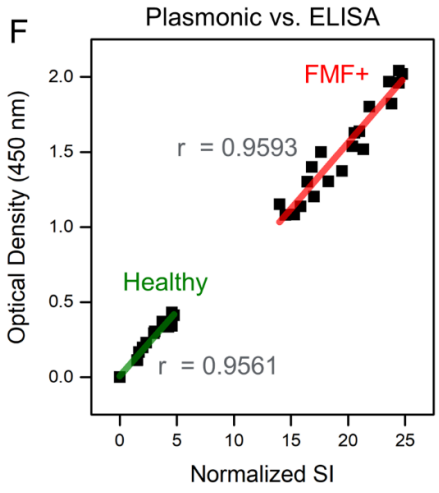
We would like to thank the reviewer for bringing this critical issue. To provide numerical values for sensitivity and specificity, a comprehensive validation study involving a large sample size of both FMF-positive and FMF-negative cases would be necessary. Sensitivity and specificity are statistical measures that evaluate the performance of a diagnostic test. Sensitivity is the ability of a test to correctly identify individuals with the condition (true positive rate), while specificity is the ability of the test to correctly identify individuals without the condition (true negative rate). These values are calculated using the following formulas:

$$Sensitivity = \frac{True\ Positives}{True\ Positives + False\ Negatives}, \quad Specificity = \frac{True\ Negatives}{True\ Negatives + False\ Positives}$$

True Positives are cases correctly identified as positive, False Negatives are cases incorrectly identified as negative, True Negatives are cases correctly identified as negative, and False Positives are cases incorrectly identified as positive. Therefore, in order to define sensitivity and specificity, we need a large patient sample pool.

Under the circumstances, if the reviewer agrees, instead of performing sensitivity and specificity tests, which rely on a large number of patient samples with different origins, potentially leading to false negative and positive values, we followed a different methodology to highlight the specificity and sensitivity of our technology. As a response to the other reviewer, we compared our methodology with an FDA-approved technique, such as ELISA, in differentiating FMF+ and FMF- cases, highlighting a high correlation between the two methods.

To validate our methodology, we compared our findings with those obtained through ELISA (enzyme-linked immunosorbent assay). Specifically, we applied Pearson calculations separately to healthy and FMF+ samples. In this analysis, we employed an ELISA kit designed for the detection of pyrin from patient samples, ensuring a rigorous evaluation of sensitivity and specificity in our assessments. Our calculations unveiled a substantial correlation between ELISA and our plasmonic biosensor, yielding Pearson's *r* values of 0.9561 and 0.9593 for healthy and FMF+ samples, respectively. The scale of Pearson's *r* ranges from -1 to 1, portraying both the strength and direction of a linear relationship. In our investigation, values approaching 1 denote an almost perfect positive linear relationship between the variables, accentuating the precision and reliability of our plasmonic technology in the analysis of clinical samples. Beyond the statistical correlation, the clinical relevance of our findings is noteworthy. The robustness of the relationship, as indicated by Pearson's *r* values surpassing the widely acknowledged threshold of 0.7 for a strong correlation, underscores the potential of our plasmonic biosensor as a steadfast and trustworthy clinical tool. This puts our technique in the same league as the commonly used ELISA method, showing that it's reliable for regular use in dealing with FMF. The validation of a strong correlation with an FDA-approved technique further affirms the clinical applicability of our plasmonic biosensor, setting the stage for its integration into the standard diagnostic concepts for FMF and potentially other medical applications.



We added a new figure (Figure 4F) to highlight the linear correlation between our methodology and ELISA.

Figure 4. *(F) Comparison between normalized SI values determined from the plasmonic pre-diagnostic technology and the optical density values determined from ELISA. In the figure, green (healthy) and red lines (FMF+) account for the linear regressions fitted to the experimental data (black squares).*

As the reviewer suggested in his/her other questions, we will evaluate this technology based on a large patient sample pool. We will use clinical samples from individuals with asymptomatic carriers, with different MEFV variants, e.g., single heterozygous, complex heterozygous, homozygous. We will also screen other rheumatic diseases, infections, and trauma. This will create a patient sample pool to ensure false negative and positive outputs for current clinical techniques based on clinical observations and genetic tests, and challenge our technology to determine its boundaries.

In order to clarify this issue, we added the below text to "Discussion" Section.

"*In our future studies, we will also conduct a comprehensive evaluation of the sensitivity and specificity of our technology. These tests necessitate a diverse range of patient samples with different origins, which could potentially result in false negative or false positive outcomes, underscoring the importance of a large and varied patient sample pool. By utilizing clinical samples from individuals with asymptomatic carriers, featuring different MEFV variants, alongside cases of other rheumatic diseases and infections, we aim to establish a robust patient sample pool. This will allow us to assess potential false outcomes in the context of current clinical techniques, relying on both clinical observations and genetic tests. Through this process, we intend to challenge our technology to ascertain its limits and capabilities.*"

8. *Discuss the potential limitations of this technique's specificity for FMF diagnosis, considering that various factors, including IL-1β, TNF-α, IFN-γ, infections, and trauma, stimulate pyrin expression. I strongly suggest authors to consider testing the technique in individuals with asymptomatic carriers, patients with different variants (single heterozygous, complex heterozygous, homozygous), and other rheumatic diseases, infections, and trauma.*

We would like to thank the reviewer for raising this critical matter. In this study, we have observed that the increased expression of pyrin in the patient group is linked to the influence of MEFV variants on pyrin synthesis, inflammatory attacks, and the impact of IL-1 levels. While previous research has indicated that cytokines like IFN-alpha and TNF can stimulate pyrin expression, the exact mechanisms remain unclear. Nevertheless, no data supports the idea of continuous pyrin expression stimulation and a sustained elevation in pyrin levels. Continuous high levels of pyrin have not been reported in these patients. However, particularly in conditions such as FMF, where pathogenic genetic mutations are present, exposure to a severe and continuous inflammatory process may lead to the detection of patients with elevated pyrin levels even during asymptomatic periods.

In order to address this issue, we added a text on this critical matter in "Discussion" Section.

"*In this study, the elevation of pyrin expression in the patient group is attributed to the influence of MEFV variants on pyrin synthesis, inflammatory attacks, and the impact of IL-1 levels. In literature, it has been shown that cytokines such as IFN-alpha and TNF, in particular, stimulate pyrin expression, although this has not been clearly elucidated.*[Ref1,Ref2] *On the other hand, there is no data regarding continuous stimulation of pyrin expression and a constant elevation in pyrin levels. There is no data available regarding the continuous high levels of pyrin in these patients. However, especially in disorders like FMF, the presence of pathogenic genetic mutations, exposure to a severe and continuous inflammatory process, and the stimulation of pyrin expression may lead to the detection of patients with high pyrin levels even during asymptomatic periods. FMF and other inflammatory diseases will be the subject of future studies regarding the relationship and differences between pyrin expression with etiology related to infection, trauma.*"

Ref1: Heilig, R. & Broz, P. Function and mechanism of the pyrin inflammasome. Eur. J. Immunol. 48, 230–238 (2018).

Ref: Simon, A., Van Der Meer, J. W. M. & Drenth, J. P. H. Familial Autoinflammatory Syndromes. Kelley and Firestein's Textbook of Rheumatology: Volumes 1-2, Tenth Edition vol. 2 (Elsevier Inc., 2016).

We also added the note below to the "Discussion" Section to highlight our future works planned on testing our technology for diverse range of FMF as the reviewer suggested.

"*Future research will delve into FMF and other inflammatory diseases, investigating the connections and distinctions between pyrin expression in relation to causes such as infection and trauma. This will necessitate testing our pre-diagnostic technology in a diverse range of individuals, including asymptomatic carriers, patients with various MEFV variants, such as single heterozygous, complex heterozygous, and*

homozygous individuals. Additionally, the study will encompass a broader scope, exploring pyrin expression in the context of other rheumatic diseases, infections, and trauma."

9. *Suggest that the primary utility of this technique may lie in revealing the pathogenicity of variants of unknown significance and distinguishing between individuals with a single heterozygous variant who are sick or carriers. Include these points in the introduction and discussion sections.*

We would like to thank the reviewer for suggesting the discussion of this critical issue. Most recently, 396 variants were detected in the MEFV gene region, which encodes pyrin on chromosome 16, and those located in exon 10 (B30.2) region were considered more pathogenic. Just as there are pathogenic features in other exon and intron regions, there are also non-pathogenic features and features of unknown significance (VUS). We also aim to provide significant benefit in terms of clarifying the identification and recognition of pathogenicity in cases carrying heterozygous features and exon 10 enhanced features other than the heterozygous pattern.

In order to address this issue, we added a text on this matter in "Discussion" Section.

"*Based on recent studies, it is well-documented that the MEFV gene, responsible for encoding the pyrin protein on chromosome 16, harbors a total of 396 variants.*[Ref1] *Notably, variants located within the exon 10 (B30.2) region have been established as having a higher pathogenic potential.*[Ref2] *While pathogenic characteristics are evident in various exon and intron regions, there exists a subset of variants categorized as "variants of unknown significance" (VUS) due to the uncertainty surrounding their clinical implications. Our research is positioned to provide a valuable contribution in elucidating the pathogenicity of these previously unclassified variants and in distinguishing individuals with heterozygous variants, particularly those displaying exon 10-enhanced features beyond the typical heterozygous pattern. These findings emphasize the utility of our technique in differentiating between affected individuals, carriers, and those with VUS.*"

Ref1: Guilaine BOURSIER. MEFV sequence variants. Available at: https://infevers.umai-montpellier.fr/web/search.php?n=1.

Ref2: Chirita, D. et al. Mutations in the B30.2 and the central helical scaffold domains of pyrin differentially affect inflammasome activation. Cell Death Dis. 14, 213 (2023).

MINOR POINTS:

1. *Remove "(FMF)" from the title.*

We would like to thank the reviewer for his/her suggestion. We removed the abbreviation for Familial Mediterranean Fever from the title.

2. *Remove the word "disease" after "FMF."*

We would like to thank the reviewer for his/her suggestion. We removed the word "disease" after "FMF" throughout the manuscript.

3. *Consider moving or removing the sentence, "Children are particularly affected by FMF, and the disease process can be distressing for them," affecting the text's flow.*

We concur with the reviewer's perspective; and accordingly, excised the mentioned sentence from the "Introduction" Section.

4. *Consider using "presumptive diagnosis" or "preliminary diagnosis" instead of "pre-diagnosis" for better terminology.*

We express our appreciation to the reviewer for his/her valuable suggestion. As per his/her recommendation, we consistently substituted "pre-diagnosis" with "preliminary diagnosis" across the entire manuscript.

We also replaced "pre-diagnosis" with "preliminary diagnosis in Figure 6 as below.

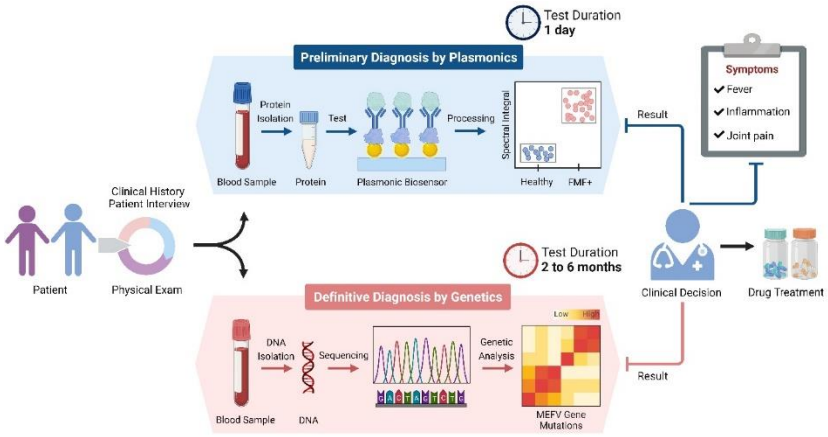


Figure 6

5. *Remove the phrase "FMF was first documented in a case report in 1908, and it was clearly described in 1945. Moreover," as it is unrelated to the primary subject and disrupts the manuscript's flow.*

We thank the reviewer for his/suggestion. We removed the mentioned phrase from “Introduction” Section, which improved the flow of the associated paragraph.

6. *On Line 62, replace "pain" with "restriction."*

As the reviewer suggested, we replaced “pain” with “restriction” in the corresponding sentence in “Introduction” Section.

7. *Remove the phrase "and serositis" from Line 62.*

As the reviewer suggested, we remove “and serositis” in the corresponding sentence in “Introduction” Section.

8. *On Line 70, change "Interleukin-1" to "interleukin-1β."*

As the reviewer suggested, we replaced “Interleukin-1” with “interleukin-1β” in the corresponding sentence in “Introduction” Section.

9. *On Lines 80-83 and 507, note that per EULAR FMF treatment recommendations, a clinical diagnosis of FMF is sufficient to initiate colchicine treatment; genetic diagnosis is not necessary for treatment initiation.*

We appreciate the reviewer's thorough examination of our article, identifying sentences that were unintentionally stated incorrectly. In order to address these mistakes, we replaces two sentences with the corrected one as below:

“*Despite the advantages of this diagnostic method in addressing an important disease, the lengthy duration of genetic analyses is a major obstacle for physicians in initiating timely FMF treatment.*”

Was replaced by

“*Despite the advantages of this diagnostic method in addressing an important disease, the lengthy duration of genetic analyses is a major obstacle for physicians aiming to use these analyses to promptly identify FMF and initiate a timely treatment. While the initiation of colchicine treatment does not require genetic analyses and a clinical diagnosis of FMF is sufficient for treatment initiation, obtaining a definitive diagnosis is critical to prevent potential unnecessary treatment resulting from a false diagnosis.*[Ref1] *FMF diagnosis is established primarily through the assessment of clinical symptoms. However, genetic screening is considered indispensable to both confirm the diagnosis and distinguish it from other akin conditions in the presence of patients exhibiting atypical or incomplete clinical presentations. For instance, there are numerous autoinflammatory diseases manifesting symptoms similar to FMF, and genetic analyses serve as a guiding tool in such cases.*

Ref1: Zadeh, N., Getzug, T. & Grody, W. W. Diagnosis and management of familial Mediterranean fever: Integrating medical genetics in a dedicated interdisciplinary clinic. Genet. Med. 13, 263–269 (2011)."

And;

"Consequently, it is crucial for patients to initiate drug therapy during the waiting period for genetic test results, as it can help alleviate disease symptoms."

Was replaced by

"*Here, genetic diagnosis is not necessary for treatment initiation, while the clinical diagnosis of FMF is sufficient to initiate colchicine treatment. In this context, our biosensor technology introduces an additional parameter to the traditional preliminary diagnosis based on symptoms and clinical findings, enabling physicians to initiate FMF treatment before receiving genetic test results.*"

10. *On Lines 101-111, consider moving this paragraph to the methods section.*

We appreciate the reviewer's suggestion. The paragraph the reviewer pointed out was meant to provide a brief introduction to the pre-diagnostic platform without going into too much detail in "Introduction" Section. We provided a more detailed explanation of each component of the technology in "Material and Methods" Section. Therefore, if the reviewer agrees, we would prefer to keep this paragraph where it is.

11. *Provide information about institutional or commercial sources of the used pyrin and anti-pyrin antibody materials.*

Based on the reviewer's suggestion, we provided this information to the "Surface Modification for Capturing Pyrin Protein" Section in the modified manuscript.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The revision has significantly improved the quality of the manuscript. However, the response to the questions #2 are mostly inaccurate justification. Here is why? Regardless of the terminal groups (-NH2 vs. SH, siloxane will form Si-O bonds with the glass surface. The average diameter of gold nanoparticles used in this current study is ~40 nm. The crystal plane of Au is Au(111). It is very unlikely that the crystal structure of Au will change. Moreover, only the bottom surface of gold nanoparticles will be functionalized with either -NH2/-SH and that should not alter the crystal structure. The thiol footprint is ~0.25 nm2. Thus, few tens of thiols will bind to spherical gold nanoparticles. Finally, the silane will form a monolayer onto the glass substrate. There is no way irregular and multilayer binding will occur. The authors have cited an article for colloidal gold nanoclusters that has nothing to do with the solid-state, LSPR-based sensor fabrication approach.

Here is the real story why aqueous dispersion of gold nanoparticles have not been used to attach on to thiolated silane surface. Thiol is a hydrophobic group and repeal water. Citrate-stabilized gold nanoparticles are commonly synthesized in water. The incubation of Au nanoparticles into MPTES-functionalized glass does not allow nanoparticle adsorption onto the surface. Nevertheless, the stability of gold nanoparticles onto APTES (i.e., -NH2-Au interaction) is very poor, and the current fabrication strategy requires multiple nanoparticle surface modification steps. Overall, the stability and reproducibility of solid-state LSPR sensors fabricated with spherical gold nanoparticles are extremely poor. Moreover, gold nanoparticles move onto APTES-functionalized glass substrates and aggregates over time if the coverslip is incubated in aqueous solution.

Although, the technology can detect some disease related antibody, which any LSPR-based sensors can do, this reviewer is skeptical about the data presented in Fig. 4B where the standard deviations for all the samples look identical. Finally, 300 pg/mL sensitivity is very unrealistic for spherical gold nanoparticles. Together, I do not support this work for publication in Nature Communications.

Reviewer #2 (Remarks to the Author):

The authors have effectively addressed my concerns. While the method shows promise in distinguishing FMF from healthy subjects within a specific FMF patient group, testing it across a broader range of patients, including those with infections and other inflammatory conditions resembling FMF, could enhance its clinical applicability. The patient group that would benefit most from this would be those with VUS. The authors noted this as a limitation in their revised article

REVIEWERS' COMMENTS

REVIEWER #1

The revision has significantly improved the quality of the manuscript.

We would like to thank the reviewer for dedicating his/her time and effort to thoroughly assess our work. His/her insightful feedback, comprising 12 detailed questions, has significantly enhanced both the qualitative and quantitative aspects of our research. We truly appreciate his/her valuable contributions.

However, the response to the questions #2 are mostly inaccurate justification. Here is why? Regardless of the terminal groups (-NH2 vs. SH, siloxane will form Si-O bonds with the glass surface. The average diameter of gold nanoparticles used in this current study is ~40 nm. The crystal plane of Au is Au(111). It is very unlikely that the crystal structure of Au will change. Moreover, only the bottom surface of gold nanoparticles will be functionalized with either -NH2/-SH and that should not alter the crystal structure. The thiol footprint is ~0.25 nm2. Thus, few tens of thiols will bind to spherical gold nanoparticles. Finally, the silane will form a monolayer onto the glass substrate. There is no way irregular and multilayer binding will occur. The authors have cited an article for colloidal gold nanoclusters that has nothing to do with the solid-state, LSPR-based sensor fabrication approach.

Here is the real story why aqueous dispersion of gold nanoparticles have not been used to attach on to thiolated silane surface. Thiol is a hydrophobic group and repeal water. Citrate-stabilized gold nanoparticles are commonly synthesized in water. The incubation of Au nanoparticles into MPTES-functionalized glass does not allow nanoparticle adsorption onto the surface. Nevertheless, the stability of gold nanoparticles onto APTES (i.e., -NH2-Au interaction) is very poor, and the current fabrication strategy requires multiple nanoparticle surface modification steps. Overall, the stability and reproducibility of solid-state LSPR sensors fabricated with spherical gold nanoparticles are extremely poor. Moreover, gold nanoparticles move onto APTES-functionalized glass substrates and aggregates over time if the coverslip is incubated in aqueous solution.

We would like to express our gratitude to the reviewer for raising his/her concern once again. We believe there might be a misunderstanding regarding our fabrication technique. In this revision, we will utilize this opportunity to provide a detailed clarification of the process. In our manuscript, we followed a fabrication methodology employed in different examples from the literature that were used for a variety of plasmonic applications.

In the scope of this study, 3-aminopropyltriethoxysilane (APTES) was used as the binding molecule for attaching gold nanoparticles to the glass surface. The structure of APTES reacts with the –OH groups present on the substrate surface to form Si-O bonds via sol-gel reaction. In summary, an amino-functional surface structure was created. Similarly to APTES, (3-mercaptopropyl) trimethoxysilane (MPTES) is an organosilane with three alkoxy groups capable of reacting with hydroxyl groups on the substrate surface to form Si-O bonds and leaving terminal functional thiol groups suitable for immobilization. However, in our study, we used APTES and obtained an amino-functional surface. Both molecules, e.g., APTES and MPTES, are commonly used for the binding of AuNPs to the surface [1]. As shown in the literature, weak interactions stem from electrostatic bonding, while strong interactions arise from covalent bonding. Furthermore, it has been also demonstrated that both binding agents effectively bind Au groups to the surface [1]. However, there is no quantitative evidence indicating which functionality is superior in the immobilization of AuNPs [1].

In the literature, numerous studies follow synthesis procedures similar to the one described in our manuscript, e.g., developing amino-functional surface modifications with APTES followed by the adsorption of gold nanoparticles (AuNP) to prepare glass surfaces for sensing applications.

In the following figure, we highlighted several examples from the literature. In Figure 1A, Vu-Dinh et al. initially modified the glass surface with APTES to impart amino functionality, facilitating the adsorption of AuNPs, akin to the approach we adopted in our manuscript [2]. Similarly, in Figure 1B, Stetsenko et al. also first rendered the glass surface amino-functional using APTES, and then carried out AuNP adsorption [3]. In Figure 1C, reflecting the article that guided our particle synthesis procedure, Hongming et al. commenced by introducing amino functionality to the glass surface via APTES, which was then followed by the adsorption of gold nanoparticles (AuNPs) and PDA polymerization, culminating in the fabrication of a plasmonic chip [4]. Similarly, in Figure 1D, Shantang et al. initially utilized APTES to achieve an amino-functional glass surface prior to the adsorption of AuNPs [5]. These examples demonstrate that our experimental approach is well-supported by existing literature, reinforcing the validity of our methodology.

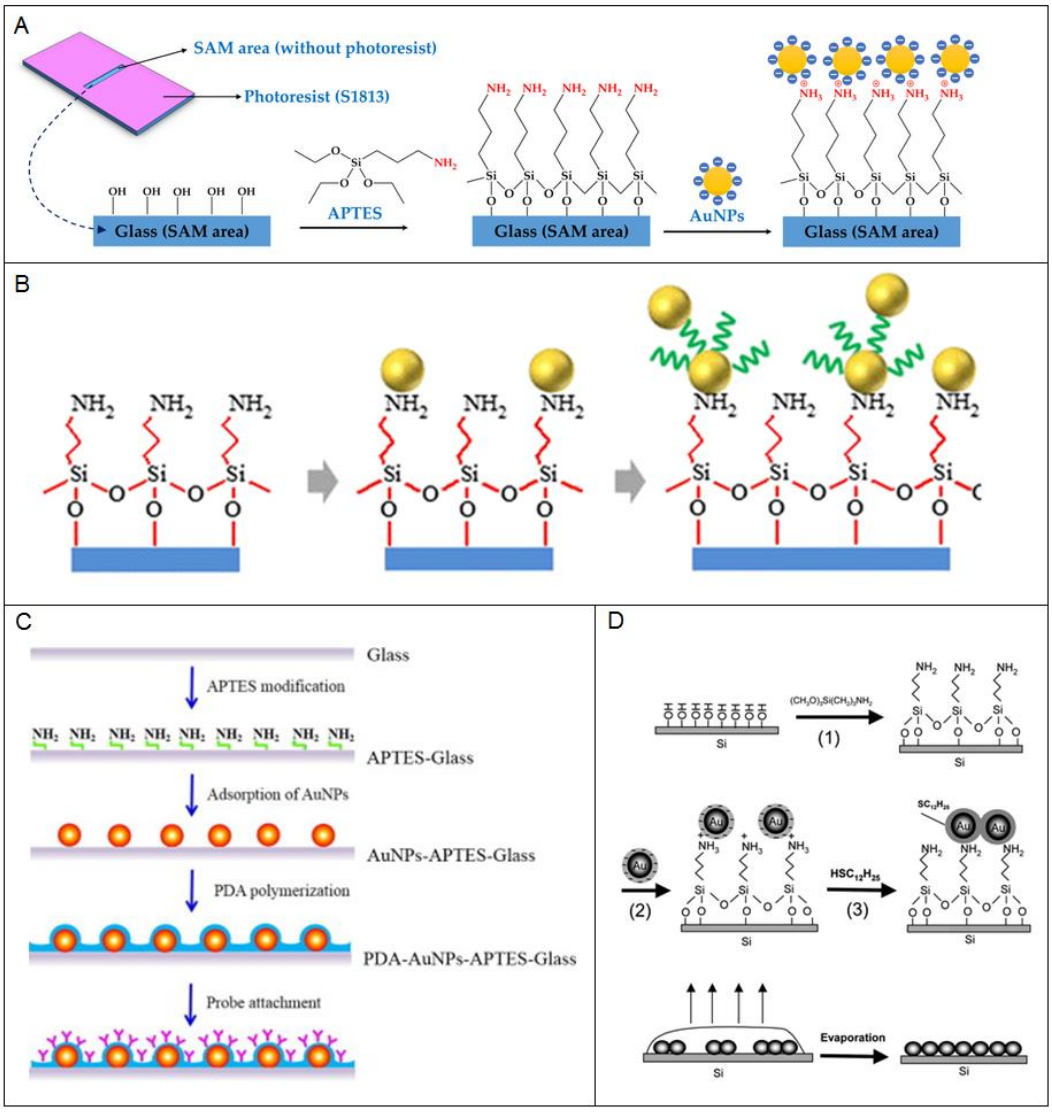


Figure 1. The significance of APTES and AuNPs in glass surface modifications [2-5].

In terms of **fabrication methodology**, APTES is preferred in this study for three primary reasons:

i. The interaction of APTES structure with Au nanoparticles is weaker compared to the interaction of 3-mercaptopropyl groups with Au nanoparticles. Therefore, there is no distortion in the crystal structure of Au nanoparticles [6]. This weak interaction also leads to a lower degree of passivation compared to thiols [7]. Consequently, since the Au surface is less passivated, subsequent modifications can be easily performed on the surface.

ii. Amino groups contain one unshared electron pair. During the attachment of APTES molecules to the glass surface via sol-gel chemistry, they bind to Au nanoparticles through electron transfers and electrostatic interactions via amino groups. However, in the case of MPTES, there are two unshared electron pairs on the S atom in the HS- groups, and the interaction between HS- groups and Au nanoparticles is stronger [8-9], which may result in not only single-row accumulation of Au but also irregular and multilayered bindings on the surface. This situation is undesirable for optical sensors.

iii. In the literature, it was also demonstrated that the accumulation of Au films on glass surfaces derived from APTES occurs more rapidly than those derived from MPTES [10], which further justifies the preference for APTES in this study.

In conclusion, the study investigated the use of an amino-functional silane that allows for the conjugation of other amines to gold surfaces.

Fabrication cost is another aspect favoring the preference for APTES.

The aim of the study is to develop a sensor system with disposable measurement units, enabling point-of-care testing (POCT) for the diagnosis of FMF as a cost-effective alternative to expensive genetic tests. With this measurement system, instant results could be obtained, thus offering a more accessible and affordable option for medical diagnosis. To ensure widespread adoption and affordability, the disposable sample unit is targeted to be low-cost. While we have the capability to fabricate the plasmonic chip in a clean room environment, we prefer manufacturing through particle synthesis for scalability and cost-effectiveness.

Furthermore, considering this goal via the use of nanoparticle synthesis, the structure of MPTES is a more expensive chemical compared to APTES (500 mL of 98% APTES costs 288 Euros, while 500 g of 95% MPTES costs 580 Euros) [11-12].

Although, the technology can detect some disease related antibody, which any LSPR-based sensors can do, ….

There seems to be a misunderstanding regarding the working principle of our methodology, contrary to the reviewer's assertion. In this study, we did not detect a disease-related antibody. Instead, we utilized a specific antibody (anti-pyrin) to detect the presence of a particular protein (pyrin), using its quantity in the blood as an indication of a genetic disease. Our technology not only detects the presence of a biomarker but also quantitatively distinguishes its presence between healthy individuals and patients, both of whom have this particular biomarker protein in different amounts. Unlike the majority of plasmonic biosensing studies in the literature, our study involved characterizing and testing our plasmonic platform with patient samples. Rather than using purified biological materials or relatively easier body fluids, e.g., saliva or urine, we directly processed a complex material, which is blood, to determine the presence of a specific protein amidst numerous others. Our results demonstrate the highly sensitive nature of our methodology.

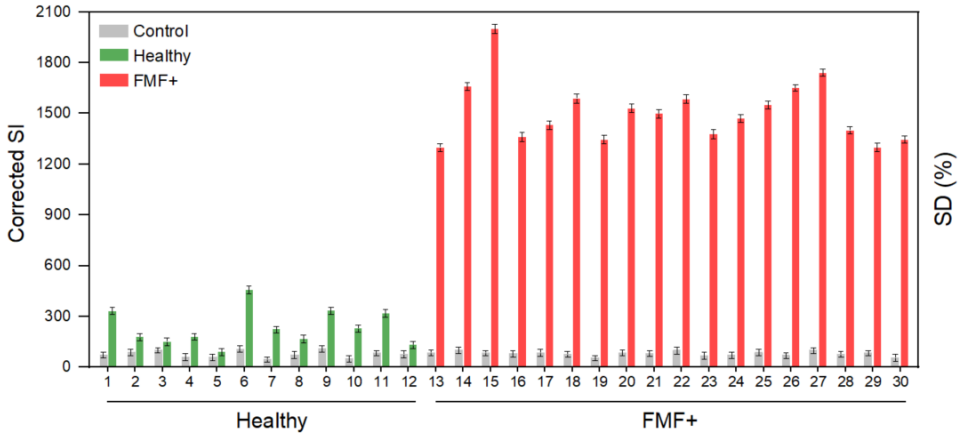
In this study, as the reviewer correctly points out, we utilized an LSPR-based plasmonic sensor, a technology that has been previously developed in the literature. However, the novelty of our study lies in its applications, specifically the utilization of a plasmonic biosensing platform to replace a genetic test used for diagnosing a rare disease with a genetic origin.

In contrast to similar examples, in addition to developing plasmonic chips, we also created the entire detection unit and the associated software for hardware control and data processing.

… this reviewer is skeptical about the data presented in Fig. 4B where the standard deviations for all the samples look identical.

We would like to thank the reviewer for carefully reading our manuscript. In the first revision, based on the reviewer comment, we repeated the test with the same patient samples that were stored at -20 °C.

For this particular dataset, in response to the reviewer's comment in the first revision, we have included the mean values of the repetition tests along with standard deviation (SD) values. In the figure, we represented the SD values as percentages. However, unfortunately, the label of the second Y-axis was remained under the adjacent subfigure by mistake (Figure 4C) as indicated below. To resolve this issue, we replaced the percentage SD values with actual values and discussed the consistency of the SD values in percentage through additional text in the manuscript.



Here, we have presented the SD values corresponding to 30 samples, divided as follows: 1-30 for the control channel and 31-60 for the sensor channel, as detailed below:

Table 1. SD values in percentages of the 60 samples used in Figure 4B in the manuscript.

#	SD (%)	#	SD (%)	#	SD (%)	#	SD (%)	#	SD (%)	#	SD (%)
1	7.8	**11**	9.6	**21**	7.6	**31**	9.6	**41**	9.4	**51**	8.2
2	10.7	**12**	11.1	**22**	11.4	**32**	13.5	**42**	12.3	**52**	9.6
3	8.8	**13**	7.9	**23**	10.2	**33**	10.8	**43**	10.7	**53**	8.7
4	9.9	**14**	9.7	**24**	10.8	**34**	13.4	**44**	13.3	**54**	11.2
5	7.1	**15**	10.4	**25**	8.9	**35**	8.3	**45**	10.8	**55**	9.2
6	11.2	**16**	11.5	**26**	11.1	**36**	13.7	**46**	13.1	**56**	11.2
7	10.3	**17**	9.2	**27**	10.2	**37**	8.1	**47**	9.2	**57**	7.7
8	10.4	**18**	10.4	**28**	12.1	**38**	12.7	**48**	11.7	**58**	12.2
9	9.1	**19**	9.3	**29**	7.6	**39**	8.8	**49**	9.8	**59**	11.4
10	10.6	**20**	10.9	**30**	13.4	**40**	12.6	**50**	11.3	**60**	10.9

The reviewer has rightly observed a remarkable consistency in the standard deviation values across a range of measurements, which is a testament to the reliability of our hardware configuration. This level of reliability has been attained through the strategic employment of multiple high-resolution spectrometers and the selection of optical components that are spectrally harmonized, all encased within a design specifically engineered to reduce optical, electrical, and mechanical disturbances. Furthermore, our approach to data processing significantly contributes to minimizing measurement errors caused by spectral setup fluctuations. By effectively removing the spectral influence of both background noise and the light source's emission profile, and by assessing spectral characteristics across various wavelengths concurrently, we significantly enhanced the precision and accuracy of our measurements.

In order to highlight this fact, we added the text below to the "*Clinical Sample Tests*" subsection in the "*Results*" section.

"*In the figure, the squares represent the mean integral values of the data, and the error bars indicate a range of twice the standard deviation from these means, showing the variability in real values. Additionally, when considering the standard deviation as a percentage of the mean, a measure of relative variability, this relative standard deviation spans from 7% to 14% across the dataset. The consistency in the relative standard deviation across various measurements underscores the robustness of our hardware approach. This robustness was achieved by employing multiple spectrometers yielding high resolution, and the use of optical components that are spectrally compatible with each other, along with a casing designed to minimize optical, electrical, and mechanical fluctuations. Furthermore, our data processing methodology plays a crucial role in reducing errors due to fluctuations in the spectral measurement setup. This was accomplished by eliminating the spectral contribution of the background noise and the emission distribution of the light source, and simultaneously evaluating spectral features at different wavelengths, thereby enhancing the reliability and accuracy of our measurements.*"

Finally, 300 pg/mL sensitivity is very unrealistic for spherical gold nanoparticles.

We appreciate the reviewer's concern regarding the reported sensitivity of 300 pg/mL for spherical gold nanoparticles. It is important to note that, in the context of surface plasmon resonance (SPR) based methodologies, sensitivities reaching 10 – 100 pg/mL have been documented [13]. Furthermore, plasmonic structures enhanced by metallic particles have demonstrated similar limits of detection (LODs) [14]. Compared to these advanced methodologies, our plasmonic chip exhibits a relatively modest LOD, which nonetheless sufficiently discriminates between FMF+ and FMF- cases based on the amount of pyrin protein. Our clinical findings affirm that the sensitivity achieved by our device is adequate for this specific application.

Moreover, our fabrication approach does not necessitate the use of costly clean-room facilities or complex and expensive sample preparation processes, an aspect that is paramount for the feasibility of commercial applications. This consideration significantly enhances the practical value of our technology.

In addition, the literature reveals several studies employing metallic nanoparticle-based plasmonic chips that achieve higher LODs compared to ours [15]. Our methodology, which combines a robust and sensitive hardware configuration with a sophisticated spectral post-processing technique, has been instrumental in further increasing the system sensitivity and achieving an LOD as low as 300 pg/mL. This accomplishment underscores the efficacy and applicability of our approach in the targeted clinical setting.

In order to highlight this fact, we added the text below to "*Limit-of-Detection of the Biosensor Platform*" subsection in the "*Results*" section.

"*A sensitivity of 0.24 ng/mL (240 pg/mL) is not considered high, especially in comparison to SPR (Surface Plasmon Resonance)-based methodologies, which can achieve an LOD as sensitive as 10 – 100 pg/mL.[13] Metallic particle-enhanced periodic plasmonic structures have also been reported to achieve similar LODs.[14] Relative to these methodologies, our plasmonic chip supports a comparatively lower LOD, yet as shown in the following section, it is sufficient to distinguish between FMF+ and FMF- clinical samples based on the concentration of pyrin protein. Our clinical results confirm that our sensitivity level is adequate for this specific application. Besides, our fabrication method does not require expensive and sophisticated clean-room facilities or complex and costly sample preparation, which is crucial for commercial applications. Furthermore, as demonstrated in the literature, there are several studies on metallic nanoparticle-based plasmonic chips that achieve higher LODs compared to ours.[15] In addition to our robust and sensitive hardware approach, our spectral post-processing methodology further improves the system's sensitivity, yielding better LODs compared to the classical nanoparticle based plasmonic chips.*"

Together, I do not support this work for publication in Nature Communications.

In response to the reviewer's decision not to support publication in Nature Communications, we respectfully contend that the reasons provided do not, in our view, justify outright rejection.

Another reviewer has highlighted the clinical significance of our work, underlining its potential impact. Furthermore, the reviewer approved other aspects of our revision, indicating an acknowledgment of the study's merits in certain areas.

Our responses aim to demonstrate the functionality of our plasmonic chip and the adequacy of our Limit of Detection (LOD). These elements are crucial in showcasing the potential applicability of our technology in a clinical setting.

Finally, we sincerely appreciate the reviewer for insightful comments and the opportunity to revise our manuscript accordingly. We have carefully addressed each point the reviewer raised, providing detailed responses and making appropriate modifications to clarify our research findings and methodology. Moreover, we have expanded on the technological innovation of our study, emphasizing its potential impact on FMF research and treatment. We believe that our revisions, coupled with the explained significance of our technology, meet the publication criteria and substantially enhance the manuscript's contribution to the field.

We hope that our revisions and responses to the reviewer's comments have elucidated the importance and novelty of our work.

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REVIEWER #2

The authors have effectively addressed my concerns. While the method shows promise in distinguishing FMF from healthy subjects within a specific FMF patient group, testing it across a broader range of patients, including those with infections and other inflammatory conditions resembling FMF, could enhance its clinical applicability.

In the previous evaluation of our manuscript, the reviewer provided very helpful feedback, which significantly improved our manuscript and allowed us to further emphasize our technology from a clinical perspective. This is also crucial for broadening our reader base.

We again would like to express our gratitude for the reviewer's valuable and positive contributions. The technology developed in this manuscript is effective in distinguishing healthy individuals from patients for the case of FMF. In the case of infections and other inflammatory diseases with similar symptomatology with FMF, sensitivity of our technology could be enhanced by supporting the method's results with clinical findings while distinguishing FMF from these diseases. The efficacy of our technology in differentiating these groups of diseases requires further investigating in more extensive studies as planned in our future studies.

In order to highlight this fact, we added the text below to the "*Discussion*" section in the modified Manuscript.

"*Our future research will also concentrate on differentiating FMF from infections and other inflammatory conditions that share similar symptoms with FMF. Our technology has proven effective in distinguishing individuals with FMF from healthy ones. However, when it comes to separating FMF from similar diseases, integrating clinical data could enhance the precision of our technology's results. The primary limitation in distinguishing these diseases lies in our technology's sensitivity, an area we aim to explore in greater depth through comprehensive future studies.*"

The patient group that would benefit most from this would be those with VUS. The authors noted this as a limitation in their revised article.

We agree with the reviewer that patients with variants of uncertain significance (VUS) would benefit the most from our technology [2]. VUS have recently become more important in the context of FMF. Approximately 400 variations have been identified in the MEFV gene, and a significant portion of these are considered VUS [3]. The relationship of these variations with clinical findings remains unclear. Our technology could also contribute to assessing the pathogenicity of variants thought to be VUS.

In order to highlight the potential use of our technology in the discrimination of VUS cases in FMF, we added the text below to the "*Discussion*" section in the modified Manuscript with the three references below:

"*Based on the recent studies, it is well-documented that the MEFV gene, responsible for encoding the pyrin protein on chromosome 16, harbors a total of 396 variants.[1] Notably, variants located within the exon 10 (B30.2) region have been established as having a higher pathogenic potential.[2] While pathogenic characteristics are evident in various exon and intron regions, in the realm of FMF, there exists a subset of variants categorized as "variants of unknown significance" (VUS) due to the uncertainty surrounding their clinical implications.[3] The clinical implications of these variations are not yet fully understood, leaving a gap in our comprehension of their impact on FMF. This uncertainty underscores the need for innovative solutions to better understand these genetic anomalies. Our technology emerges as a promising tool in this context, offering the potential to elucidate the pathogenicity of variants previously deemed uncertain and distinguishing individuals with heterozygous variants, particularly those displaying exon 10-*

enhanced features beyond the typical heterozygous pattern. In the future, by leveraging our technology, we could bridge the gap in knowledge regarding VUS, providing a clearer picture of their role in FMF and enhancing our ability to tailor more effective treatment strategies for affected individuals. Our findings emphasize the utility of our technique in differentiating between affected individuals, carriers, and those with VUS."

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Reviewers' comments:

Reviewer #1 (Remarks to the Author):

I carefully read the author's response letter. The authors clearly think that I have very little knowledge about the LSPR-based sensing. I will take it. Anyway, the manuscript has many drawbacks that can be difficult to overcome, thus a successful transformation of this technology to clinical diagnostic may not be feasible at this stage.

REVIEWERS' COMMENTS

Reviewer #3 (Remarks to the Author):

The manuscript by Cetin and coworkers reports on the development of a rapid pre-diagnostic sensing platform for the detection of Familial Mediterranean Fever through the identification and quantification of pyrin.

There are several points to be raised.

1. The clinical relevance of this work is high, as FMF is a genetic disease affecting only a small fraction of world population, thus not commonly targeted with specific sensors. The importance of the platform is that it will undoubtedly provide positive repercussions from the clinical standpoint and on the life of patients affected by it.

2. The experiments have in general been carried out with detail and the data analysis is appropriate. However, in Figure 3D I see that the points in the calibration curve have been fit linearly when clearly they shouldn't have been. It would be preferable if the authors fit the points with the proper data interpolation function rather than with a linear interpolation for convenience. Furthermore, from the rebuttal letter I can see how previous reviewers might have commented on the appropriateness of the surface functionalization. While I believe that the authors may have optimized their process to increase shelf life, I find that neither APTES nor the use of biotin-streptavidin or adsorption for antibody functionalization will lead to much more optimization as these approaches have limitations that have been clearly demonstrated by the scientific community and now replaced by more reliable functionalization approaches, that produce better target recognition and more stability.

3. In general, I do not think that this manuscript is appropriate for Nature Communications, as the fundamental science is not new and thus the work does not add anything novel in that respect. I believe that it should be published in a more sectorial journal.

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Reviewer #3:

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We appreciate the referee's feedback and acknowledgment of the clinical relevance of our work. While Familial Mediterranean Fever (FMF) may indeed affect a smaller fraction of the global population, it is highly prevalent in certain countries, particularly in the Mediterranean region, the Middle East, and parts of Armenia and Turkey, where it poses a significant public health challenge. In these regions, FMF has a substantial impact on the quality of life of affected individuals, often leading to complications such as amyloidosis when not diagnosed or treated in a timely manner. Our biosensor platform addresses a critical need in these countries by providing a more accessible, cost-effective, and accurate diagnostic tool, which is especially important for improving early detection and management of FMF. We believe that the development of disease-specific sensors, even for conditions with regional concentrations, can have a profound impact on healthcare systems and patient outcomes in those areas.

We are confident that our technology will provide significant clinical benefits for FMF patients and potentially serve as a model for similar applications in other rare genetic disorders.

2. *The experiments have in general been carried out with detail and the data analysis is appropriate. However, in Figure 3D I see that the points in the calibration curve have been fit linearly when clearly they shouldn't have been. It would be preferable if the authors fit the points with the proper data interpolation function rather than with a linear interpolation for convenience.*

We appreciate the reviewer's observation regarding the calibration curve in Figure 3D. It is true that at higher pyrin protein concentrations, the behaviour deviates from linearity. However, for the purpose of determining the limit of detection (LOD), it is essential to achieve linearity, as this is required by the theoretical modelling involved. To ensure this, our data analysis focused on small protein concentration ranges where the relationship between protein concentration and spectral shift exhibited linear behaviour. By concentrating on these lower concentration values, we ensured that the linear fit was both valid and appropriate for calculating the LOD. Higher concentrations were beyond the range necessary for this specific analysis, and thus the linear approximation holds in the range where it is most relevant for the LOD determination.

Furthermore, from the rebuttal letter I can see how previous reviewers might have commented on the appropriateness of the surface functionalization. While I believe that the authors may have optimized their process to increase shelf life, I find that neither APTES nor the use of biotin-streptavidin or adsorption for antibody functionalization will lead to much more optimization as these approaches have limitations that have been clearly demonstrated by the scientific community and now replaced by more reliable functionalization approaches, that produce better target recognition and more stability.

We thank the reviewer for his/her insightful comments regarding surface functionalization. We acknowledge the limitations of using APTES, biotin-streptavidin, or adsorption for antibody functionalization, as highlighted by the scientific community. While our current approach was optimized to enhance shelf life, we agree that more advanced

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functionalization strategies may offer better target recognition and stability. In our future work, we plan to explore and integrate alternative surface chemistry models to further optimize our platform's performance.

3. *In general, I do not think that this manuscript is appropriate for Nature Communications, as the fundamental science is not new and thus the work does not add anything novel in that respect. I believe that it should be published in a more sectorial journal.*

We sincerely appreciate the reviewer's feedback and his/her assessment of our manuscript. While we acknowledge that the fundamental principles of plasmonics may not be entirely novel, we believe the true novelty of our work lies in the application of plasmonic technology to diagnose a genetic disorder like FMF, a disease that has been historically diagnosed through genetic testing. FMF primarily affects populations in the Mediterranean, and current genetic testing often yields inconclusive results, particularly for heterozygous mutations, where approximately 50% of cases may remain unresolved.

Our platform not only introduces a more accessible and cost-effective method for diagnosing FMF but also holds the potential to eventually replace or complement genetic tests that currently dominate the diagnostic landscape. Given the shortcomings of genetic tests in yielding conclusive results for all patients, the ability to directly detect pyrin protein levels—an essential indicator of FMF—could offer a more definitive diagnostic approach for individuals whose genetic test results are unclear.

Moreover, the device we have developed goes beyond being just a simple sensor. It is a complex and fully integrated system that combines optofluidics, real-time spectroscopy, and a stable surface functionalization approach. This level of integration is not commonly seen in the context of plasmonic biosensing for genetic diseases. By offering stability for up to six months and an impressive detection limit of 0.24 ng/mL, our biosensor platform provides both high specificity and practical usability in a clinical setting.

In summary, while the plasmonic technology itself may not be entirely new, the combination of this technology with our novel application in FMF diagnosis, the high-performance characteristics of our system, and its potential to serve as a more effective alternative to genetic testing represent significant contributions to the field. We believe that these elements render our work highly relevant and novel, with broad implications for both clinical practice and the future of diagnostic tools for genetic diseases.

We appreciate the reviewer's suggestion to consider a more sectorial journal, but we respectfully believe that the innovation demonstrated through the integration of this technology into a clinically impactful and broadly applicable platform justifies its potential fit for Nature Communications.