Anti PF-4 antibody tests and other laboratory diagnosis in VITT: a literature review

林正修

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Characteristics of Platelet Factor 4

- Released from alpha-granules of activated platelets during platelet aggregation
- A positively-charged (cationic) tetrameric protein (70-amino acid) binds with high affinity to heparin (polyanionic), belonging to the CXC chemokine family
- Neutralization of heparin-like molecules on the endothelial surface of blood vessels, thereby inhibiting local antithrombin activity and promoting coagulation
- As a strong chemoattractant for neutrophils and fibroblasts, PF4 probably has a role in inflammation and wound repair (contribution to high thrombosis risk)

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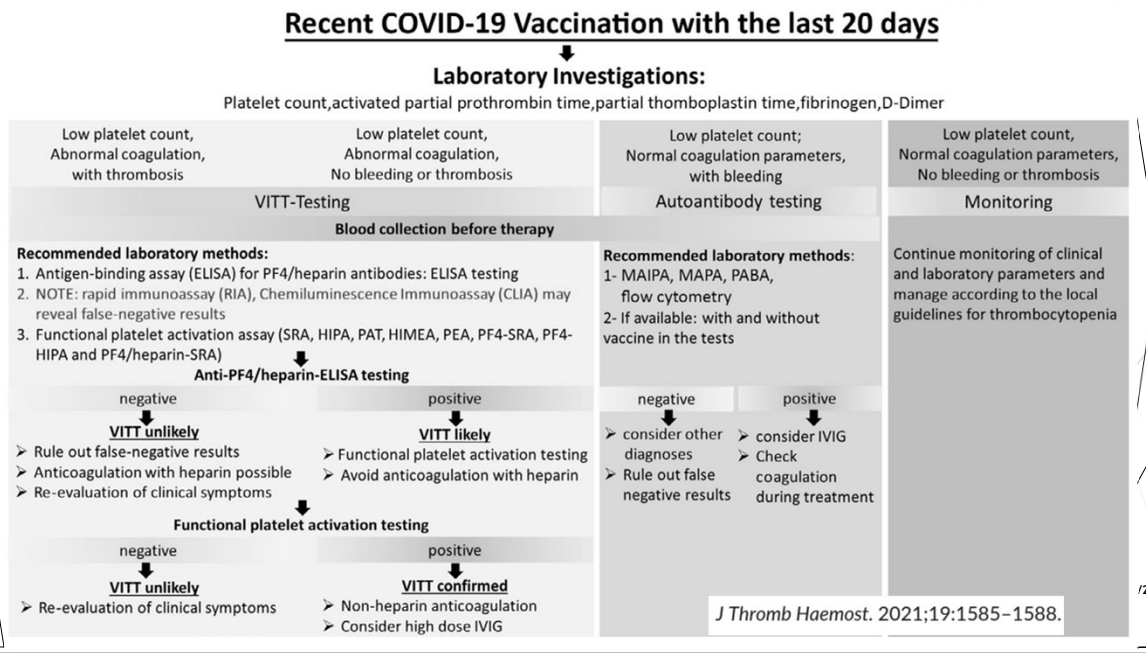
ISTH recommendations for laboratory diagnosis of VITT (2021 Apr.)



Abbreviation for functional platelet activation assays

- SRA : serotonin release assay
- HIPA : heparin-induced platelet aggregation
- PAT : platelet aggregation test
- HIMEA : heparin-induced multiple electrode aggregometry
- PEA : PF4-dependent P selectin expression assay
- PF4-SRA : PF4-serotonin release assay
- PF4-HIPA
- PF4/Heparin-SRA

Platelet-activating antibodies against platelet factor 4

Classic HIT / Autoimmune HIT / VITT

Heparin-dependent vs Heparin-independent

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VITT -UpToDate

Autoimmune heparin-induced thrombocytopenia (HIT) syndromes

Clinical entity	Description
Delayed-onset HIT	HIT that begins or worsens after stopping of heparin
Refractory (also called persistent or persisting) HIT	HIT that persists for >1 week despite stopping of heparin
Spontaneous HIT	HIT that occurs in the absence of proximate (recent) heparin exposure
Heparin flush HIT	HIT that is induced by exposure to heparin flushes
Fondaparinux-associated HIT	HIT that is believed to be triggered by exposure to fondaparinux
Severe HIT (eg, platelet count <20,000/microL) with overt DIC	HIT that is associated with DIC, with one or more of the following: relative/absolute hypofibrinogenemia, elevated INR (without another explanation), normoblastemia (circulating nucleated RBCs)
VITT	Anti-PF4 antibodies that occur in response to certain COVID-19 vaccines and activate platelets in the absence of heparin

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Autoimmune HIT
(Greinacher A., JTH 2017; 15:2099-114)

Table 1 Autoimmune heparin-induced thrombocytopenia (aHIT) syndromes

Clinical entity	Description
Delayed-onset HIT	HIT that begins or worsens after stopping of heparin
Persisting HIT	HIT that persists for > 1 week despite stopping of heparin
Spontaneous HIT syndrome	HIT without proximate heparin exposure
Flush heparin HIT	HIT induced by exposure to heparin flushes
Fondaparinux-associated HIT	HIT that is believed to be triggered by exposure to fondaparinux
Severe HIT (e.g. platelet count of $< 20 \times 10^9\ L^{-1}$) with overt DIC	Overt HIT-associated DIC defined as proven HIT with one or more of the following: relative/absolute hypofibrinogenemia, elevated INR (without another explanation), and normoblastemia (circulating nucleated red blood cells)

DIC, disseminated intravascular coagulation; INR, International Normalized Ratio.

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Autoimmune HIT described by Greinacher A (JTH 2017; 15:2099-114)

- Some HIT patient without heparin exposure in the past (spontaneous HIT syndrome).
- Sera from these patients contain heparin-independent antibodies
  - Not unique but also found in sera of a minority of (heparin-dependent) typical (classical) HIT patients.
  - More likely to have unusual HIT syndromes such as delayed-onset HIT, persisting HIT, fondaparinux-associated HIT, and HIT induced by exposure to heparin 'flushes'.

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Characteristics of anti-PF4 antibodies described by Greinacher A (JTH 2017; 15:2099-114)

- Heparin-dependent
  - Activating platelets more strongly at pharmacologic unfractionated heparin (UFH) concentrations (0.1–0.3 IU/mL) than in the absence of heparin, i.e. 0 IU/mL UFH or 'buffer control'
  - Suprapharmacologic concentrations of heparin (e.g. 10–100 IU/mL) inhibit serum induced platelet activation, an effect caused by disruption of PF4-containing multimolecular complexes at very high heparin concentrations.
- Heparin-independent
  - Platelet activation at buffer control.
  - If only perform platelet activation assays at 0.1–0.5 IU mL1 UFH and at 100 IU mL1 heparin,the phenomenon of heparin-independent platelet activation underlying autoimmune HIT is underrecognized.

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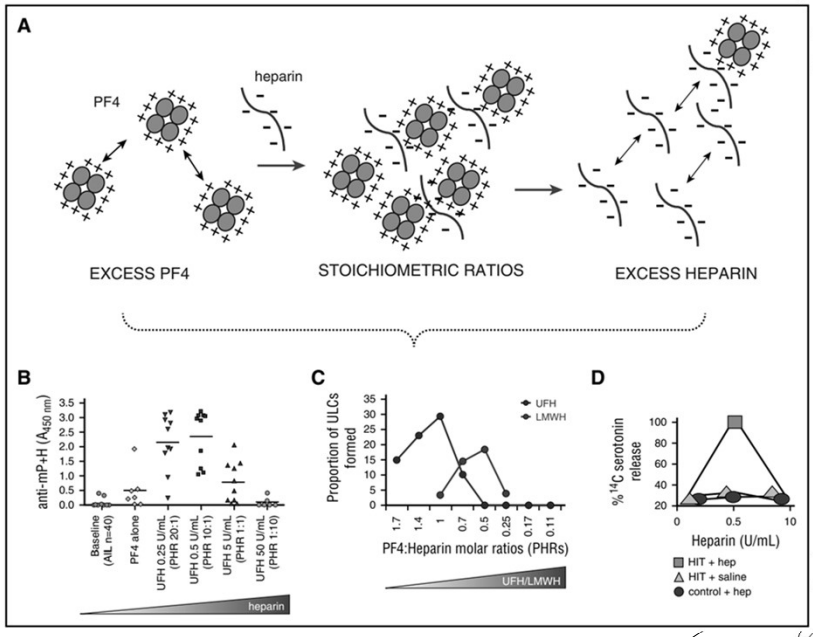
HIT patients



Arepally, G. M. (2017). Heparin-induced thrombocytopenia. Blood, 129(21), 2864–2872

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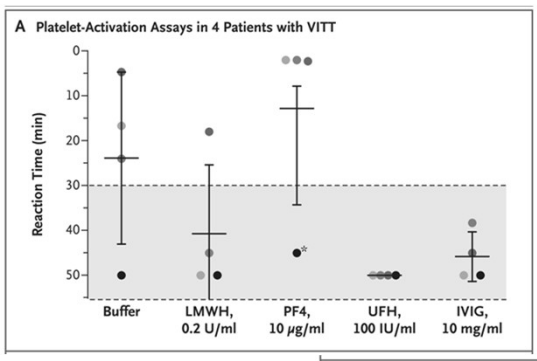
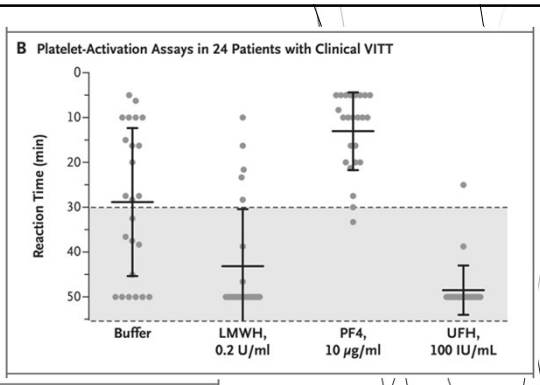
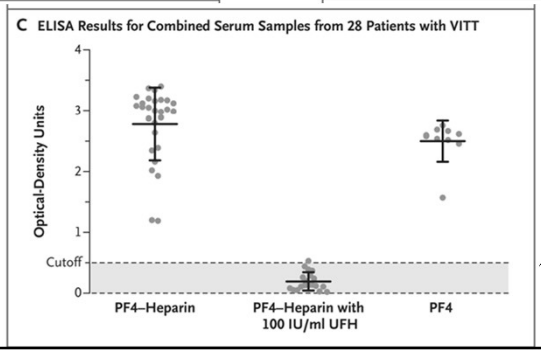






VITT patients

Greinacher A et al (April)

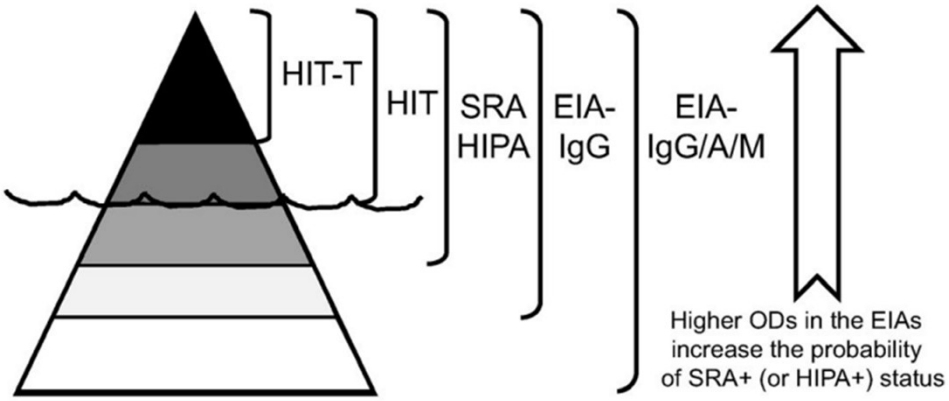
N Engl J Med 2021;384:2092-101.

Greinacher A.
NEJM 2021; 384 (22): 2092-101

CONCLUSIONS

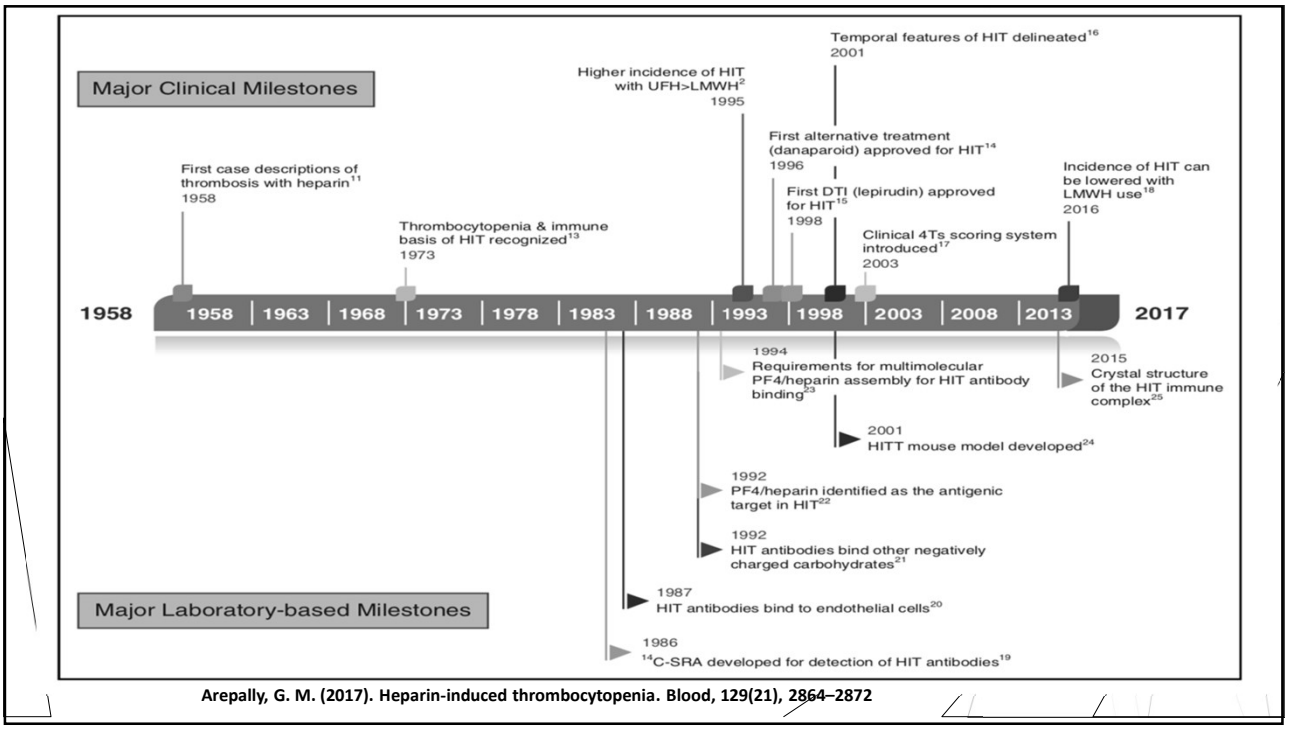
Vaccination with ChAdOx1 nCov-19 can result in the rare development of immune thrombotic thrombocytopenia mediated by platelet-activating antibodies against PF4, which clinically mimics autoimmune heparin-induced thrombocytopenia. (Funded by the German Research Foundation.)

"Iceberg model" of HIT (for VITT ?)



EIA-IgG/A/M result (OD units):	≤0.4	0.4-1.0	1.0-1.5	1.5-2.0	≥2.0
Probability of SRA+ status:	~0%	~5%	~25%	~50%	~90%

Warkentin TE. How I diagnose and manage HIT. Hematology Am Soc Hematol Educ Program. 2011;2011:143-149.



Arepally, G. M. (2017). Heparin-induced thrombocytopenia. Blood, 129(21), 2864–2872

Laboratory diagnosis of HIT

- Immunoassay (measuring the presence of anti-PF4/Heparin antibodies)
  - Enzyme-linked immunosorbent assay (ELISA)
  - Particle gel immunoassay (PGIA)
  - Particle filtration immunoassay (PFIA)
  - Lateral flow immunoassay (LFIA)
  - Latex agglutination (immunoturbidimetric) assay (LAIA)
  - Chemiluminescent immunoassay (CLIA)

High sensitivity (>99%)
Low specificity (30%-70%)

- Functional platelet activation assay (detecting anti-PF4 antibodies capable of binding and cross-linking platelet FcγRIIA)
  - ^{14}C-serotonin release assay
  - Platelet aggregation test
  - Flow-based platelet activation test

High specificity (>95%)
PPV (89%-100%)
Low sensitivity (56%-100%)

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Table 1. Immunoassays for diagnosis of HIT: available classes of assays, antibody specificities, thresholds, test variations, and manufacturers

Classes of assays	Antibody specificity	Threshold	Test variation	Manufacturer (names of tests)
ELISA	Polyspecific	Low*	High heparin dose confirmation step	In-house assays
	IgG specific	Intermediate†		GTI Diagnostics, Waukesha, WI (GTI-PF4; HAT; PF4-Enhanced; GTI-IgG)
		High‡		Hyphen-BioMed, Neuville-Sur-Oise, France (Zymutest HIA IgGAM; Zymutest HIA IgG)
				Diagnostica Stago, Asnières-sur-Seine, France (Asserachrom HIPA)
				Gen-Probe (Gen-Probe PF4)§
				Technoclone GmbH, Vienna, Austria (Technozym)
PaGIA	Polyspecific	Low‖		Diamed, Cressier sur Morat, Switzerland (ID-H/PF4 PaGIA)
		Intermediate¶		
PIFA	Polyspecific	Positive/negative		Akers Biosciences Inc, Thorofare, NJ (HealthTEST)
Lateral flow immunoassay	IgG specific	Positive/negative		Diagnostica Stago (STic EXPERT HIT); Milenia Biotec, Giessen, Germany (Milenia QuickLine HIT)
CLIA	Polyspecific	Low#		Instrumentation Laboratory, Bedford, MA (HemosIL AcuStar HIT-Ab; HemosIL AcuStar HIT-IgG)
	IgG specific	Intermediate**		
		High††		
Latex agglutination assay	Polyspecific	Low‡‡		Instrumentation Laboratory (HemosIL HIT-Ab)

Systematic review & meta-analysis. Blood. 2016;127(5):546-557

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Diagnostic value of immunoassays for heparin-induced thrombocytopenia: a systematic review and meta-analysis

Michael Nagler,[1,2] Lucas M. Bachmann,[3] Hugo ten Cate,[1] and Arina ten Cate-Hoek[1]

[1]Laboratory of Clinical Thrombosis and Haemostasis and Cardiovascular Research Institute, Maastricht University Medical Center, Maastricht, The Netherlands; [2]Division of Haematology and Central Haematology Laboratory, Luzerner Kantonsspital, Lucerne, Switzerland; and [3]Medignition Inc., Zurich, Switzerland

Key Points

- Immunoassays used to diagnose heparin-induced thrombocytopenia vary substantially with regard to the specific test characteristics.
- High sensitivity (>95%) in combination with high specificity (>90%) was found in only 5 tests.

Immunoassays are essential in the workup of patients with suspected heparin-induced thrombocytopenia. However, the diagnostic accuracy is uncertain with regard to different classes of assays, antibody specificities, thresholds, test variations, and manufacturers. We aimed to assess diagnostic accuracy measures of available immunoassays and to explore sources of heterogeneity. We performed comprehensive literature searches and applied strict inclusion criteria. Finally, 49 publications comprising 128 test evaluations in 15 199 patients were included in the analysis. Methodological quality according to the revised tool for quality assessment of diagnostic accuracy studies was moderate. Diagnostic accuracy measures were calculated with the unified model (comprising a bivariate random-effects model and a hierarchical summary receiver operating characteristics model). Important differences were observed between classes of immunoassays, type of antibody specificity, thresholds, application of confirmation step, and manufacturers. Combination of high sensitivity (>95%) and high specificity (>90%) was found in 5 tests only: polyspecific enzyme-linked immunosorbent assay (ELISA) with intermediate threshold (Genetic Testing Institute, Asserachrom), particle gel immunoassay, lateral flow immunoassay, polyspecific chemiluminescent immunoassay (CLIA) with a high threshold, and immunoglobulin G (IgG)-specific CLIA with low threshold. Borderline results (sensitivity, 99.6%; specificity, 89.9%) were observed for IgG-specific Genetic Testing Institute-ELISA with low threshold. Diagnostic accuracy appears to be inadequate in tests with high thresholds (ELISA; IgG-specific CLIA), combination of IgG specificity and intermediate thresholds (ELISA, CLIA), high-dose heparin confirmation step (ELISA), and particle immunofiltration assay. When making treatment decisions, clinicians should be a aware of diagnostic characteristics of the tests used and it is recommended they estimate posttest probabilities according to likelihood ratios as well as pretest probabilities using clinical scoring tools. (*Blood.* 2016;127(5):546-557)

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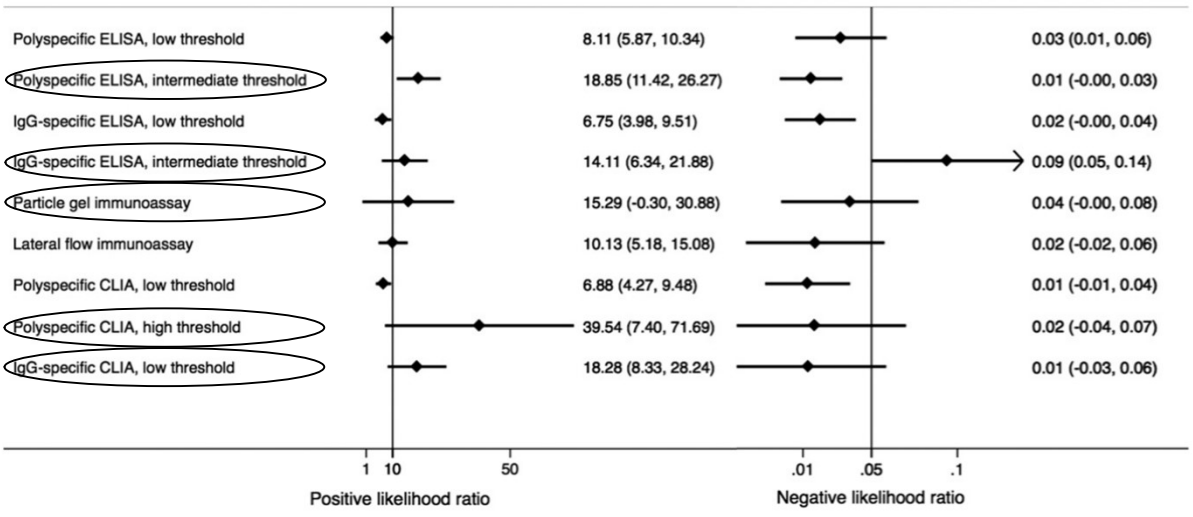

Figure 4. Diagnostic accuracy of different immunoassay classes as characterized by positive and negative likelihood ratios. Likelihood ratios (LR) are powerful measures describing how many times more (or less) likely a test result is in patients with the disease in contrast to patients without the disease.[80] In the context of HIT, a test with a +LR above 10 (corresponding to a specificity of 90%) and a –LR below 0.05 (corresponding to a sensitivity of 95%) is considered favorable.

Systematic review & meta-analysis. Blood. 2016;127(5):546-557

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Immucor Kits to detect anti-PF4 antibodies

Product	Quantity	Catalog #
LIFECODES PF4 IgG (45) Designed to detect heparin-associated PF4 antibodies of the IgG type	45 Tests/Kit	HAT45G
Additional Reagents		Multiple - PF4
LIFECODES PF4 Enhanced (45) Designed to detect IgG, IgA and IgM heparin-associated PF4 antibodies	45 Tests/Kit	X-HAT45

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Performance Evaluation

		Comparative Method		
		Positive	Negative	Total
PF4 ENHANCED®	Positive	144	51*	195
	Negative	2	452	454
	Total	146	503	649

Agreement: 91.8%

Co-positivity: 98.6% Co-negativity: 89.9%

Comparative Method: Serotonin Release Assay

* Blood 1997 (suppl 1); 90: 460a

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Evaluation of laboratory assays for anti-Platelet Factor 4 antibodies in UK patients suspected of VITT (Platton S et al, JTH 2021 (May); 19(8): 2007-13)

- **IgG-specific ELISAs**
  - Asserachrome HPIA IgG (Stago)
  - Lifecodes PF4 IgG (Immucor)
  - Hyphen Biomed Zymutest HIA IgG (Quadratech Diagnostics)
  - Aeskulisa HiT II (Aesku)
- **Polyspecific ELISAs (IgG/IgA/IgM)**
  - Asserachrome HPIA (Stago)
  - Lifecodes PF4 Enhanced (Immucor)
- **Rapid Assays**
  - Polyspecific Diamed PAGIA gel (BioRad Laboratories)
  - IgG-specific Stic expert lateral flow device (Stago, LFIA)
  - IgG-specific HemosIL AcuStar HIT-IgG $_{(PF4-H)}$ (Werfen, CLIA)
  - IgG-specific HemosIL HIT Ab $_{(PF4-H)}$ (Werfen, LAIA)

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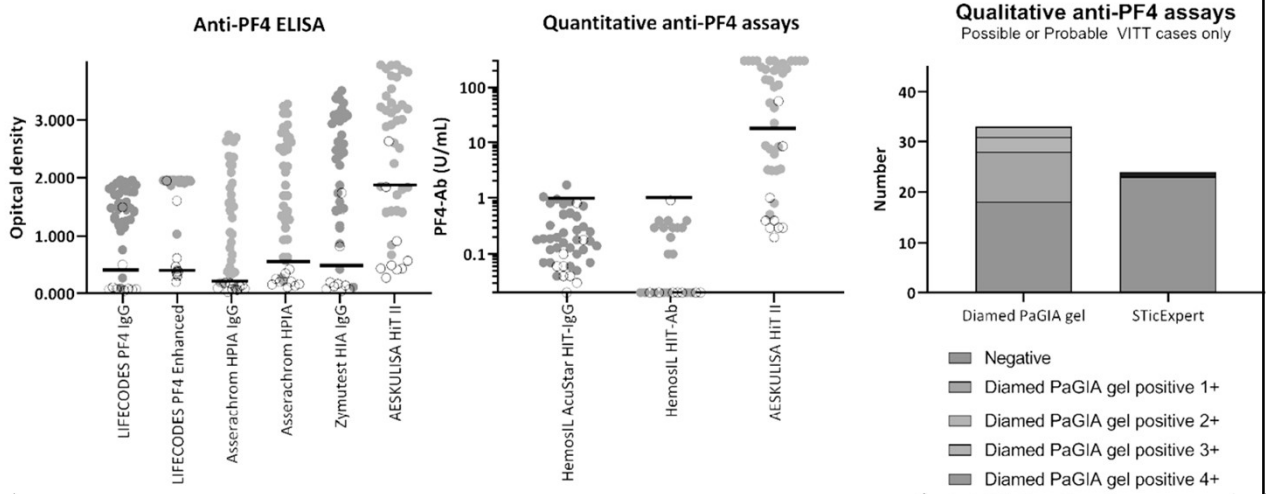
Platton S et al, JTH 2021; 19(8): 2007-13



Solid circles: VITT possible (n=23) or probable (n=11)
Empty circles: VITT unlikely (n=9)
Solid black line: assay-specific cutoff for assay

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Platton S et al, JTH 2021; 19(8): 2007-13

TABLE 2 Sensitivity and specificity of assays for possible and probable VITT and for HIT

Assay	Sensitivity for VITT % (95% CI)	Specificity for VITT % (95% CI)	Sensitivity for HIT % (95% CI)	Specificity for HIT % (95% CI)
IgG-specific ELISAs				
AEKSULISA HiT II	70.6 (53.8–83.2)	88.9 (56.5–99.4)	91[a]	97[a]
Asserachrom HPIA IgG	91.1 (77.0–97.0)	100.0 (70.1–100.0)	72.0 (68.4–75.5)[7]	93.8 (90.3–97.4)[7]
Lifecodes PF4 IgG	94.1 (80.9–99.0)	77.8 (45.3–96.1)	99.6 (22.7–100.0)[7]	89.9 (86.2–92.6)[7]
Zymutest HIA IgG	94.1 (80.9–99.0)	77.8 (45.3–96.1)	99.2 (86.4–100.0)[7]	85.8 (77.1–91.5)[7]
Polyspecific ELISAs				
Asserachrom HPIA	94.1 (80.9–99.0)	100.0 (70.1–100.0)	92.7 (73.6–98.3)[7]	87.3 (79.9–92.3)[7]
Lifecodes PF4 Enhanced	100.0 (89.9–100.0)	55.6 (26.7–81.1)	99.9 (90.9–100.0)[7]	87.4 (79.2–92.7)[7]
Rapid tests				
Diamed PaGIA gel	45.5 (29.8–62.0)	66.7 (35.4–87.9)	96.5 (89.8–98.9)[7]	93.7 (83.1–97.8)[7]
HemosIL AcuStar HIT-$IgG_{(PF4-H)}$	5.9 (1.0–19.1)	100.0 (70.1–100.0)	98.8 (69.2–100.0)[7]	94.6 (90.7–96.9)[7]
HemosIL HIT-$Ab_{(PF4-H)}$	0.0 (0.0–17.6)	100.0 (67.6–100.0)	100.0[7]	84.3[7]
STic expert	4.2 (0.2–20.2)	100.0 (17.8–100.0)	98.4 (85.3–99.9)[7]	90.3 (84.4–94.1)[7]

Abbreviations: 95% CI, 95% confidence interval; ELISA, enzyme-linked immunosorbent assay; HIT, heparin-induced thrombocytopenia; IG, immunoglobulin; VITT, vaccine-induced immune thrombocytopenia and thrombosis.

aManufacturer's data.

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Summary

- None of the rapid assays tested, which may be suitable for the exclusion of HIT, is suitable for the exclusion of VITT.
- No single ELISA method appears to detect all cases of VITT
  - If a single ELISA test is negative, a second ELISA or platelet activation assay should be considered where there is strong clinical suspicion.

Platton S et al, JTH 2021; 19(8): 2007-13

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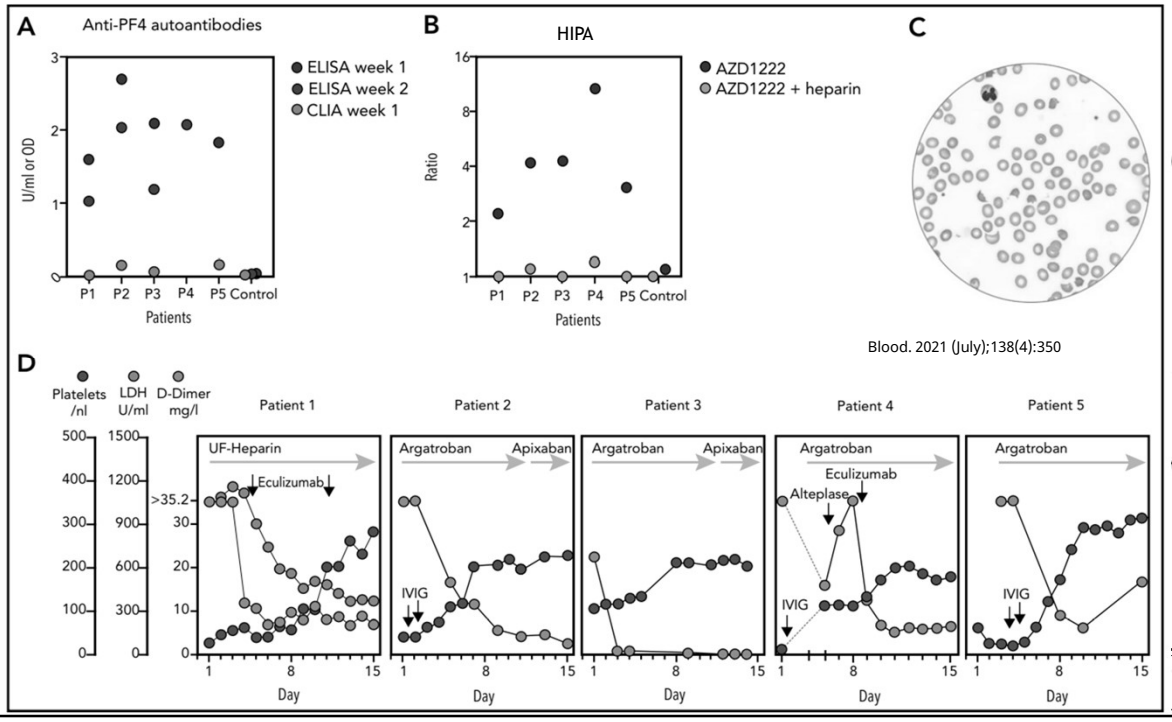

Blood. 2021 (July);138(4):350

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Characteristics of VITT antibodies (UpToDate)

- IgG class
- Recognize PF4 bound to platelets; the epitope on PF4 differs from the epitope recognized by heparin-induced thrombocytopenia (HIT) antibodies
- Detectable in PF4/polyanion and PF4 enzyme-linked immunosorbent assay (ELISA) and in functional assays.
- Cause platelet activation via low affinity platelet Fcγlla receptors
- Not heparin dependent (not induced by heparin exposure; do not require heparin for detection in in vitro platelet activation assays)

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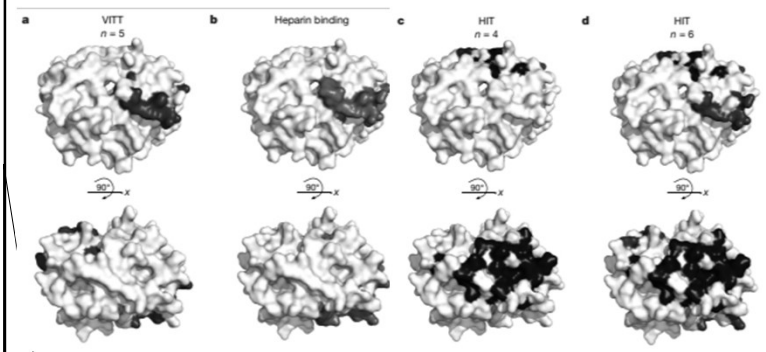
Antibody epitopes in vaccine-induced immune thrombotic thrombocytopenia
Nature 2021 (July);596:565-69



e

HIT with HD abs: EAEEDGD[redacted]VKTTSQVRPRHITSLEVIKAGPHCPT[redacted]LIATLKNGRK[redacted]APL[redacted]KIIKKLLES

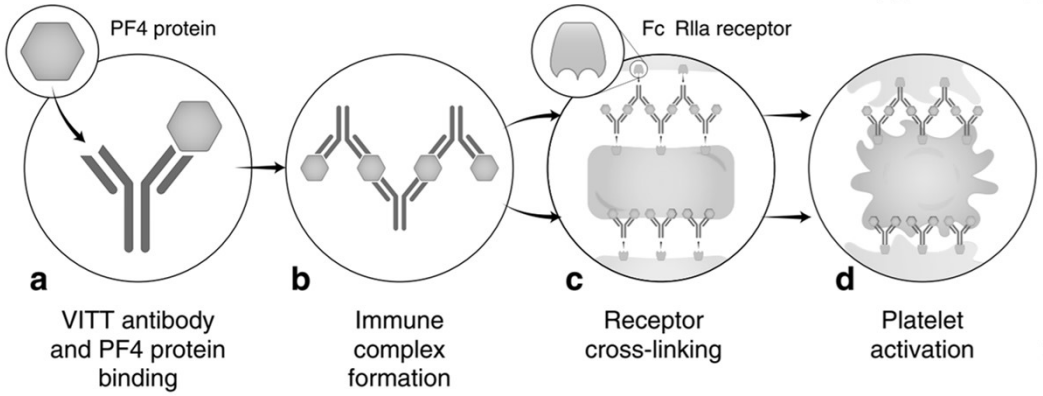
HIT with HI abs: EAEEDGD[redacted]VKT[redacted]SQVRP[redacted]TSLEVIKAGPHCPTA[redacted]LIATLK[redacted]GRK[redacted]LQAPLY[redacted]KIIK[redacted]LLES

VITT: EAEEDGDLQCLCVKTTSQVRP[redacted]TSL[redacted]VIKAGPHCPTAQLIATL[redacted]GR[redacted]CLDLQAPLYK[redacted]IIK[redacted]LLES

Fig. 1 | Identification of amino acids in PF4 that are critical for the binding of antibodies using alanine-scanning mutagenesis. a, **b**, For VITT samples ($n = 5$), the binding site (coloured in red; **a**) aligns within the heparin-binding site on PF4 (coloured in brown; **b**). **c**, For HIT samples ($n = 10$), one main binding site was identified for all samples (coloured in blue). **d**, An additional binding site was found in 6 out of 10 (60%) of the HIT samples (coloured in red), which aligns within the heparin-binding site on PF4. **e**, The amino acids that are predicted to be part of the binding sites for VITT and heparin-dependent (HD) and heparin-independent (HI) antibodies (abs) in HIT are highlighted on the primary sequence of PF4. Images are modified from the Protein Data Bank (PDB) entry 1RHP.

Antibody epitopes in vaccine-induced immune thrombotic thrombocytopenia
Nature 2021 (July);596:565-69

- VITT antibodies bind to platelets via an eight amino acid region of PF4 on the platelet surface, located within the heparin binding site.
- VITT antibody binding is blocked by heparin.
- The amino acids bound by VITT antibodies overlap with but differ from the amino acids bound by HIT antibodies, and VITT antibody binding to platelets is stronger than HIT antibody binding.



Extended Data Fig. 5 | Proposed mechanism for VITT antibodies binding to and clustering PF4 tetramers, independent of heparin, and forming platelet-activating immune complexes. a, **b**, We postulate that VITT antibodies bind the antigen, PF4 (**a**), which in turn can cluster PF4 tetramers and create platelet-activating immune complexes in the absence of heparin (**b**). **c**, **d**, The immune complexes can be found on the platelet surface and in solution, resulting in the aligning and close proximity of the Fc part of these antibodies, which then are able to cross-link FcγRIIa receptors (**c**) and lead to platelet activation (**d**).

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三、檢驗確認： 疾管署/血液病學會: VITT臨床指引 (2010.06.09)

1. 血液凝固檢驗：PT, aPTT, d-dimer（常見嚴重上升）、fibrinogen（常見嚴重下降）。
2. Anti-Platelet Factor 4/heparin ELISA 檢驗。
3. 血小板活化試驗。

TTS(VITT)診斷標準：影像確認之血栓＋血小板低下＋anti-platelet factor 4/heparin 抗體強陽性。
理想上，同時以血小板活化試驗佐證，病患血漿中抗體具活化血小板能力。
臨床上，若無 2.3 檢查，d-dimer 高出正常值上限四倍以上，可考慮當作 TTS（VITT）治療。

國內目前無醫療院所常規執行 anti-PF4/heparin 檢查，而血小板活化試驗則無商業化試劑，亦鮮少有醫療或研究單位能夠執行。可依 ISTH 建議，若 d-dimer 數值升高超過正常值上限四倍，即可依臨床狀況決定當作 TTS（VITT）治療。但務必在使用抗凝藥物治療及免疫球蛋白之前，留下以檸檬酸鈉（sodium citrate）抗凝離心後之-80 度冷凍血漿檢體，以供後續回溯執行確認性檢查。Anti-PF4/heparin ELISA 屬於篩檢性，雖然 TTS（VITT）病患報告皆有此抗體，但此檢驗仍有為數不少的偽陽性或偽陰性問題，不應以此結果當作 TTS（VITT）診斷的唯一標準。**唯因應 TTS 個案通報至疾病管制署「疫苗不良事件通報系統 (VAERS)」後，此檢測可輔助臨床診斷與疫苗安全性訊號偵測，疾管署已委請相關研究單位協助檢驗（請完成附件送驗單填寫，並上傳至 VAERS 系統）。**

最後，要強調的是，TTS (VITT)的診斷仍存在一定的臆測性與不確定性，其他原因引起的血栓或血小板低下，仍然必須加以排除，臨床整體評估仍為必要。

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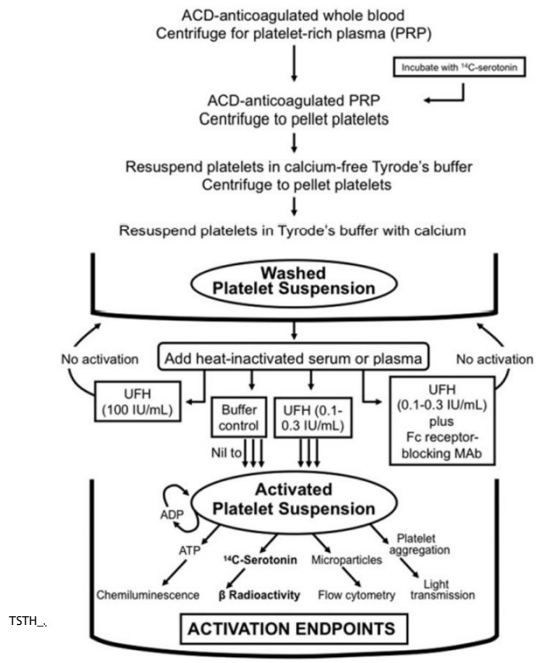
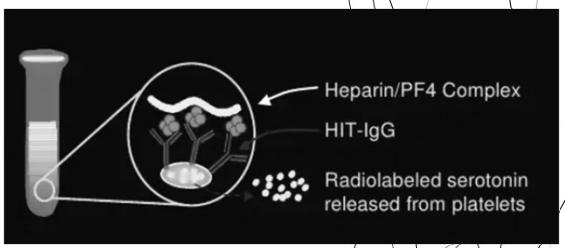
Functional platelet activation assays (ISTH recommendation, 2021/Apr)

- ^{14}C-Serotonin release assay (SRA) - Sheridan D et al, Blood 1986;67:27-30
- Heparin-induced platelet activation assay - Greinacher A et al, Thromb Haemost. 1991;66:734-736
- Platelet aggregation test - Fratantoni JC et al, Blood 1975;45:395-401
- Heparin induced multiple electrode aggregometry - Galea V et al Platelets 2013;24:441-447
- PF4-dependant P selectin expression assay - Padmanabhan A et al, Chest. 2016;150:506-515
- PF4-SRA - Nazi I et al, Thromb Haemost. 2015;13:1900-1907
- PF4/heparin-SRA – Vayne C et al, Br J Haematol 2017;179:811-819





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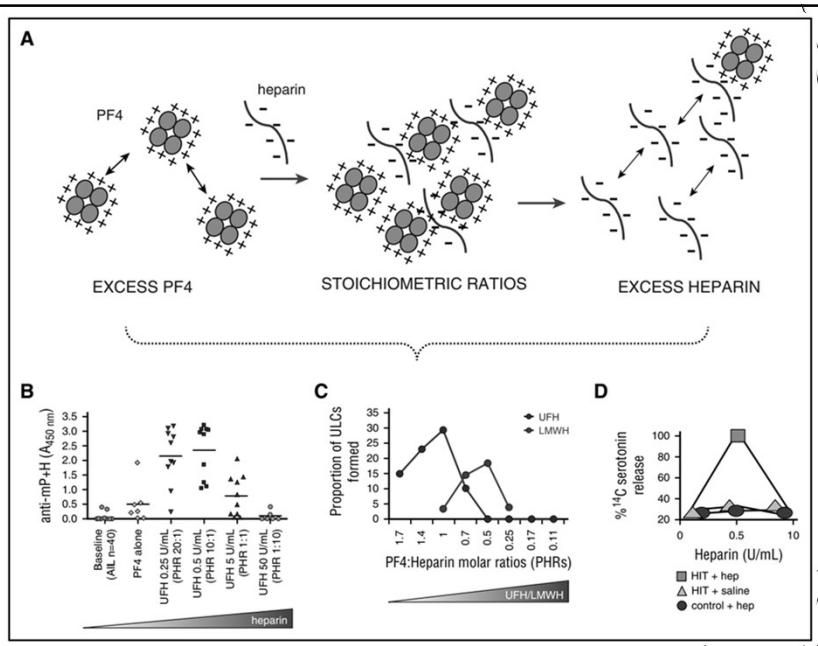

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HITAlert (Diapharma): A functional HIT assay – flow cytometry

HITAlert™ Test Principle:

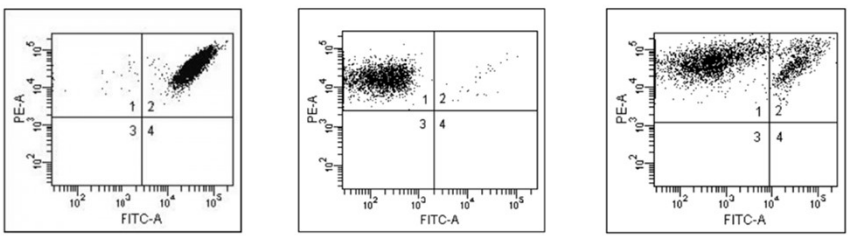
Donor platelets (PRP) + test sample + heparin = platelet activation?

Samples

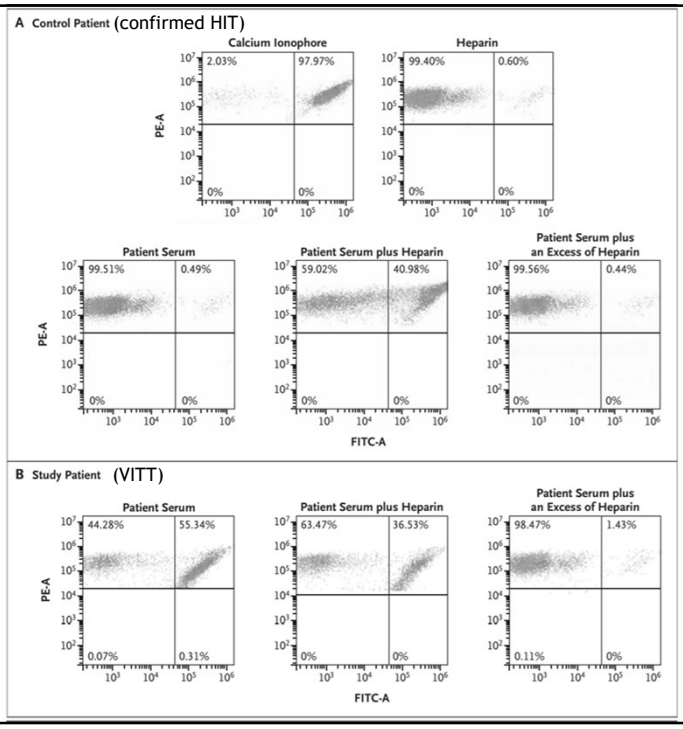
(1) PRP + heparin (blank)
(2) Positive control (chemical)
(3) PRP + test sample
(4) PRP + test sample + heparin
(5) PRP + test sample + high dose heparin

- Functional assessment of platelet activation where reactivity is independent of PF4
- Complete kit with ready-to-use reagents & controls
- Non-radioactive approach utilizing standard flow cytometry
- Rapid results (<2 hours)
- Specifically detects heparin-complexed antibodies, includingIL-8/heparin & NAP-2/heparin complexed antibodies

Anti-CD41



Anti-Annexin-V



N Engl J Med 2021;384:2202-11

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Thank you for your attention !