11° Riunione Gruppo Studio Piastrine

Gazzada, 3 - 5 Ottobre 2010

PREPARAZIONI PIASTRINICHE PER STUDI DI TRASCRITTOMICA:

CACCIA AL LEUCOCITA

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IT'S TIME TO TAKE STOCK OF.....

1-

TISSUE FACTOR and PLATELETS

2-

Leukocyte contamination in platelet RNA preparations

BLOOD, 5 AUGUST 2010 VOLUME 116, NUMBER 5

To the editor:

No evidence for tissue factor on platelets

Beth A. Bouchard
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Kenneth G. Mann
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.....to resolve this ONGOING CONTROVERSY....

.... platelets express active TF after de novo synthesis,[2,3] transfer from monocytes,[4] or release from -granules.[5]

2. Panes O *et al*. **Human platelets synthesize and express functional tissue factor**. *Blood.* 2007

3. Schwertz H, et al. **Signal-dependent splicing of tissue factor pre-mRNA modulates the thrombogenicity of human platelets**. *J Exp Med.* 2006

4. Falati S, Liu Q, Gross P, et al. **Accumulation of tissue factor into developing thrombi in vivo is dependent upon microparticle P-selectin glycoprotein ligand1 and platelet P-selectin**. *J Exp Med.* 2003

5. Siddiqui FA et al. **The presence and release of tissue factor from human platelets**. Platelets. 2002

In other studies,[6,7] however, the existence of platelet TF could not be demonstrated.

6. Butenas S, et al. **Tissue factor activity in whole blood**. *Blood.* 2005.

7. Osterud B et al. **What is blood borne tissue factor?** *Thromb Res.* 2009.

RESULTS:

Flow cytometric analyses indicated that no TF was expressed by PAR1- and PAR4-activated platelets compared with unactivated platelets despite maximal -granule release (98% P-selectin–positive platelets)….

…no TF antigen was detected by immunoassay after prolonged stimulation of platelets with LPS.

No FXa was generated by extrinsic FXase using unstimulated or stimulated platelets as a possible TF source. Similarly, no clot was formed in a plasma-based clotting assay.

TF on monocytes or monocyte-derived microparticles is not transferred to platelets.

CONCLUSION:

Based on these observations, we conclude that platelets do not express detectable TF antigen or activity.

Discrepancies between our data and those published by others may be a result of the assays used to quantify TF antigen and activity in different laboratories.

Our assays use specific and highly sensitive anti-TF monoclonal antibodies and physiologically relevant standards and controls, whereas other reported assays use combinations of monoclonal and polyclonal antibodies, which may recognize TF fragments or cross-react with other proteins…..

? ? ? ?

METHODS in figure legend:

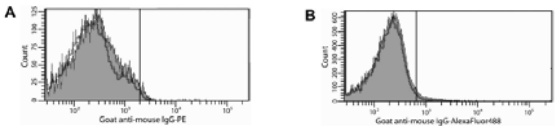

Figure 1. Search for TF on platelets by flow cytometry. (A) **Washed platelets were activated with PAR1 (100μM) and PAR4 (500 μM) agonist peptides (2 hours, 37°C).** (B) Platelet-rich plasma was incubated with THP-1 cells in the presence or absence of 250 ng/mL LPS (4 hours, 37°C)……

Eureka! ! ! !

To the Editor:

Tissue factor expression on platelets is a *dynamic* event

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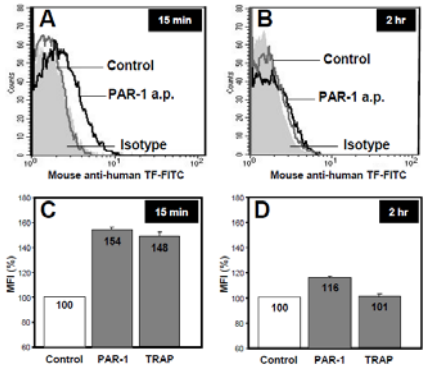
Vincenzo Toschi
Division of Haemat and Blood Transfusion and Thrombosis Centre, AO Ospedale San Carlo Borromeo, Milan
Elena Tremoli
Dept. of Pharmacological Sciences, Univ. degli Studi di Milano and Centro Cardiologico Monzino IRCCS, Milan

RESULTS:



Our results confirm that the amount of TF present on the platelet surface, after 2 hours stimulation with PAR-1 agonist peptide and TRAP-6, is negligible (..) (Figure 1). After a 15 minutes stimulation, however, PAR-1 and TRAP-6 significantly increase platelet TF expression (..) compared with resting platelets (...)

CONCLUSION:

• This indicates that TF expression by platelets in response to certain agonists is an early event, confirming data already published by our group[2,3].

(REF: Camera et al, ATVB 2003 and ATVB 2008)

• Our data support the evidence that platelets do express TF as a result of a rapid and dynamic process. Thus its detection may considerably vary if observed at different time points after *in vitro* cell stimulation.

Oggetto: Decision on your Letter to the Editor #BLOOD/2010/307306

Title: Tissue factor expression on platelets is a *dynamic* event

Dear Dr. Camera:

I am pleased to inform you that your Letter to the Editor has been accepted for publication **in the next available issue of Blood Journal**.

……

Sincerely,

Cynthia E. Dunbar, M.D.
Editor-in-Chief, Blood

IT'S TIME TO TAKE STOCK OF…..

1-

TISSUE FACTOR and PLATELETS

2-

Leukocyte contamination in platelet RNA preparations

Manuscript Number: TR-D-10-00225R1

Gene expression profiling reveals multiple differences in platelets from patients with stable angina or non-ST elevation acute coronary syndrome

REVISION

Gualtiero Colombo, Karl Gertow, Giancarlo Marenzi, Marta Brambilla, Monica De Metrio, Elena Tremoli and Marina Camera

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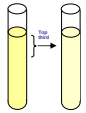
Reviewer comment:

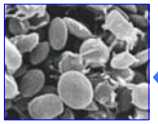
"The biggest challenge with platelet RNA WGE studies is the contamination with leukocyte RNA. *The RNA content of a single leukocyte is estimated to be **3-4 log higher** than that of a platelet. The authors are aware of this drawback and comment on this several times. They have chosen an arbitrary limit for leukocyte contamination with **not more than 5 leucocytes per 10^5 platelets**. This is a rather liberal limit and the results of Q-PCR tests with myeloid and lymphoid markers have not been shown (bottom of page 8). There are other means to inspect platelet WGE studies for signals that have been produced by RNA from contaminating leucocytes.*

Each blood cell type has its own set of "strictly lineage-specific transcripts" and these could have been used to provide reassurance that leukocyte contamination is low enough to not be a confounding factor in the analysis."

Methods – Platelet isolation

- 40 ml blood in ACD/patient
- Platelet isolation by sequential centrifugation of PRP (top third) with PGE_1 and EDTA



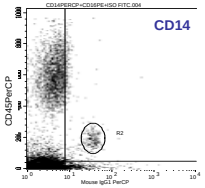


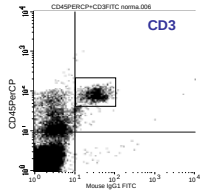
- **Assessment of platelet activation (FACS)**
- **Assessment of leukocyte contamination**
- **RNA extraction and quality control**

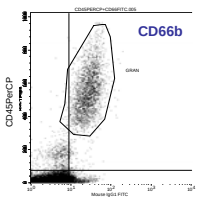
Leukocyte population markers

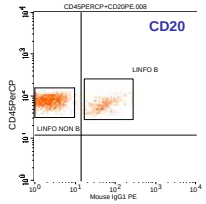
Monocytes

Granulocytes





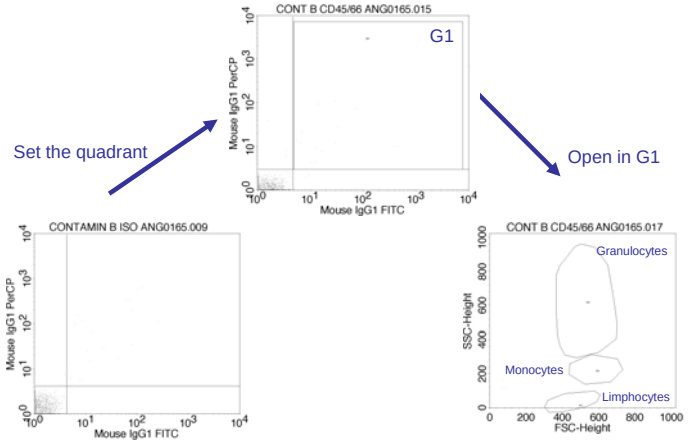




Lymphocytes T

Lymphocytes B

Assessment of leukocyte contamination by flow cytometry



*Only preparations with less than **5 leukocytes/10^5 platelets** were used for RNA isolation*

Mean number of contaminating WBC in 10^5 platelets

Platelet Preparations used for Microarray, PCR and Western blot experiments			
	Mean number of contaminating WBC in 10^5 platelets, (% of patients)		
	Granulocytes ($CD66b^+$)	**Monocytes** ($CD14^+$)	**Lymphocytes** ($CD2^+ + CD20^+$)
Stable angina patients	**0** (70%)	**0** (76%)	**0** (76%)
	2.4 (30%)	**1** (24%)	**1** (24%)
NSTE-ACS patients	**0** (65%)	**0** (76%)	**0** (71%)
	1.3 (35%)	**0.6** (24%)	**1.3** (29%)

Mean number of contaminating WBC in 10^5 platelets

Platelet Preparations used for Microarray, PCR and Western blot experiments			
	Mean number of contaminating WBC in 10^5 platelets, (% of patients)		
	Granulocytes ($CD66b^+$)	**Monocytes** ($CD14^+$)	**Lymphocytes** ($CD2^+ + CD20^+$)
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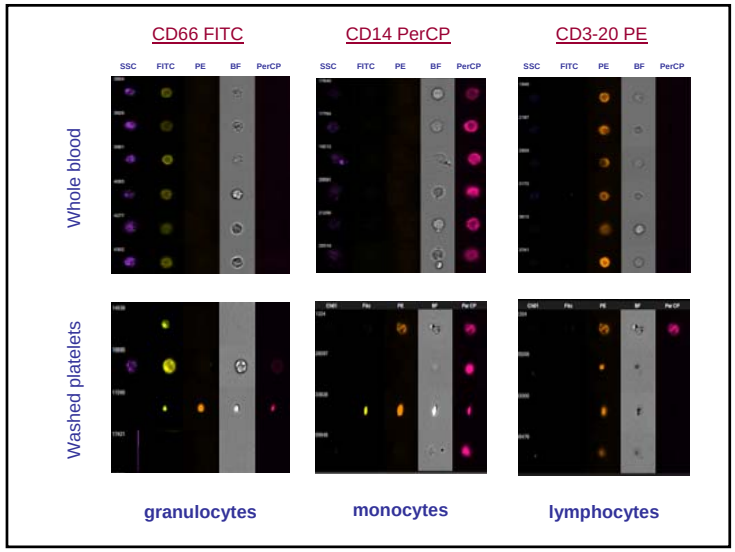
NSTE-ACS patients	**0** (65%)	**0** (76%)	**0** (71%)
	1.3 (35%)	**0.6** (24%)	**1.3** (29%)

Discarded Platelet Preparations			
	Mean number of contaminating WBC in 10^5 platelets, (% of patients)		
	Granulocytes ($CD66b^+$)	**Monocytes** ($CD14^+$)	**Lymphocytes** ($CD2^+ + CD20^+$)
Stable angina patients	**0** (0%)	**0** (64%)	**0** (38%)
	5.6 (100%)	**0.8** (38%)	**1.7** (64%)
NSTE-ACS patients	**0** (0%)	**0** (33%)	**0** (0%)
	5.2 (100%)	**2.5** (67%)	**0.7** (100%)

ImageStream^X: multispectral imaging flow cytometer (AMNIS)

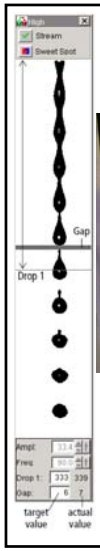
"By **combining** the speed, sensitivity, and phenotyping abilities of **flow cytometry** with the detailed imagery and functional insight of **microscopy**, the ImageStreamX overcomes the limitations of both techniques (…)"

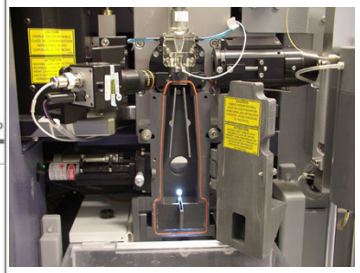


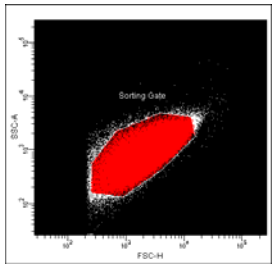
FILTRI *Purecell* PL (PALL)

	Tyrode buffer	*Purecell* filter
- Standardization	*NI*	YES
-Time	1 hour	30 min
- Platelet recovery	400 x10^6	500 x10^6
- Efficiency of leukocyte removal	<5 leuko /10^5 plt	<5 leuko /10^5 plt
- Cost	1 euro/each	18.40 euro/each
- Aggregates removal	NO	YES

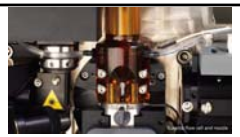
Cell sorting







NOZZLE 70 µm
PRESSIONE 70 PSI
MASCHERA 0 32 0

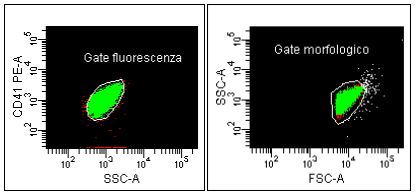


THRESHOLD → 20.000 ev/sec

100 mil ev/ ~2ore

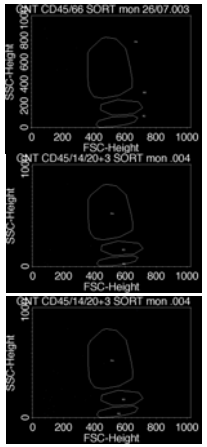
GATING STRATEGY

- *Frequence*: 78,1
- *Drop 1*: 260
- *Gap*: 8
- *Drop Delay*: 36,24
- *Voltage plate*: 6000



Population	#Events	%Parent
All Events	10.000	
Gate morfologico	9.953	99,5
Gate fluorescenza	9.605	98,3

LEUKOCYTE CONTAMINATION



Granulocyte gate (CD45+/CD66+): 0 events

Limphocyte gate (CD45+/CD20+/CD3+): 0 events

Monocyte gate (CD45+/CD14+): 0 events

CELL SORTING *vs* WASHING PROCEDURE

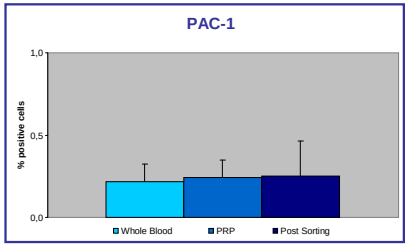
	Tyrode buffer	*Sorting*
- Standardization	*NI*	YES
- Blood volume	*30 ml*	5 ml
- Time	1 hour	3 hours
- Platelet recovery	700 x10^6	100 x10^6
- Efficiency of leukocyte removal	<5 leuko /10^5 plt	0 leuko /10^5 plt
- Cost	1 euro	>100 euro
- Specialized technician	NO	YES

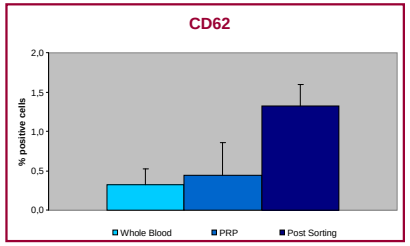
POST-SORTING platelet activation

NO GP IIbIIIa activation

but

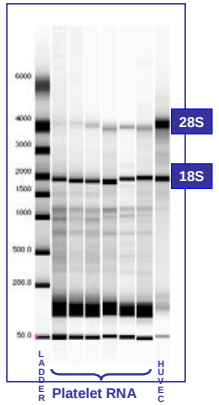
degranulation



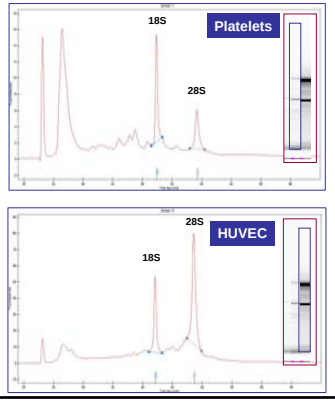


Assessment of platelet RNA quality

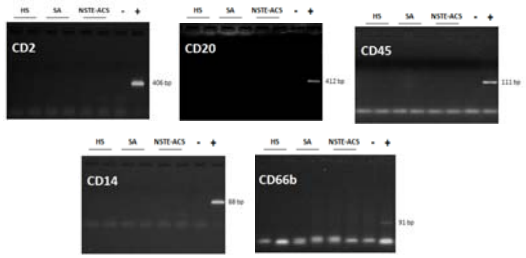
Virtual gel



Elettropherograms



RT-PCR



Leukocyte contamination of the platelet RNA preparations was assessed by amplification of specific markers for T- and B-lymphocytes (CD2 and CD20, respectively), monocytes (CD14), granulocytes (CD66b), and pan-leukocytes (CD45) by RT-PCR.

✓Platelet isolation

↓

✓Assessment of platelet activation (FACS)

↓

✓Assessment of leukocyte contamination

↓

✓RNA extraction and quality control

↓

Microarray experiments

Microarray experiments

		PLATELETS									PBMC			
SYMBOL	Gene	1	2	3	4	5	6	7	8	9	1	2	PLTavg	PBMCavg
CD14	CD14	177,37	127,28	173,98	160,45	135,79	246,94	116,53	238,39	294,25	32191,74	30427,67	185,66	31309,71
DUSP1	dual specificity phosphatase 1	71,62	86,98	122,31	106,37	102,45	111,64	102,94	171,63	274,06	21102,30	23133,63	127,78	22117,97
FCN1	ficolin	135,49	123,21	187,48	151,90	129,41	216,99	126,45	289,58	418,81	25122,01	28195,81	197,70	26658,91
HLA-DRB4	HLA-DRB4	153,01	116,47	174,56	127,97	115,80	181,80	128,24	178,44	429,92	22961,42	18029,95	178,47	20495,69
ITGB2	integrin, beta 2 (antigen CD18 (p95)	189,66	132,15	202,59	155,13	126,41	205,24	125,22	328,13	527,22	27826,67	32995,37	221,31	30411,02
LYZ	lysozime	361,70	156,90	440,73	154,19	200,61	353,89	256,93	408,17	689,61	51601,36	51003,24	335,86	51302,30

Highest PBMC expressed genes defined as signal intensity > 20000: 227

Background value=100

Positive signal threshold: at least 3 times the background value.

No PBMC signal was detected in PLT preparations