Has it already been 3 years? NETs in APS and COVID and back again

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@jasonsknight

1

Disclosures

- None relevant to this talk

2

Outline

- Brief background including some history of APS
- Let's talk about neutrophils and NETs!
- APS pathogenesis—and what it can potentially teach us about COVID-19
- For fun: some ginger with that?

3

4

Antiphospholipid syndrome (APS)

1999 → **2006** → 2023?

Journal of Thrombosis and Haemostasis, 4: 295-306

30-40% of cases linked to lupus

Clinical criteria 😔

1. **Thrombosis:** arterial / venous / microvascular, *or*
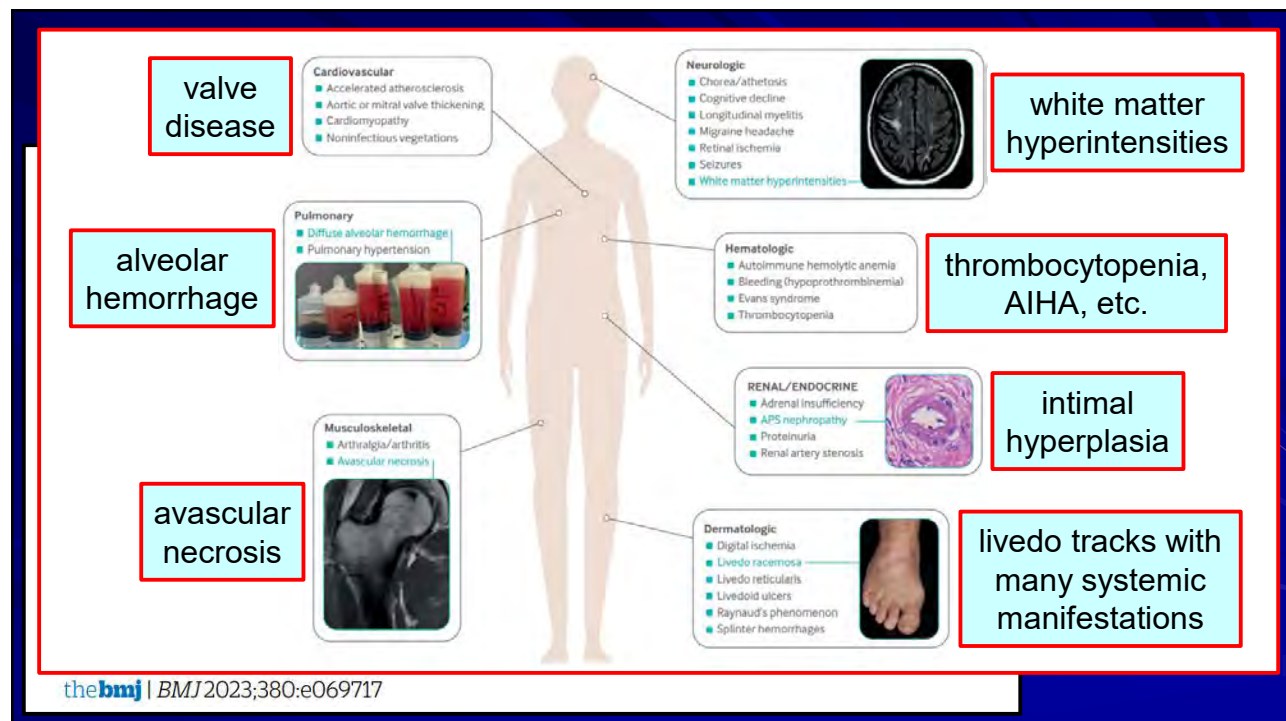2. **Obstetric morbidity:** especially after the first trimester

AND

Laboratory criteria

1. **Lupus anticoagulant:** by functional assay, *or*
2. **Anticardiolipin antibodies:** IgG or IgM, *or*
3. **Anti-beta-2 glycoprotein-I antibodies:** IgG or IgM

5

valve disease

Cardiovascular
- Accelerated atherosclerosis
- Aortic or mitral valve thickening
- Cardiomyopathy
- Noninfectious vegetations

Neurologic
- Chorea/athetosis
- Cognitive decline
- Longitudinal myelitis
- Migraine headache
- Retinal ischemia
- Seizures
- White matter hyperintensities

white matter hyperintensities

Pulmonary
- Diffuse alveolar hemorrhage
- Pulmonary hypertension

alveolar hemorrhage

Hematologic
- Autoimmune hemolytic anemia
- Bleeding (hypoprothrombinemia)
- Evans syndrome
- Thrombocytopenia

thrombocytopenia, AIHA, etc.

RENAL/ENDOCRINE
- Adrenal insufficiency
- APS nephropathy
- Proteinuria
- Renal artery stenosis

intimal hyperplasia

Musculoskeletal
- Arthralgia/arthritis
- Avascular necrosis

avascular necrosis

Dermatologic
- Digital ischemia
- Livedo racemosa
- Livedo reticularis
- Livedoid ulcers
- Raynaud's phenomenon
- Splinter hemorrhages

livedo tracks with many systemic manifestations

the**bmj** | *BMJ* 2023;380:e069717

6

Antiphospholipid syndrome (APS)

1999 → **2006** → 2023?

Journal of Thrombosis and Haemostasis, 4: 295–306

Clinical criteria

1. **Blood clots:** arterial / venous / microvascular, ***or***

2. **Pregnancy loss:** especially after the first trimester

AND

Laboratory criteria 😬

1. **Lupus anticoagulant:** by functional assay, ***or***

2. **Cardiolipin antibodies:** IgG or IgM, ***or***

3. **Beta-2 glycoprotein-I antibodies:** IgG or IgM

7

# The best stories start or end with syphilis 😐

Proc Soc Exper Biol Med 1941

A New Serologically Active Phospholipid from Beef Heart.

MARY C. PANGBORN. (Introduced by Augustus B. Wadsworth.)

Alcoholic extracts of beef heart have long been used as antigens in the complement-fixation test for syphilis, but the substances responsible for the serologic activity of such extracts have not been identified. The isolation of a new phospholipid is here reported and evidence is presented that the new substance is the active component of the antigenic extracts.
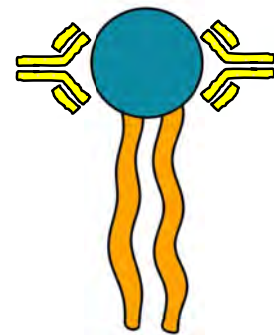
Cardiolipin: a new phospholipid isolated from beef heart

Syphilis patients develop antibodies to cardiolipin

8

False-positive syphilis test

TABLE 1.—*Approximate Incidence of Biologically False Positive Reactions in Various Nonsyphilitic Conditions*

BIOLOGICA

Infections

Disease	Approximate Incidence of BFP* Reactions, %	Disease	Approximate Incidence of BFP Reactions, %
Bacterial		**Rickettsial**	
Leprosy	60	Typhus	20
Tuberculosis, advanced	3–5	**Protozoal**	
Pneumonia, pneumococcal	2–5	Trypanosomiasis	10
Subacute bacterial endocarditis	5	**Viral**	
Chancroid	5	Vaccinia	20
Scarlatina	5	Pneumonia, "atypical"	20
Spirochetal		Measles	5
Leptospirosis	10	Chickenpox	5
Relapsing fever	20	Lymphogranuloma venereum	20
Rat-bite fever	20		

So, many lupus patients have antibodies to cardiolipin too

Noninfectious Diseases or Conditions	Approximate Incidence of BFP Reactions
Lupus erythematosus (disseminated or discoid)	20
Rheumatoid arthritis	5
Blood loss, repeated (as in multiple donations for transfusion)	? low
Pregnancy	? low

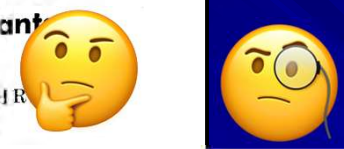* BFP = biologically false positive.

9

Parallel story: circulating "anticoagulants" in lupus

J Lab Clin Med (1963) 62:416

Thrombosis in systemic lupus

Blood seems too "thin" in the test tube…

…but too "thick" in the person

It turns out that laboratory clotting tests contain lots of phospholipids…

…as an artificial cell surface where the clotting process can begin

Antiphospholipid antibodies → artifactually prolong clotting times

Eventually standardized to create the "lupus anticoagulant" test

10

Hughes and colleagues

TABLE I—*Serological and clinical findings in patients with and without lupus anticoagulant*

	Patients with lupus anticoagulant (n = 31)	Patients without lupus anticoagulant (n = 29)	p value
Serological findings:			
Biological false positive reaction for syphilis	7	0	<0·05
DNA binding (>30%)	11	16	NS
Extractable nuclear antigens (ribonucleoprotein, Sm, Ro, La)	13	16	NS
Clinical findings:			
Thrombosis	18	3	<0·01
Previous abortions*	9	5	NS
Thrombocytopenia	9	1	<0·02
Disease of central nervous system	17	10	NS

NS = Not significant.
* 26 women in group with anticoagulant, 27 in group without.

BRITISH MEDICAL J

Thromb
associati
anticoag

M L BOEY, C

11

By the mid 1980s, we knew that…

■ The *lupus anticoagulant* phenotype associated with thrombosis and pregnancy loss.

■ And, patients with positive lupus anticoagulant were also likely to have *anticardiolipin* antibodies.

■ However… purified *"antiphospholipid"* antibodies often failed to bind phospholipids in the absence of serum!

■ Were we missing protein cofactors in serum?

12

Cofactor #1 = beta-2 glycoprotein I (β_2GPI)

Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 4120–4124, June 1990
Medical Sciences

Anti-phospholipid antibodies are ...
that includes a lipid-binding inh...
β_2-Glycoprotein I (apolipoprotei...

(anti-cardiolipin antibodies/lipid–protein complex/thrombosis...

H. Patrick McNeil*, Richard J. Simpson†, Coli...

*University of New South Wales, School of Medicine, Saint George Hospi...
Institute for Cancer Research and The Walter and Eliza Hall Institute for M...

Communicated by K. Frank Austen, March 15, 1990

β_2GPI (in blood)

negatively-charged phospholipids
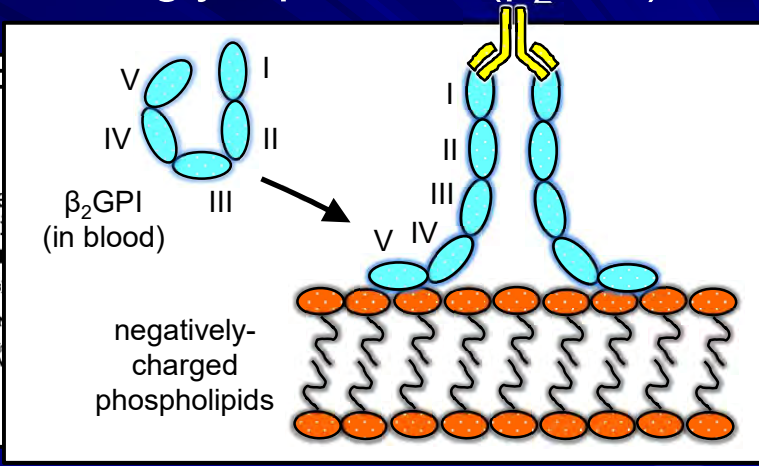
- **Oh wow—so it turns out that many relevant *"antiphospholipid"* antibodies actually bind β_2GPI**

13

Cofactor #2 = prothrombin

prothrombin bound to a phosphatidylserine-coated plate (PS/PT)

IgG aPS/PT

IgG aPT

prothrombin alone

IgG aPS/PT or aPT (U)

dRVVT-LA (+) 24/49 (49%)

dRVVT-LA (−) 5/204 (3%)

dRVVT-LA (+) 15/48 (31%)

dRVVT-LA (−) 22/192 (12%)
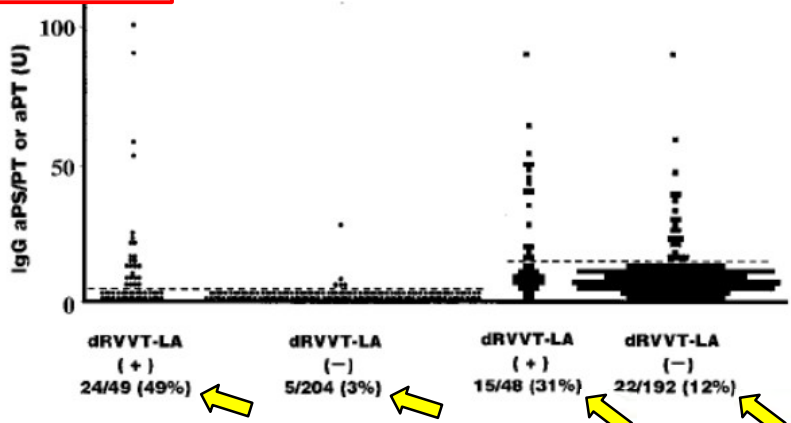
14

...

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Why research APS?

- Stroke and pulmonary embolism and post-thrombotic syndrome and stillbirth are major causes of morbidity in the general population. As the *prototypical thrombo-inflammatory disease*, APS may teach us a lot.

- We hear a lot about personalized medicine for cancer and diabetes and lupus—APS needs this too!

- It's more common than you think (1 in 2000) 🇺🇸 🇬🇧

- But how common are antiphospholipid antibodies?

15

Dallas Heart Study + antiphospholipid antibodies

Among the 2427 participants in DHS2 (blood samples collected between 2007 and 2009) included in this study, 1399 (57.6%) were female, 1244 (51.3%) were Black, 339 (14.0%) were Hispanic, and 796 (32.8%) were White; the mean (SD) age at the time of sampling was 50.6 (10.3) years.

aPL	No. positive (%) Manufacturer's threshold[a]	Titer ≥40 units
aCL IgG[b]	26 (1.0)	7 (0.3)
aCL IgM[b]	156 (6.4)	36 (1.5)
aCL IgA	11 (0.5)	6 (0.3)
aβ2GPI IgG[b]	21 (0.9)	10 (0.4)
aβ2GPI IgM[b]	63 (2.6)	26 (1.0)
aβ2GPI IgA	62 (2.5)	29 (1.2)
aPS/PT IgG	18 (0.7)	11 (0.5)
aPS/PT IgM	88 (3.4)	48 (2.0)
Any positive	353 (14.5)	153 (6.3)
Three positive aPL	17 (0.7)	2 (0.08)

More likely to find IgM and IgA antibodies than IgG

JAMA Network Open. 2023;6(4):e236530. doi:10.1001/jamanetworkopen.2023.6530

16

4/21/2023

8

Dallas Heart Study + antiphospholipid antibodies

Figure 1. Association of aPL Subtypes With Future Atherosclerotic Cardiovascular Disease (ASCVD) Events

A | Positivity based on manufacturer's threshold

aPL subtype	HR (95% CI)
aCL IgG	0.83 (0.12-5.95)
aCL IgM	0.68 (0.25-1.86)
aCL IgA	4.92 (1.52-15.98)
aβ2GPI IgG	NA
aβ2GPI IgM	1.36 (0.50-3.71)
aβ2GPI IgA	2.91 (1.32-6.41)
aPS/PT IgG	NA
aPS/PT IgM	0.95 (0.40-2.36)
Any aPL	1.28 (0.79-2.09)

Favors no future ASCVD event — Favors future ASCVD event

HR (95% CI): 0.01, 0.1, 1, 10

Compositive of stroke, MI, coronary revascularization, and cardiovascular-related death

17

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18

Neutrophils

- A type of white blood cell that is already fully armed when released into the bloodstream

- First responders against many types of infection

- The human body produces 2×10^{11} neutrophils every day *(I'm contractually obligated to tell you this)*

- Potentially problematic in the circulation and/or tissues *(i.e., the double-edged sword)*

19

Neutrophils do <u>not</u>…

- …proliferate in the periphery

- …have true subsets defined by different transcription factors; therefore, functional differences are likely mediated by stage of maturation or activation

- …need oxygen for energy production (although they do need for fullest antimicrobial functions)

- …make things easy on researchers (rapid apoptosis *in vitro*, low RNA content, mice=imperfect models)

20

Neutrophil extracellular traps (NETs)

Neutrophil Extracellular Traps Kill Bacteria
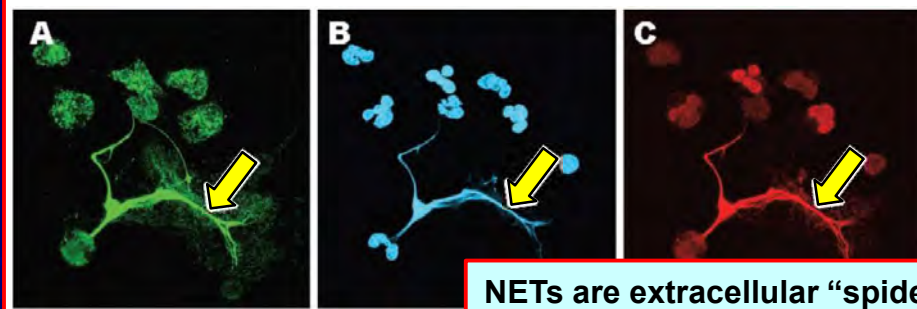
NETs are extracellular "spider webs" made of...
A, Antimicrobial proteins (kill bacteria)
B, DNA itself (the backbone of NETs)
C, Other proteins from the nucleus (e.g., histones)

Science 2004

21

Activation

Mixing of chromatin and effector proteins

Nuclear envelope rupture

Plasma membrane rupture

Decondensation

Proc Natl Acad Sci USA. 2020;117(13):7326-7337
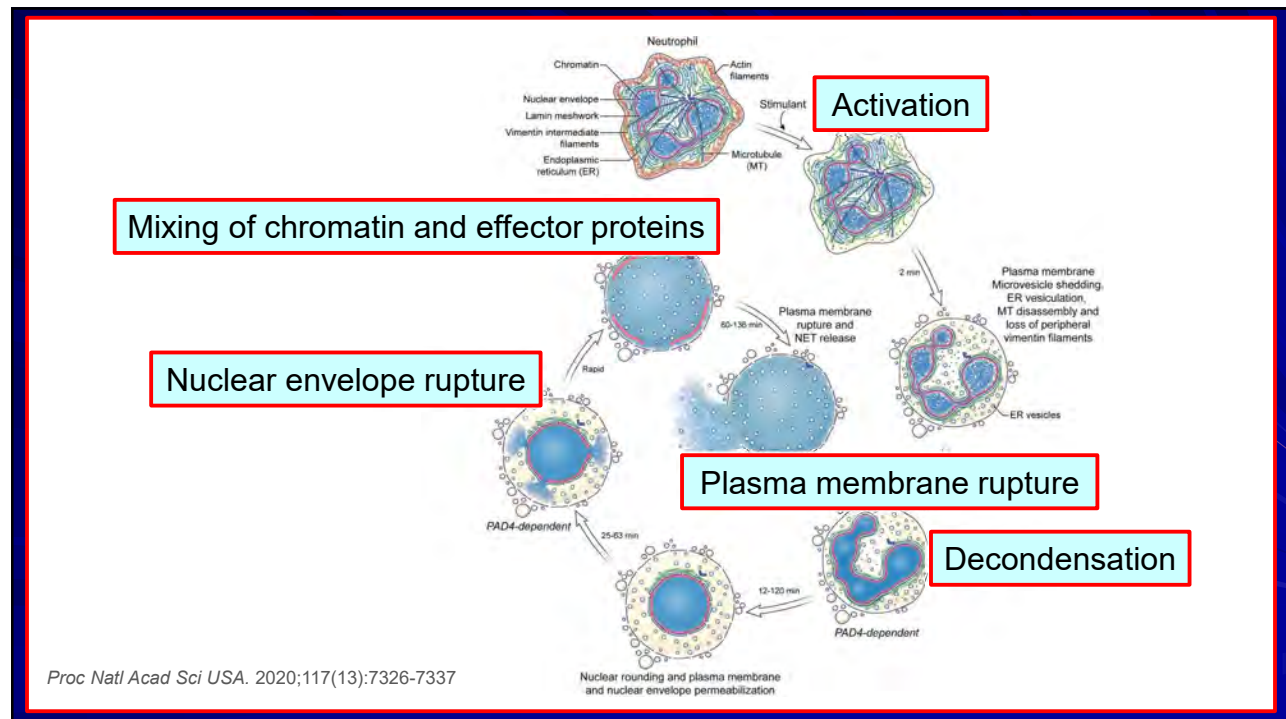
22

With great power comes great responsibility

23

Nat Rev Immunol. 2018;18(2):134-147

Nature Reviews | Immunology

24

NET remnants in APS patient blood

anti-β_2GPI are good at triggering NETosis

spontaneous NETosis

neutrophil

NETosis

β_2GPI

aPL

control neutrophils exposed to APS patient sera
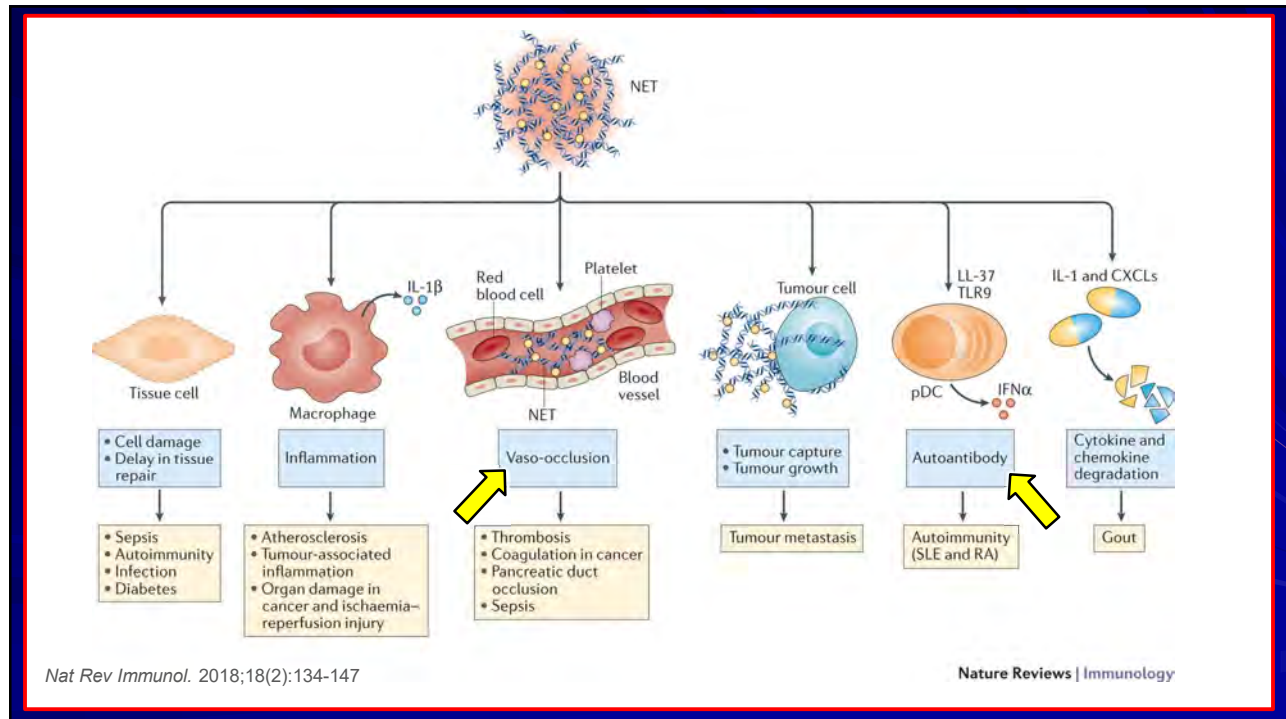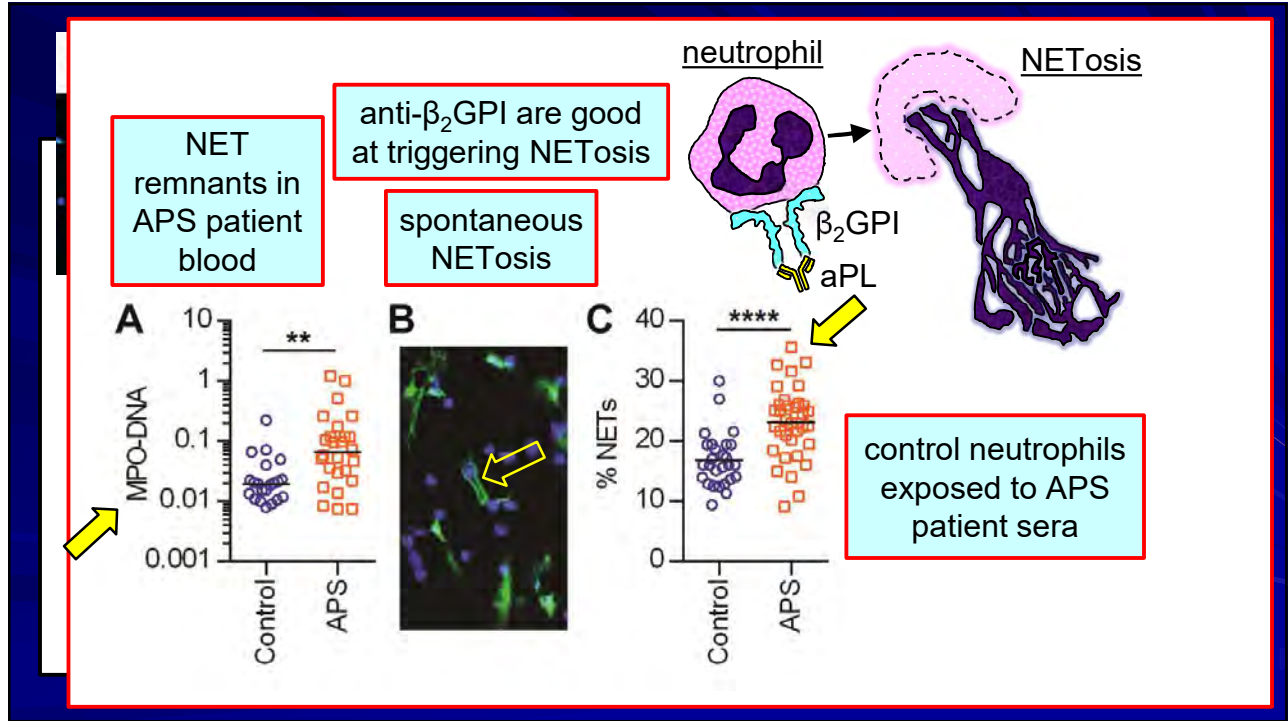
25

APS IgG (human) accelerates venous thrombosis

Which can be blocked by neutrophil depletion

And by DNase, which dissolves NETs

And by disrupting adhesion to the endothelium (PSGL-1, Mac-1)

And by boosting intracellular cAMP (dipyridamole, defibrotide, ginger)

DNA   NETs

APS thrombus

26

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- For fun: some ginger with that?

27

More aspects of APS pathogenesis
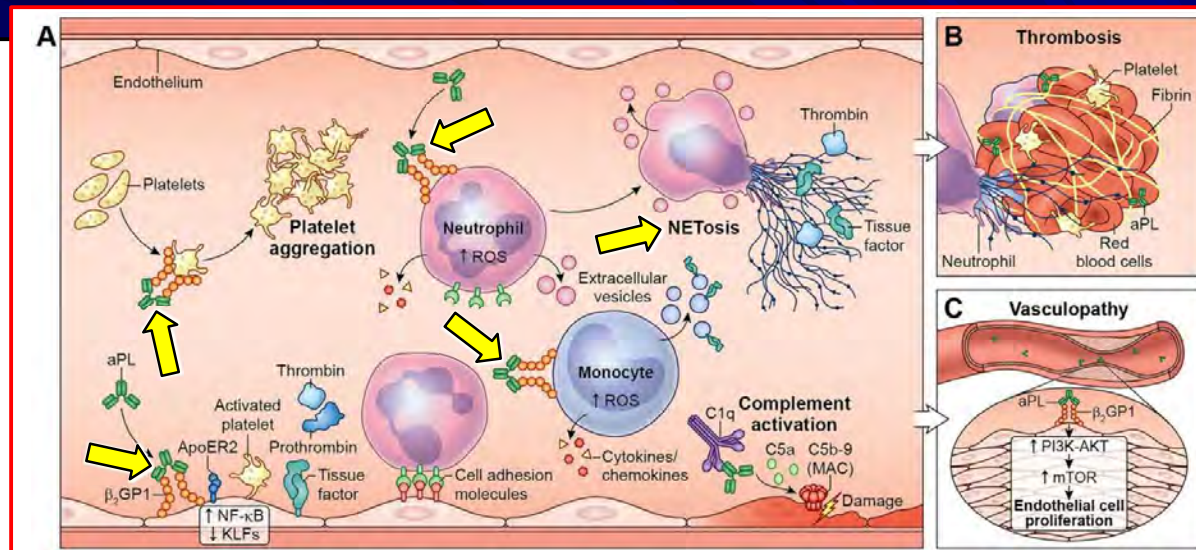
Seminars in Immunopathology (2022) 44:347–362

28

Could we "cure" APS by eliminating aPL?
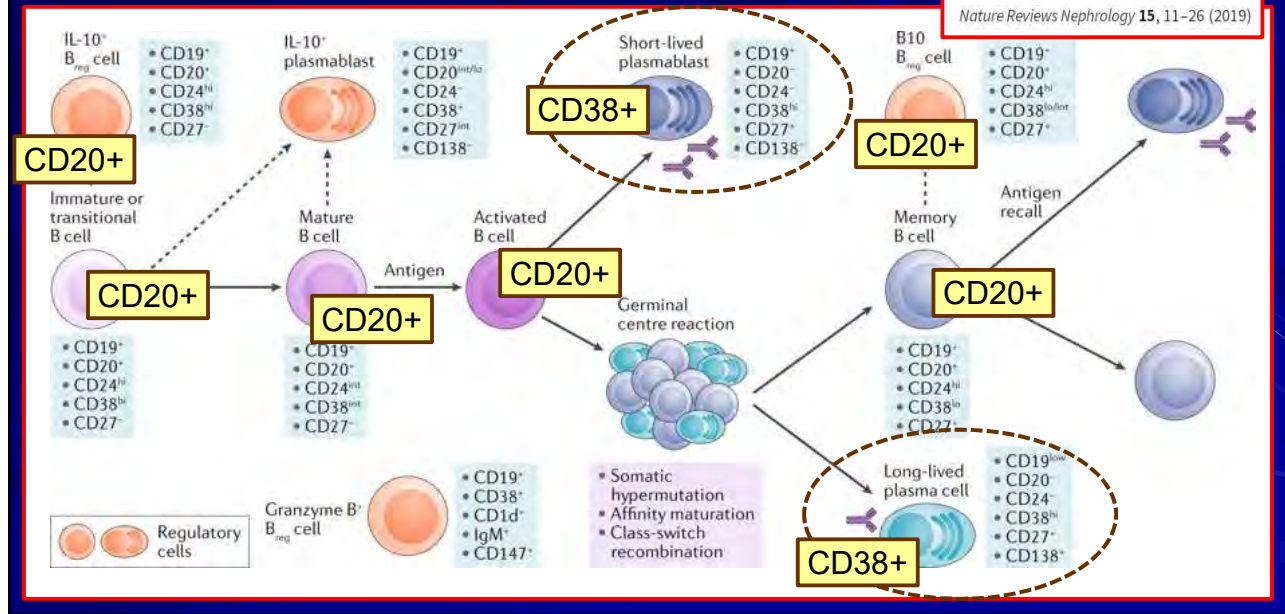
Nature Reviews Nephrology **15**, 11–26 (2019)

29

Anti-CD38 for plasma cell depletion in APS?

30
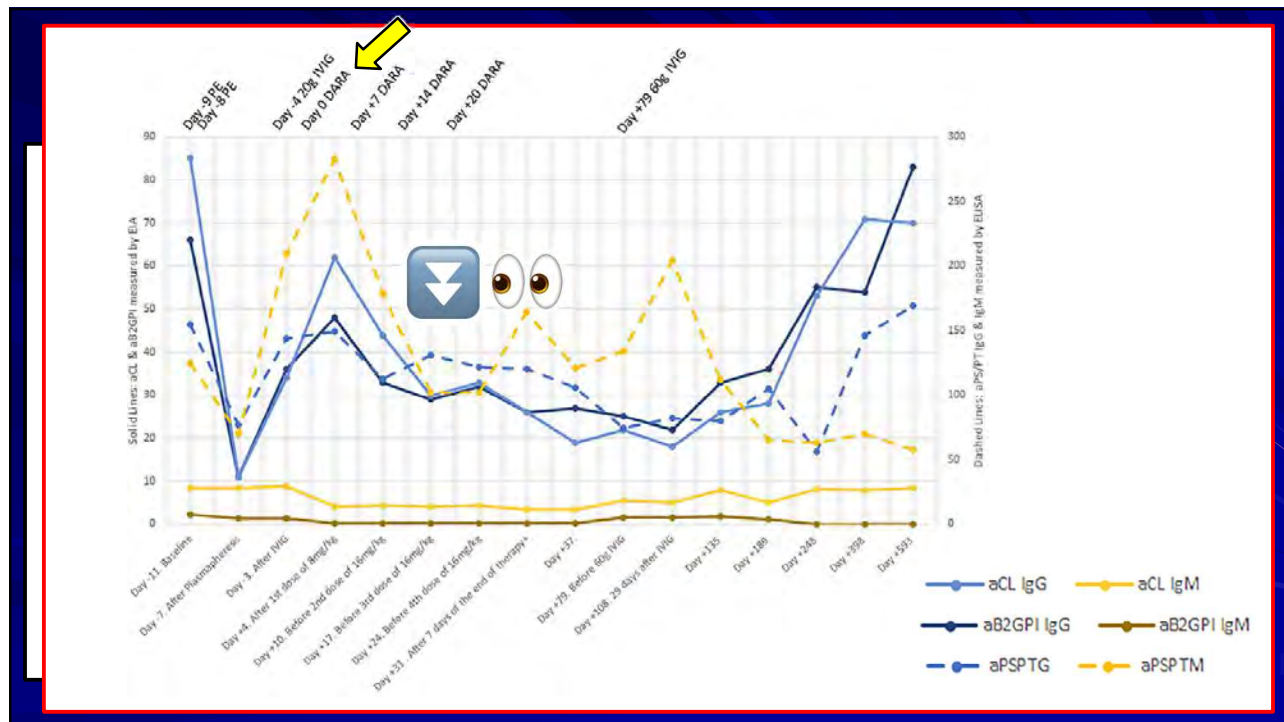
31

Anti-CD38 for plasma cell depletion in APS?

- Targeting CD38 with Daratumumab in Primary APS: A Phase 1b Dose Escalation Safety Trial **(NCT05671757)**

- **Objective:** To assess the safety and tolerability of daratumumab in APS (via dose-escalation cohorts)

- **Objective:** To assess changes in aCL and aβ2GPI levels, and lupus anticoagulant (LA) test positivity

32

Anti-neutrophil therapy? 🫧 🙅

- Depleting neutrophils = not a credible solution

- Lessons from other diseases? DNase in cystic fibrosis? Selectin inhibitors in sickle-cell crisis?

- Are there drugs we already use that have under-recognized anti-neutrophil properties?

33

Adenosine, cAMP, and neutrophils
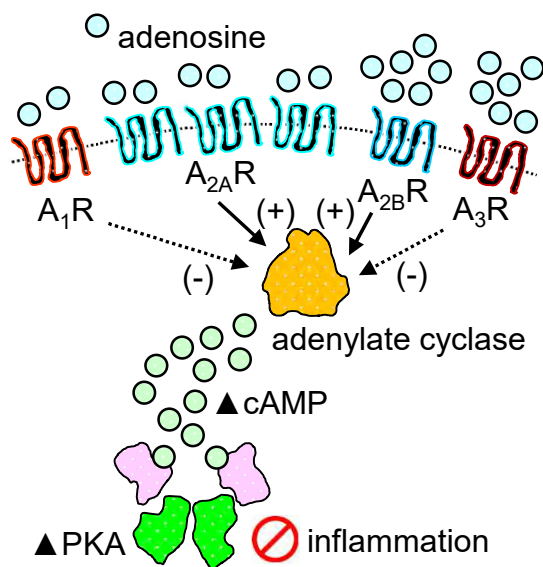
34

Adenosine receptor agonists

35

Dipyridamole

36

Dipyridamole

NETosis *in vitro*

37

	Total (n=191)	Non-survivor (n=54)	Survivor (n=137)	p value
Comorbidity	91 (48%)	36 (67%)	55 (40%)	0·0010
Hypertension	58 (3?)	26 (48%)	32 (23%)	0·0008
Diabetes	36 (1?)	17 (31%)	19 (14%)	0·0051
White blood cell count, ×10⁹ per L	6·2 (4·5–9·5)	9·8 (6·9–13·9)	5·2 (4·3–7·7)	<0·0001
<4	32 (17%)	5 (9%)	27 (20%)	<0·0001*
4–10	119 (62%)	24 (44%)	95 (69%)	..
>10	40 (21%)	25 (46%)	15 (11%)	..
Lymphocyte count, ×10⁹ per L	1·0 (0·6–1·3)	0·6 (0·5–0·8)	1·1 (0·8–1·5)	<0·0001
<0·8	77 (40%)	41 (76%)	36 (26%)	<0·0001
Haemoglobin, g/L	128·0 (119·0–140·0)	126·0 (115·0–138·0)	128·0 (120·0–140·0)	0·30

38

Using discarded serum from the clinical biochemistry lab…
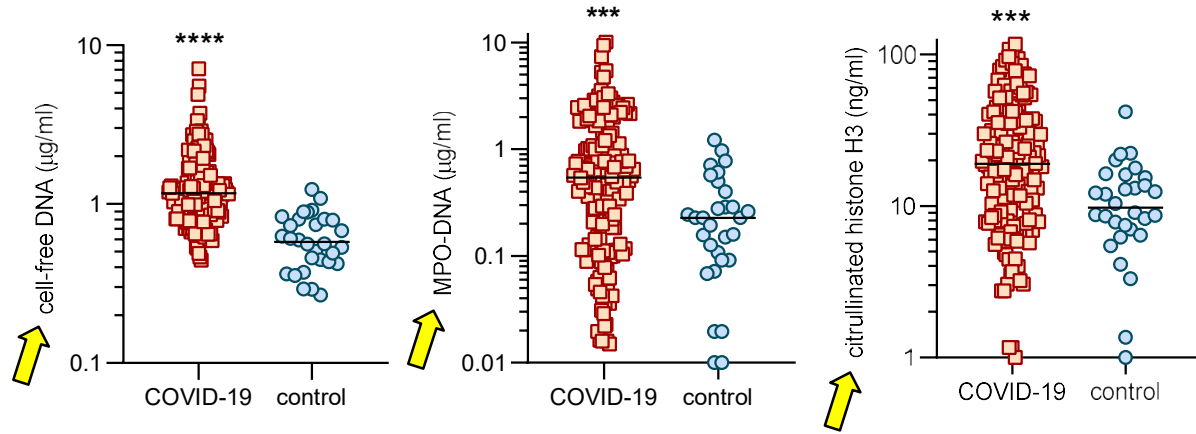
NETs correlate with clinical biomarkers…

…and respiratory status

39

Meanwhile, also in the first half of 2020…

(B) Cut sections of lung showing thrombi present within peripheral small vessels (green arrows)
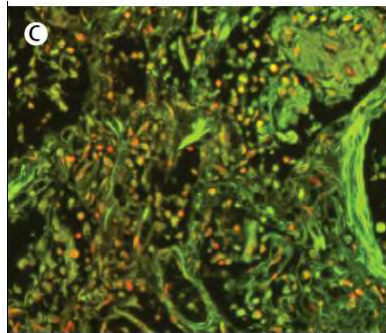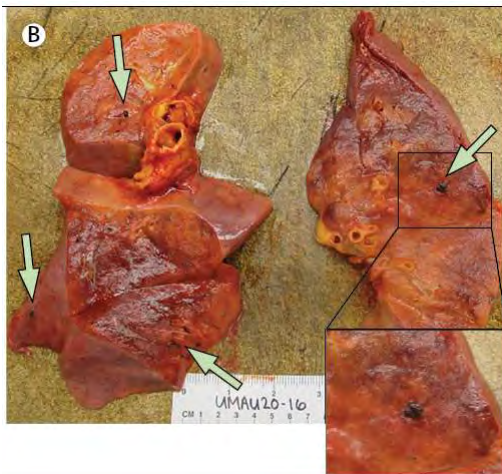
(C) Entrapment of immune cells, including degenerated neutrophils, within fibrin, and strands of extracellular material with weak DNA staining.

40

Meanwhile, also in the first half of 2020…

SCIENCE TRANSLATIONAL MEDICINE | RESEARCH ARTICLE

antiphospholipid syndrome

Can Autoimmune Antibodies Explain Blood Clots in COVID-19?

Posted on November 17th, 2020 by Dr. Francis Collins

41

aPL in COVID-19: n=172 patients

	aPL correlate with clinical biomarkers…		…and respiratory status	
		(factory cut-off)		(titer ≥40)
anti-CL IgG	8	4.7%	2	1.2%
anti-CL IgM	39	22.7%	13	7.6%
anti-CL IgA	6	3.5%	1	0.6%
anti-β_2GPI IgG	5	2.9%	3	1.7%
anti-β_2GPI IgM	9	5.2%	7	4.1%
anti-β_2GPI IgA	7	4.1%	3	1.7%
anti-PS/PT IgG	42	24.4%	21	12.2%
anti-PS/PT IgM	31	18.0%	22	12.8%
any positive	88	51.2%	53	30.8%

42

21

aPL in COVID-19: transfer into mice

patient IgG

COVID IgG also triggered more NETosis *in vitro* and *in vivo*
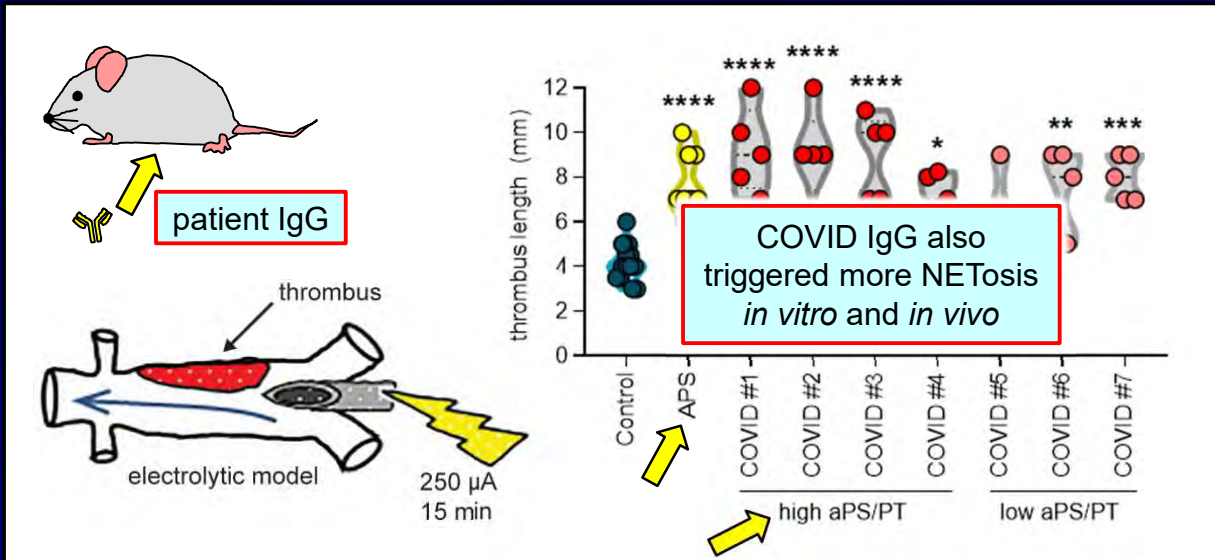
43

aPL in COVID-19: endothelial cell activation
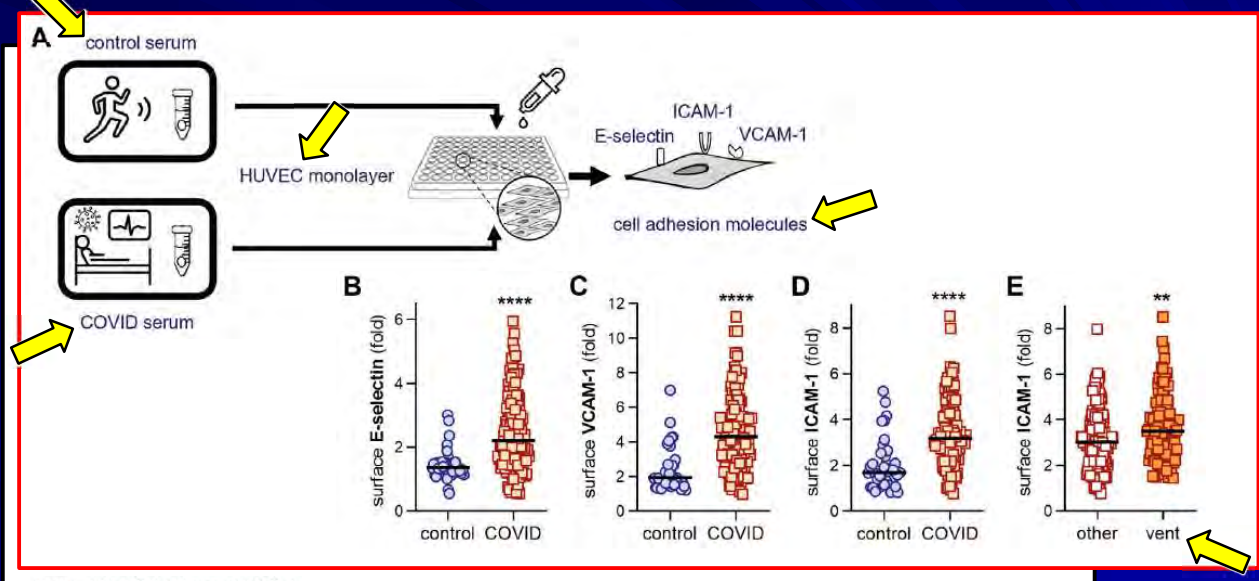
44

aPL in COVID-19: endothelial cell activation

There was a correlation between aPL-positivity and endothelial cell activation…

…which was blunted by total IgG depletion
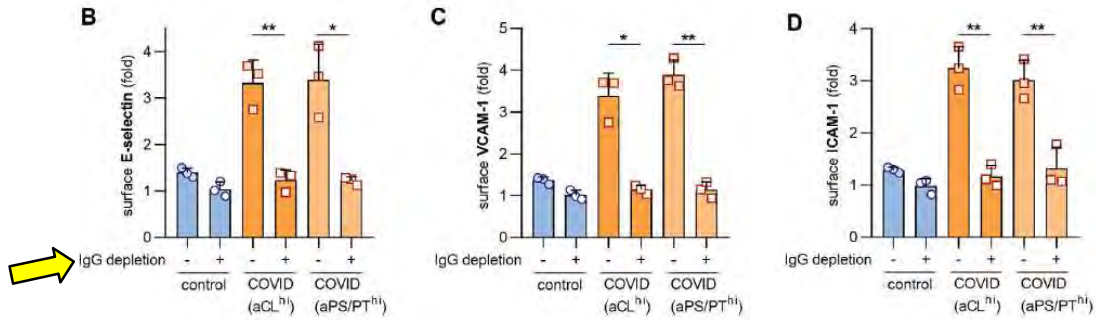
DOI 10.1002/art.42094

45

in COVID-19

ARTICLES — nature immunology

Extrafollicular B cell responses correlate with neutralizing antibodies and morbidity in COVID-19

Expansion of lupus-like B cells that appear to bypass normal germinal center checkpoints

46

Higher anti-NET IgG in serum associates with impaired ability of that serum to degrade NETs

47

Anti-NET antibodies in APS

Samples from APS ACTION: especially Brazil, Italy, and USA

48

24

Anti-NET antibodies in APS

Compare anti-NET data with n=120 "autoantigen microarray"

IgG array

Centromere protein A

Heparan sulfate proteoglycan

MPO/DNA complex

Laminin

Nucleosome

Collagen VI

$-\log_{10}$(P-Value)

$\log_2$(Fold Change)

IgM array

Proliferating cell nuclear antigen

Double-stranded DNA

Single-stranded DNA

$-\log_{10}$(P-Value)

$\log_2$(Fold Change)
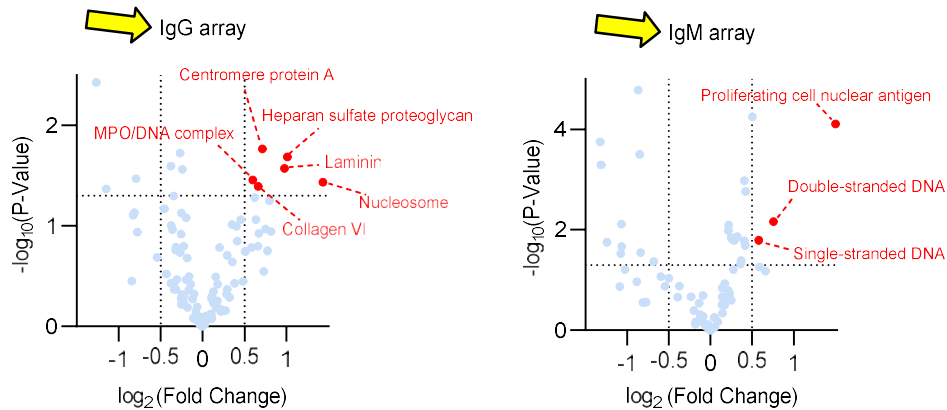
49

What about neutrophils and long-COVID?

	Non-COVID	COVID-19 Mild/moderate	Severe/critical	Convalescents	P value
Study subjects, n	54	52	74	66	
Age, years, mean ± SEM	48 ± 1.9	52 ± 2.8	67.0 ± 1.7	52.4 ± 2.2	<0.0001
Sex					
Men, n	29	28	46	35	0.5349[h]
Women, n	25	24	28	31	
Clinical Data					
Survival rate, %	-	100%	74%	-	
BMI	27.2 ± 0.6	27.2 ± 0.6[c]	31.1± 1.0[e]	29.8 ± 2.1[g]	0.0196
Peak D-dimer, ng/ml, mean ± SEM	-	1118 ± 223[d]	3007 ± 352[f]	-	0.0002
Peak CRP[a], mg/dL, mean ± SEM	-	8.3 ± 1.1	19.5 ± 1.3	-	<0.0001
Peak WBCs[b], 10³/uL, mean ± SEM	-	8.3 ± 0.5	13.2 ± 0.9	-	<0.0001
Peak Platelet count, 10³/uL, mean ± SEM	-	291 ± 22	322 ± 19	-	0.2094

50

What about neutrophils and long-COVID?
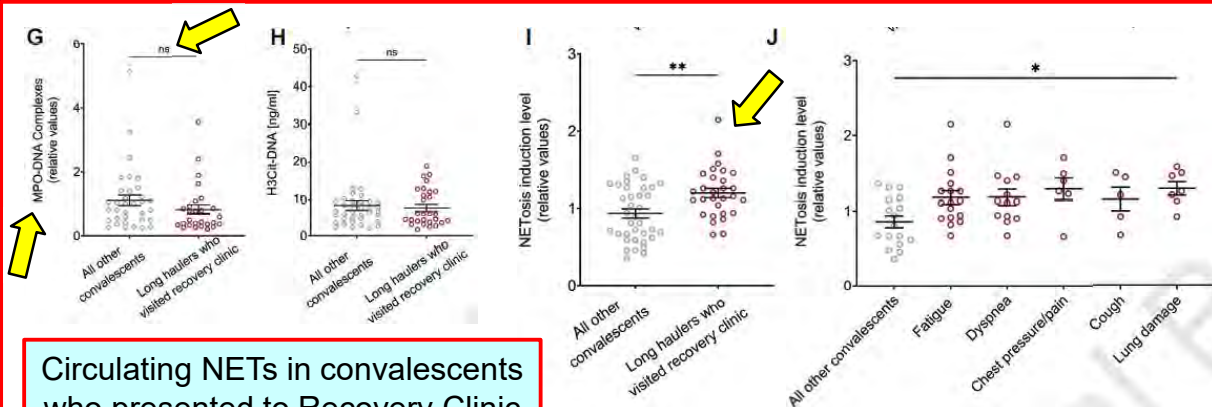
Circulating NETs in convalescents who presented to Recovery Clinic versus those who did not

Interesting results when the focus is on the NET-stimulating potential of patient serum

51

# What about neutrophils and long-COVID?

Table 1. Patient Data Table

Characteristics	PASC Patients (n = 97)	Uncomplicated COVID recovery (n = 26)
Sex		
Female	71 (73%)	10 (50 %)
Male	26 (27%)	13 (50%)
Acute COVID-19 Severity		
Asymptomatic	0	0
Outpatient	57 (59%)	23 (88%)
Hospitalized	37 (39%)	3 (12%)
ICU-admitted	3 (3%)	0
Collection DPSO. Mean (range)	140 (22-446)	110 (18-304)
0-3 months	39 (41%)	13 (50%)
3-6 months	34 (36%)	8 (31%)
6-12 months	20 (21%)	5 (19%)
>12 months	2 (2%))	0 (0%)

| PASC symptoms (self-reported)	
Dyspnea	65 (68%)
Fatigue	61 (64%)
Brain Fog	45 (47%)
Cough	31 (33%)
Headache	29 (31%)
Chest Pain	23 (24%)
Depression	20 (21%)
Myalgias	19 (20%)
Weakness	19 (20%)
Anxiety	18 (19%)
Anosmia/Dysguesia	15 (16%)
Arthralgias	14 (15%)

52

What about neutrophils and long-COVID?
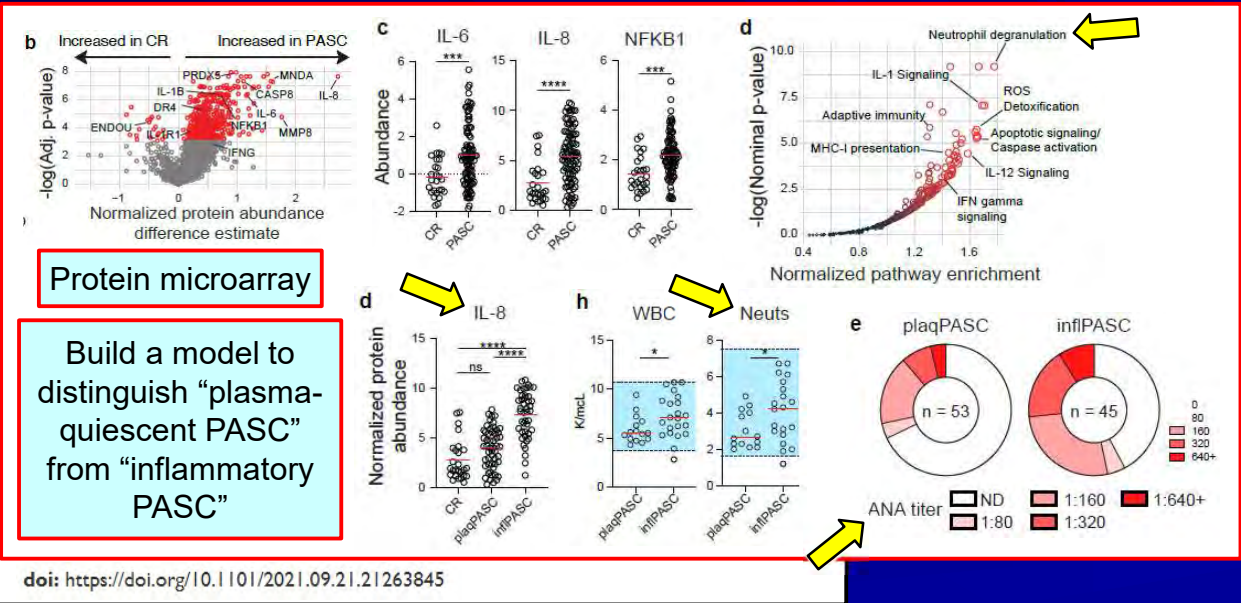
Protein microarray

Build a model to distinguish "plasma-quiescent PASC" from "inflammatory PASC"

doi: https://doi.org/10.1101/2021.09.21.21263845

53
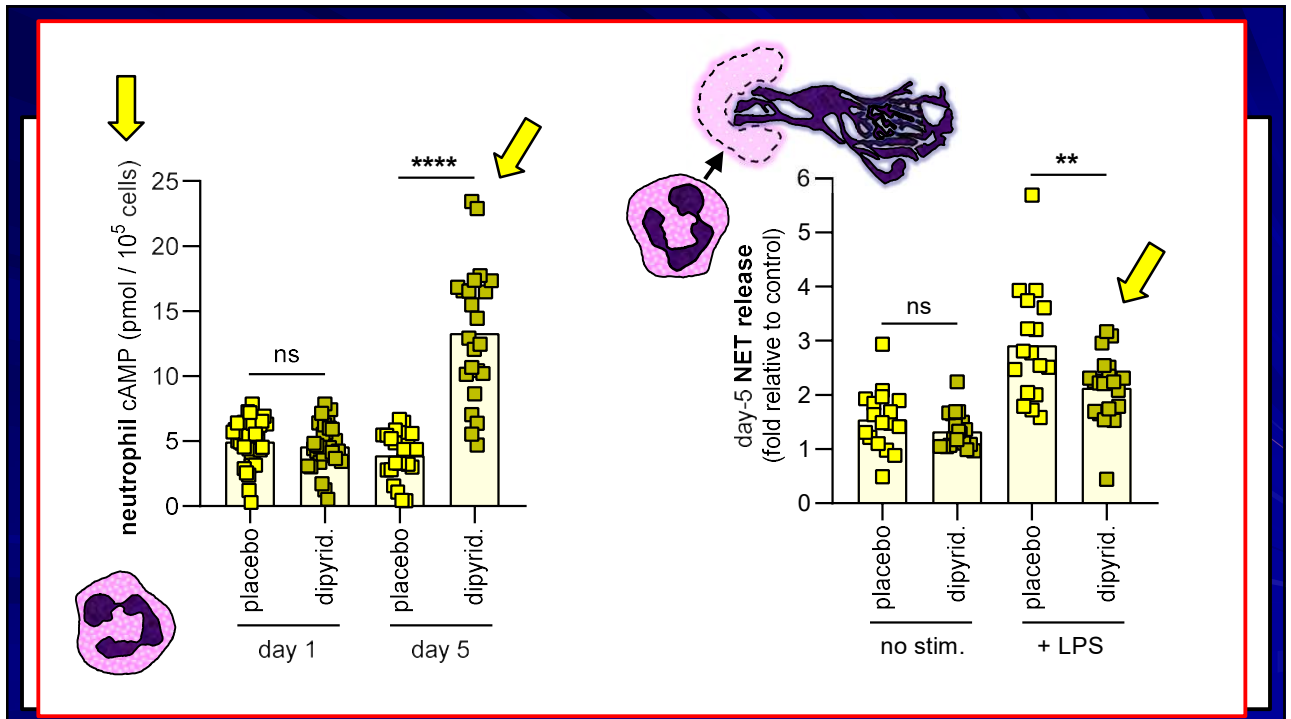
54

Outline

- Brief background including some history of APS
- Let's talk about neutrophils and NETs!
- APS pathogenesis—and what it can potentially teach us about COVID-19
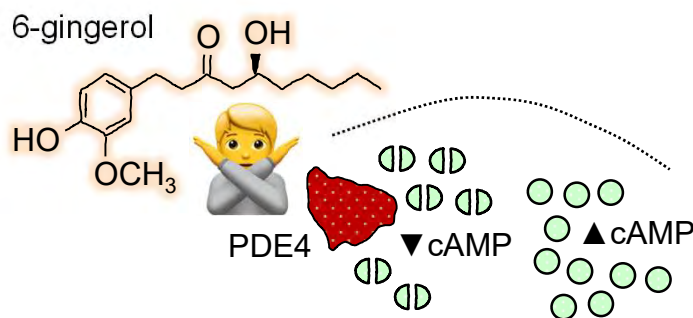- For fun: some ginger with that?

55

6-gingerol boosts neutrophil cAMP

6-gingerol is the most abundant bioactive compound in ginger root

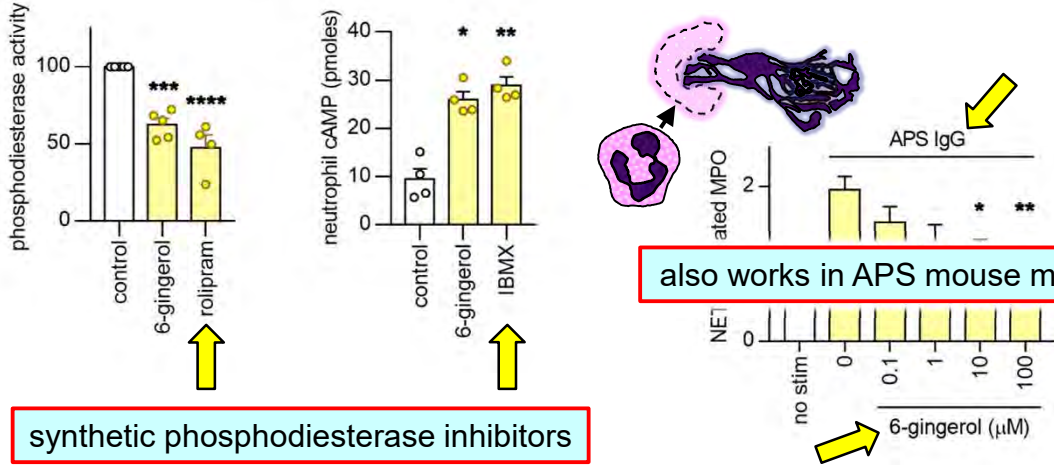~100 mg 6-gingerol in 4 grams of ginger supplement

A natural phosphodiesterase inhibitor?

6-gingerol

PDE4   ▼cAMP   ▲cAMP

JCI Insight 2021;6(3):e138385 https://doi.org/10.1172/jci.insight.138385

56

6-gingerol boosts neutrophil cAMP

also works in APS mouse models

synthetic phosphodiesterase inhibitors

JCI Insight 2021;6(3):e138385 https://doi.org/10.1172/jci.insight.138385

57

What about a whole ginger extract?
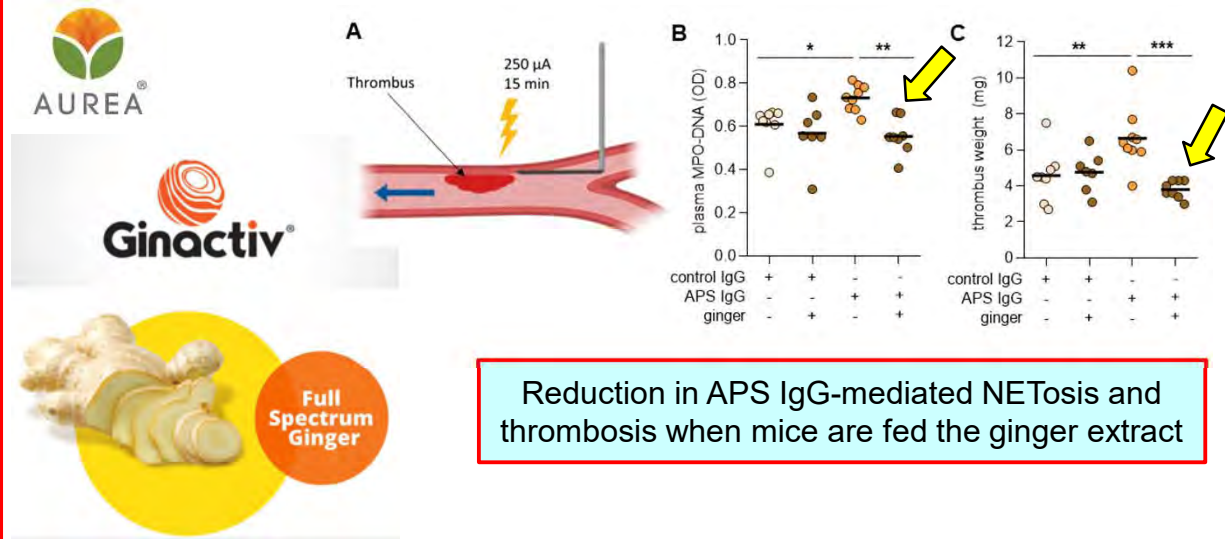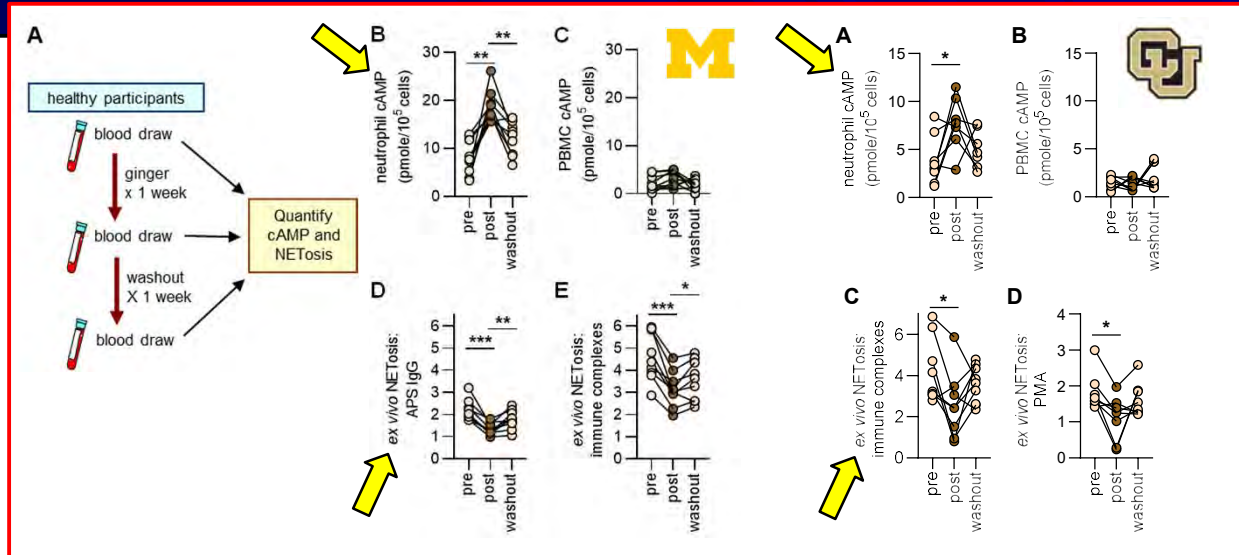

Reduction in APS IgG-mediated NETosis and thrombosis when mice are fed the ginger extract

Meeting: ACR Convergence 2022

58

What about a whole ginger extract?

Meeting: ACR Convergence 2022

59

Summary

- Brief background including some history of APS
- Let's talk about neutrophils and NETs!
- APS pathogenesis—and what it can potentially teach us about COVID-19
- For fun: some ginger with that?

60

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– David Pinsky (U-M Cardiology)
– **Hui Shi (Shanghai)**
– Sub Pennathur (U-M Nephrology)
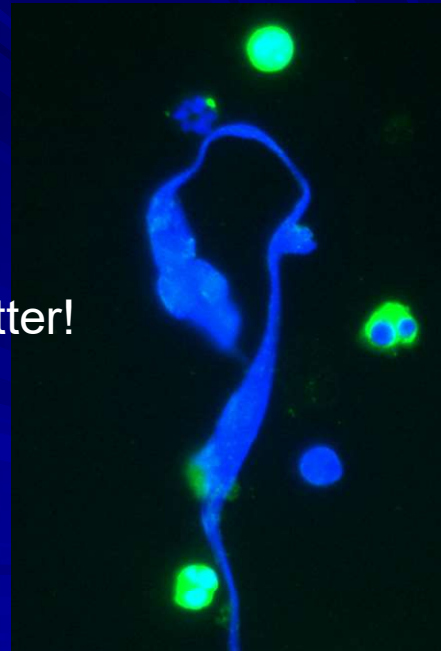– Eliza Tsou (U-M Rheum)
– John Varga (U-M Rheum)

61

Questions

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62