LAPORAN AKHIR

A Study to Develop in Vivo Determination of Platelet Activation Markers by Flow Cytometer

USM Jangka Pendek (304 / PPSP / 6131213)

PROF. MADYA NORMAH JAMALUDIN

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Jabatan Hematologi / *Department of Haematology*

Tarikh : 14 Julai 2003

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Puan,

Laporan Komprehensif Penyelidikan Jangka Pendek

Dengan hormatnya bersama ini dikepilkan Laporan Komprehensif Penyelidikan :

Tajuk : **"A Study to Develop in Vivo Determination of Platelet Activation Markers by Flow Cytometer"**

Geran : **USM Jangka Pendek (304/PPSP/6131213)**

Jangka Masa Projek : **Mula 1 April 2002 hingga 31 Mac 2003**

Hasil keputusan kajian ini telah dibentangkan dalam :

1) 4th Malaysian Society of Haematology Meeting 15-17 March 2002.
(Best Oral Presentation Award)

2) 31st Annual Meeting of International Society of Experimental Haematology, Montreal, Canada 5-9 July 2002.
(Award a Travel Grant from the ISEH)

Abstrak pembentangan ada dikepilkan.

Terima kasih.

"BERSAING DI PERINGKAT DUNIA : KOMITMEN KITA"
"BERKHIDMAT UNTUK NEGARA"

(PROF. MADYA DR. NORMAH JAMALUDIN)
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Semua laporan kemajuan dan laporan akhir yang dikemukakan kepada Bahagian Penyelidikan dan Pembangunan perlu terlebih dahulu disampaikan untuk penelitian dan perakuan Jawatankuasa Penyelidikan di Pusat Pengajian.

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LAPORAN AKHIR PROJEK PENYELIDIKAN R&D JANGKA PENDEK

A. MAKLUMAT AM

Tajuk Projek: A Study to Develop in Vivo Determination of Platelet Activation Markers by Flow Cytometer

Tajuk Program: _____

Tarikh Mula: 1 April 2002

Nama Penyelidik Utama: Prof Madya Norman Jamaladin
(berserta No. K/P)

Nama Penyelidik Lain: Dr Tariq M. Roshan
(berserta No. K/P)

B. PENCAPAIAN PROJEK:

(Sila tandakan [/] pada kotak yang bersesuaian dan terangkan secara ringkas di dalam ruang di bawah ini. Sekiranya perlu, sila gunakan kertas yang berasingan)

[/] Penemuan asli/peningkatan pengetahuan

Projek ini telah berjaya membangunkan kaedah ujian Petanda ransangan platelet menggunakan alat Flow cytometer. Melalui kaedah ini didapati bahawa jelas menunjukkan wanita telah putus haid mengalami ransangan platelet yang luar biasa dibandingkan dengan wanita yang belum putus haid. Dengan perkembangan pengetahuan ini rawatan untuk mengelakkan thrombosis mungkin diambil kira untuk golongan wanita tersebut.

☐ **Rekaan atau perkembangan produk baru,**
(*Sila beri penjelasan/makluman agar mudah dikomputerkan*)

(1) _____

_____

(2) _____

_____

(3) _____

_____

☑ **Mengembangkan proses atau teknik baru,**
(*Sila beri penjelasan/makluman agar mudah dikomputerkan*)

(1) Kaedah ujian ini boleh dijadikan ujian rutin untuk wanita yang telah putus haid.

(2) Wanita yang mempunyai ransangan platelet di beri "anti platelet" sebagai rawatan penghindaran.

(3) Boleh dijadi basis untuk 'drug trial' produk atau ubatan 'anti-platelet'

☐ **Memperbaiki/meningkatkan produk/proses/teknik yang <u>sedia ada</u>**
(*Sila beri penjelasan/makluman agar mudah dikomputerkan*)

(1) Pengambilan spesimen perlu lebih berhati hati untuk mengelakan 'in vitro activation'

(2) _____

_____

(3) _____

_____

C. PEMINDAHAN TEKNOLOGI

☐ Berjaya memindahkan teknologi.

Nama Klien: (1) _____
(Nyatakan nama penerima pemindahan teknologi ini dan sama ada daripada pihak swasta ataupun sektor awam)
(2) _____
(3) _____

☑ Berpotensi untuk pemindahan teknologi.
(Nyatakan jenis klien yang mungkin berminat)
Syarikat Farmakutikal yang mempunyai drug "anti-platelet" terutama untuk pencegahan gangguan pembekuan atau "Thrombosis"

D. KOMERSIALISASI

☐ Berjaya dikomersialkan.

Nama Klien: (1) _____
(2) _____
(3) _____

☐ Berpotensi untuk dikomersialkan.
(Nyatakan jenis klien yang mungkin berminat)

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E. PERKHIDMATAN PERUNDINGAN BERBANGKIT DARIPADA PROJEK

(*Klien dan jenis perundingan*)

(1) _____

(2) _____

(3) _____

(4) _____

F. PATEN/SIJIL INOVASI UTILITI

(*Nyatakan nombor dan tarikh pendaftaran paten. Sekiranya paten/sijil inovasi utiliti telah dipohon tetapi masih belum didaftarkan, sila berikan nombor dan tarikh fail paten*).

(1) _____

(2) _____

(3) _____

G. PENERBITAN HASIL DARIPADA PROJEK

(i) LAPORAN/KERTAS PERSIDANGAN ATAU SEMINAR

(1) Pembentangan dalam negeri (abstrak dilampirkan)

(2) Pembentangan luar negeri (abstrak dilampirkan)

(3) _____

_____

(4) _____

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(5) _____

_____

(ii) **PENERBITAN SAINTIFIK**

(1) dalam proses

(2)

(3)

(4)

(5)

(6)

H. <u>HUBUNGAN DENGAN PENYELIDIK LAIN</u>

(sama ada dengan institusi tempatan ataupun di luar negara)

(1) Penyarah dari University Malaya Medical Centre.

(2)

(3)

(4)

I. SUMBANGAN KEWANGAN DARI PIHAK LUAR

(Nyatakan nama agensi dan nilai atau peralatan yang telah diberi)

(1) _____

(2) _____

(3) _____

J. PELAJAR IJAZAH LANJUTAN

(Nyatakan jumlah yang telah dilatih di dalam bidang berkaitan dan sama ada diperingkat sarjana atau Ph.D).

	Nama Pelajar
Sarjana	Dr Tariq M. Roshan
M.Med (Hematologi)	
Ph.D	

K. MAKLUMAT LAIN YANG BERKAITAN

_____

_____

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_____

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_____

13/11/03
Tarikh

Tandatangan

Professor Zabidi Azhar Mohd. Hussin
Chairman of Research & Ethics Committee
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16150 Kubang Kerian,
KELANTAN, MALAYSIA

TANDATANGAN PENGERUSI
JAWATANKUASA PENYELIDIKAN
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FINAL REPORT OF THE STUDY OF METHODOLOGICAL DEVELOPMENT AND DETERMINATION OF PLATELET ACTIVATION MARKERS IN PRE- AND POST MENOPAUSAL WOMEN BY FLOW CYTOMETER.

IRPA SHORT TERM GRANT: 304/PPSP/6131213
RESEARCHERS: Associate Prof. Dr. Normah Jamalludin
Dr. Tariq Mahmood Roshan

INTRODUCTION:

Cardiovascular disorders are a major cause of mortality and morbidity not only among industrialized and developed nations but are also emerging as a major contributory factor to overall morbidity and mortality in developing countries like Malaysia. According to Malaysia's book of health records (Ministry of health, 1999) the rate of death due to cardiovascular events is 20.33% and cerebrovascular events constituting 7.74% of the total. Various factors contributing to these cardiovascular events include aging, obesity, smoking, hyperlipidemias, hypertension and diabetes mellitus. In many instances of cardiovascular events occurring as a result of the aforementioned factors, thrombogenesis is an important single most common underlying pathological process. While females are generally protected from cardiovascular diseases before menopause, this population is at a higher risk of such events compared to their male counter parts due to the loss of protection by estrogen after menopause (Wenger et. al. 1993). Coronary artery disease is then a major cause of death in postmenopausal women as it is more age dependent in women than in men. A big proportion of all cardiovascular events in either sex are thrombotic in nature. Therefore it is essential to critically understand the structure and function of not only the constituents of blood but also various events stimulating such thrombogenesis.

Platelet activation either disturbed and / or increased plays a major role in the pathophysiology of thrombosis. In many instances of cardiovascular events, thrombogenesis is an important single most common underlying pathological process. Different techniques show changes in platelet reactivity but cannot establish its association with the particular condition. Diagnostic value of flow-cytometric analysis of platelets activation in thrombotic events is being established. At the same time whole blood flow-cytometry has none of the limitations, which are encountered by other techniques of platelet activation analysis. With platelet activation there is conformation changes in GP structure resulting in neo-epitopes while at the same time some new surface receptors are secreted. These neo-epitopes can be targeted by monoclonal antibodies.

Whole blood flowcytometry in the absence of an added exogenous platelet agonist determines the activation state of circulating platelets. In addition to this inclusion of exogenous agonist in the assay enables analysis of the reactivity of the circulating platelets *in vitro*. In our study we did not perform the later part to see the reactivity of the circulating platelet.

Pre-menopausal women have lower risk of cardiovascular disease compared to post-menopausal women, which is more age dependent in women than in men. This might be due to the loss of protection by estrogen after menopause (Wenger et. al. 1993). The association of platelet activation and cardiovascular thrombotic events is well established. Therefore it is

essential to critically understand the structure and function of not only the constituents of blood but also various events stimulating such thrombogenesis.

OBJECTIVES:

(i) Primary objectives.

1 To develop and optimize method for *in vivo* determination of platelet activation markers by flow cytometer.

2 To determine platelet activation markers by the developed technique in pre and postmenopausal women.

(ii) Secondary objectives.

1 To establish a relationship between platelet activation markers serum cholesterol, BMI, age in both groups and also estradiol levels in post menopausal women.

MATERIALS & METHODS:

Materials

Calibrite™ Beads

Becton Deckinson CaliBRITE beads are designed for use with FACS® family of flow cytometers. The beads are used to adjust instrument settings, set fluorescence compensation, and check instrument sensitivity. For our study we use calibration beads with every run of samples for monitoring instrument performance.

Three-color kit was used in this study. It contains fluorescein isothiocyanate (FITC)-labeled bead, a phycoerythrin (PE)-labeled beads, a peridinin chlorophyll protein (PerCP)-labeled bead, and unlabeled beads. The following list illustrates PMT light signal detection:

1. Fluorescence-1 (FL1) FITC (yellow-green)
2. Fluorescence-2 (FL2) PE (red-orange)
3. Fluorescence-3 (FL3) PerCP (red)
4. Fluorescence-4 (FL4) APC (red)

Each fluorochrome emits light over a range of wavelengths when excited by the laser bean. Thus, a portion of the FITC signal is detected by the FL2 PMT, a portion of the PE signal is detected by the FL1 and FL3 PMTs; a portion of the PerCP signal is detected by the FL4 PMT (PerCP signal is not detected by the FL2 PMT); and a portion of the APC signal is detected by the FL# PMT. This "spectral overlap" must be corrected using electronic compensation. CaliBRITE beads are used to determine the appropriate compensation settings.

After the instrument settings have been determined, CaliBRITE beads are used to evaluate instrument sensitivity. Forward scatter (FSC) and side scatter (SSC) instrument sensitivity are measured by the mean channel separation between the light-scatter signal of the beads and background signal (electronic and optical). FL1, FL2, and FL3 fluorescence sensitivity is determined by measuring the mean channel separation between the signal of the labeled beads and the unlabeled beads. A minimum channel separation must be met for the scatter and fluorescence parameters. This allows cells to be distinguished from sample debris or background signal and for dimly stained cells to be distinguished from unstained cells (Becton Dickson CaliBRITE™ beads package insert, 1998).

Preparation of Test Suspension

Suspensions were prepared in two tubes (Falcon disposable 12X75 mm capped polystyrene test tubes, BD catalogue number 2058) immediately prior to use. Beads vials were used after gentle mixing. Tubes were labeled as unlabeled and labeled. In unlabeled tube 1ml of sheath fluid added and then one drop of unlabeled beads were added. In the second tube (labeled) 3ml of sheath fluid was added with one drop each of unlabeled, FITC, PE, and PerCP beads. PMT voltage was adjusted using unlabeled tube while labeled tube was used for fluorescence compensation, and sensitivity test. Manual optimization was done before running the cells.

MONOCLONAL ANTIBODIES USED

I. CD61

CD61 recognizes an 110-kdalton (kDa) protein, also known as gpIIIa, the common β-subunit (integrin β_3-chain) of the gpIIb/IIIa complex and the vitronectin receptor (VNR) (von dem Borne et. al. 1989 & Modderman, 1989). The gpIIb/IIIa complex and the VNR are integrins, ie, α/β-heterodimeric glycoprotein complexes that are involved in cell adhesion (Springer, 1990, Hynes, 1992 & Parmentier, 1990). With the CD41 antigen (gpIIb or α_{IIb}), CD61 antigen forms the gpIIb/IIIa complex, which acts as a receptor for fibrinogen, von Willebrand factor (vWf), fibronectin, and vitronectin on activated platelets (Fijnheer et. al. 1990). With CD51 antigen, the CD61 antigen forms the VNR, which mediates activation-independent cell adhesion to vitronectin, vWf, fibrinogen, and thrombospondin.

The CD61 antigen is found on all normal resting and activated platelets (Modderman, 1989). This is further supported by the individuals with Glanzmann's thromboasthenia which have >90% reduction of binding of CD61, and heterozygote carriers of the disorder show approximately 50% reduction (Jennings et.al., 1986). The CD61 antigen is also found on endothelial cells, megakaryocytes, and on some myeloid, erythroid, and T-lmphoid leukemic cell lines (von dem Borne et. al. 1989 & Modderman, 1989). CD61 is used in following research applications

Platelet and platelet-derived microparticles in blood.
Megakaryocytes and megakaryocytic leukemias
Idiopathic thrombocytopenia in blood and tissue.
Cell adhesion.

II. PAC-1

PAC-1 recognizes an epitope on the glycoprotein IIb/IIIa (gpIIb/IIIa,$\alpha_{IIb}.\beta_3$) complex of activated platelets at or near the platelet fibrinogen receptor (Shattil et.al., 1985, Abrams et.al., 1990 & Shattil et.al., 1987). The gpIIb/IIIa complex is located on the surface membrane of resting platelets (Abrans & Shattil, 1991). Platelet activation induces a calcium-dependent conformational change in gpIIb/IIIa that exposes a ligand binding site (Ginsberg et. al., 1990 & Shattil et. al., 1993). Four adhesive macromolecules are capable of interacting with the activated form of gpIIb/IIIa: fibrinogen, vWf, fibronectin, and vitronectin (Bennet et.al., 1988). PAC-1 binds only to activated platelets and appears to be specific for this recognition site within gpIIb/IIIa (Michelson, 1996a, Taub et. al., 1989 & Abrams et. al., 1992). Approximately 45,000 to 50,000 gp IIb/IIIa receptors appear on the platelet surface upon activation (Shattil, 1985 & Abrams, 1991). The binding of fibrinogen to gpIIb/IIIa receptor is required for platelet aggregation and PAC-1 inhibits fibrinogen mediated platelet aggregation. PAC-1 is a pentameric IgM κ-immunoglobulin. Activation-dependent antibody binding can be affected by fixation and choice of anticoagulant. PAC-1 will not bind fixed platelets or EDTA-treated blood.

III. CD62P

The CD62P antigen, also known as platelet activation-dependent granule-external membrane (PADGEM) protein or granule membrane protein (GMP-140), is a 140-kdalton single-chain polypeptide (Larsen et. al., 1989). The CD62P antigen is a member of the selectin family of adhesion molecules and mediates the adhesion of activated platelets to neutrophils and monocytes in hemostasis (Larsen et.al., 1989, Hamburger & McEver, 1990 & Parmentier et.al., 1990).

The CD62P antigen is an integral membrane protein associated with α-granules of platelets, Weibel-Palade bodies of endothelial cells, and megakaryocytes. It is expressed on the internal α-granule membrane of resting platelets. Upon platelet activation and granule secretion, the α-granules membrane fuses with the external plasma membrane and the CD62P antigen is expressed on the surface of the activated platelets (Larsen et. al., 1989, Stenberg et. al., 1985, Hamburger & McEver, 1990, Metzelaar et. al., 1990 & Bonfanti et. al., 1989). CD62P antibody is composed of IgG_1 heavy chains and kappa light chains. It is being used in identification of in vivo-activated platelets, studies of platelet aggregation and also for in vitro platelet activation.

Analysis and Data acquisition

Logarithmic amplification for forward scatter (FSC) and side scatter (SSC) was selected. CD 61 PerCP positive events were acquired. Platelets stained with isotype control were used to adjust FL1 and FL2 voltage so that FL1/FL2 baseline signals are depicted squarely in the first decade in FL1 vs FL2 dot plot. Platelets stained with CD 62 PerCP and activation dependent monoclonal antibodies were used to adjust compensation. Ten thousand activation independent platelet events were acquired for each sample. Total platelet population was displayed as two color dot plots and the results were used for statistical analysis. For PAC-1 positive events cell percentage present in quadrant 2 and 4 were added. Similarly for CD 62P positive events quadrant 3 and 4 were added. Events, which were present in quadrant 4, were those platelets, which were positive for both PAC-1 and CD 62P.

CV of both activation dependent antibodies was calculated with standard formula and it was 5.22 and 3.8% for both CD62P and PAC-1 respectively. After standardizing the technique, we evaluated platelet activation markers in 49 post-menopausal (56.16 ± 0.083 years; mean ± SEM) and 42 pre-menopausal women (39.38 ± 1.09). Informed verbal consent was taken before recruiting subjects. Already standardized technique was used for *in vivo* evaluation of platelet activation markers by flow cytometer using 3-color analysis (CD61 PerCP, CD 62P & PAC-1). Care was taken during blood sampling and processing to minimize *in vitro* activation of platelets.

RESULTS:

The aim of the study one was to optimize the method for detection of platelet activation markers by flow cytometer. Accuracy and precision of the technique was first determined. Blood samples from 21 subjects were divided into two and stained and ran separately for the determination of CD 62P and PAC-1. Means and standard deviations of readings were calculated which were used to calculate the co-efficient of variation. The co-efficient of variation for both CD 62P and PAC-1 were 5.2 and 3.8% respectively. The mean values and CV calculated for both CD 62P and PAC-1 respectively.

To achieve our secondary objectives, a total of 91 women, 49 post and 42 pre-menopausal recruited. Clinical characteristics and variable of interests of volunteers are given in tables.

Calculation of standard deviation and coefficient of variation of CD62P

No.	Observation 1 (x)	Observation 2 (y)	(x-y)	$(x-y)^2$
1	1.00	1.04	-0.04	0.0016
2	0.25	0.28	-0.03	0.0009
3	0.49	0.40	0.09	0.0081
4	0.55	0.58	-0.03	0.0009
5	0.85	0.75	0.10	0.01
6	0.12	0.15	-0.03	0.0009
7	0.55	0.49	0.06	0.0036
8	0.37	0.32	0.05	0.0025
9	0.33	0.37	-0.04	0.0016
10	0.04	0.06	-0.02	0.0004
11	0.36	0.28	0.08	0.0064
12	0.33	0.34	-0.01	0.0001
13	4.53	4.66	-0.13	0.0169
14	3.25	3.33	-0.08	0.0064
15	0.74	0.69	0.05	0.0025
16	0.35	0.25	0.10	0.01
17	0.19	0.23	-0.04	0.0016
18	0.57	0.61	-0.04	0.0016
19	1.35	1.24	-0.11	0.0121
20	1.22	1.19	0.03	0.0009
21	1.05	1.09	-0.04	0.0016
Total	**18.49**		**1.2**	**0.0906**
Mean	**0.8804**		**0.057**	

$$SD = \sqrt{1/2n \sum (x-y)^2}$$
$$= \sqrt{1/2 \text{ X } 21 \text{ (0.0906)}}$$
$$= \sqrt{0.0021}$$
$$SD = 0.046$$
$$CV = SD / Mean \text{ X } 100$$
$$= 0.046 / 0.8804 \text{ X } 100$$
$$CV = 5.22\ \%$$

Calculation of standard deviation and coefficient of variation of PAC 1

No	Observation 1 (x)	Observation 2 (y)	(x-y)	$(x-y)^2$
1	4.69	5.22	-0.53	0.2809
2	5.52	5.28	0.24	0.0576
3	2.77	2.67	0.1	0.01
4	5.77	5.21	0.56	0.3136
5	4.19	4.09	0.1	0.01
6	1.59	1.70	-0.11	0.0121
7	4.40	4.22	0.18	0.0324
8	1.35	1.51	-0.16	0.0256
9	1.51	1.55	-0.04	0.0016
10	0.89	0.74	0.05	0.0025
11	0.74	0.75	-0.01	0.0001
12	1.47	1.50	-0.03	0.0009
13	19.12	19.51	-0.39	0.1521
14	9.19	9.74	-0.55	0.3025
15	4.38	4.46	-0.08	0.0064
16	1.43	1.32	0.11	0.0121
17	1.11	1.01	0.1	0.01
18	3.81	3.84	-0.03	0.0009
19	18.42	18.51	-0.09	0.0081
20	11.69	11.87	-0.18	0.0324
21	12.18	11.36	0.82	0.6724
Total	**116.22**		**4.46**	**1.944**
Mean	**5.534**		**0.212**	

$$SD = \sqrt{1/2n \Sigma (x-y)^2}$$
$$= \sqrt{1/2 \text{ X } 21 \ (1.944)}$$
$$= \sqrt{0.046}$$
$$SD = 0.215$$
$$CV = SD / Mean \text{ X } 100$$
$$= 0.215 / 5.534 \text{ X } 100$$
$$CV = 3.8\%$$

Clinical characteristics of Pre-menopausal women

VARIABLE	MINIMUM	MAXIMUM	MEDIAN	STANDARD DEVIATION
AGE	22	51	40.00	7.07
BMI	15.61	35.10	23.95	4.21
CHOLESTEROL	3.65	7.06	5.53	0.85

Clinical characteristics of Post-menopausal women

VARIABLE	MINIMUM	MAXIMUM	MEDIAN	STANDARD DEVIATION
AGE	47	65	55.00	5.79
BMI	16.70	35.10	25.80	4.37
CHOLESTEROL	4.45	10.04	6.10	1.26
ESTRADIOL	18.40	66.60	35.46	11.58

(Age in years; BMI in kg / meter square; Cholesterol (Total cholesterol) in mmol/L; Estradiol levels in pmol/L)

To compare different parameters between the two groups one way ANOVA was done. Age and total cholesterol was significantly different between the two groups while it was not the case with BMI which was not significantly different between the two groups.

Results from study 2 showed that platelet activation markers CD62P and PAC1 were significantly low in Premenopausal groups as compared to post menopausal group. In post menopausal women CD62P was 7.2855± 1.6354 vs. 0.8887 ± 1.922 (mean ± SEM, $p < 0.001$) of the pre-menopausal women. PAC-1 in post menopausal women was significantly higher (29.4448 ± 3.0456, $p = 0.001$) as compared to pre-menopausal women (5.2201 ± 0.7101, $p = 0.000$).

One of the secondary objectives of the study was to correlate platelet activation markers with age, cholesterol and BMI in both the groups and also to correlate estradiol level with activation markers in post-menopausal group of volunteers.

Comparison of clinical characteristics of both pre- and post- menopausal women.

VARIABLE	PRE-MENOPAUSAL	POST-MENOPAUSAL	p value
AGE	39.38±1.09	56.16±0.83	0.000
BMI	25.09±0.64	25.35±0.62	0.775
CHOLESTEROL	5.53±0.13	6.41±0.18	0.000

(Age in years; BMI in kg / meter square; Cholesterol (Total cholesterol) in mmol/L)

Correlation between age, cholesterol, BMI and CD62P

Variables	Correlation co-efficient r	p value	Significance
Age	r = 0.373	p = 0.000	S
BMI	r = - 0.150	p = 0.15	NS
Cholesterol	r = 0.285	p = 0.007	S

Correlation between age, cholesterol, BMI and PAC-1

Variables	Correlation co-efficient r	p value	Significance
Age	r = 0.586	p = 0.000	S
BMI	r = - 0.107	p = 0.31	NS
Cholesterol	r = 0.375	p = 0.000	S

DISCUSSION:

To contribute in haemostasis, platelets need to be activated. However pathological activation may result in pathological thrombosis. This pathological platelet activation has been noted in many clinical conditions like DVT, unstable angina and stroke. There are many assays to monitor the status of the patients platelets, however they all measure different things and therefore give a different picture of platelets. Physiologic assays in particular can certainly show that platelet function has been inhibited but cannot effectively show that platelets have become activated. Also these are crude techniques which cannot detect the subtle changes. Platelet count should be normal for these assays and these tests cannot be performed in thrombocytopenic patients. Biochemical assays on the other hand can detect platelet activation long before they can be detected in physiologic assays. There are two principal assay system, ELISA and flow cytometry and ELISA has the advantage of being easy to perform and does not require expensive equipment. While on the other hand flow cytometry allows us to see, changes in individual platelets although flow cytometer is probably the most accurate assay. The ease of using ELISA makes the later most common assay system used. In flow cytometry another important though not a major factor is what antibodies are used from many available in the market. Different antibodies have different significance as e.g. PAC-1 induction occurs with weak stimulation while CD63 antigen requires very strong stimulation. On the other hand, GMP 140 (P selectin , CD62P) does not preserve well and is unlikely to exist in the circulation because it is cleared from the circulation by spleen.

In our study blood sampling was done by the investigator to avoid artifactual activation of platelets. Subjects with difficult or traumatic venepuncture were not included for further platelet activation studies. Tourniquet was not used for drawing blood and where necessary was used only for venepuncture. This was done since Ritche et. al. (2000b) in their

study suggested effects of tourniquet pressure on plasma fibrinogen, platelet P-selectin, monocyte tissue factor and concluded that tourniquet pressure can be used while drawing blood sample for these three heamostatic variables. However in their study they did not study the change in GPIIb/IIIa with use of tourniquet. Due to these factors the use of tourniquet pressure was kept to the minimal.

To meet with objective 1 of primary objectives, co-efficient of variation calculated for the study I for both CD62P and PAC-1; 5.2 and 3.8 % respectively showed that the technique was good and comparable with other studies. A relatively high CV for CD 62P was partially due to high variation in the percentage of this activation marker resulting from high sensitivity of the technique.

Later part of the study also showed that the platelet activation markers PAC-1 and CD 62P were significantly higher in post menopausal women, as compared to the pre-menopausal group. The levels of platelet activation markers in this study were compared with the control group since every study sample was run with control group sample to avoid over interpretation of results by faulty technique. The finding of increased platelet activation markers in post menopausal age group may correlate with the frequent thromboembolic events and may suggest a role of platelet activation in an increased incidence of cardiovascular disease in this population. Other studies have also shown an association between platelet activation or dysfunction and pulmonary embolism, deep vein thrombosis and disseminated intravascular coagulation.

Negative correlation was noted between estradiol and CD 62P in post-menopausal women showing the beneficial effect of estradiol on platelets. Similarly PAC-1 has negative correlation in our study with estradiol. Other studies, which have shown the effect of hormones on platelets, showed indirect evidence of beneficial effect of HRT on platelets. This is the first study, which showed the beneficial effect of estradiol on platelets determined by activation dependent markers on flow cytometer. There was also positive correlation observed between age and activation markers showing the effect of age on platelets and this might be explained by age related changes in vessel wall and its function. Serum cholesterol also showed positive correlation while there was no positive correlation between activation markers and BMI. This might be explained due to difficulty in venepuncture in volunteers with high BMI. The samples with difficult venepuncture were discarded and were not included in the study to avoid artifactual platelet activation.

When we studied our volunteers there was no clinical evidence of cardiovascular and thrombotic disease. However increase platelet activation was noted in post-menopausal women, this is why we can say there are other factors apart from the activation markers which results in clinical complications and out come in particular patient. However platelets play an important role in arterial thrombosis

Our study has several limitations, one of which is lack of measurement of platelet function. Platelet function was not measured in our study due to lack of availability of required facilities for such measurements. One limitation of the study is that we have not looked into the reactivity of the platelet in both groups of population that is no *in vitro* stimulation was done. In other studies platelet reactivity was done with platelet activation markers. However these studies increase activation markers as determine *in vivo* technique.

CONCLUSION:

Results of this work demonstrated the effect of menopause on platelet activation markers. Following conclusions can be made from the results of our study.

a) Determination of platelet activation markers by flow cytometer is a sensitive accurate technique.

b) Platelet activation markers CD62P and PAC-1 were increased in post-menopausal women as compared to pre-menopausal women
b) With decrease in estradiol levels there is increase in activation markers in post-menopausal group.
c) Also, there is increase in the activation markers with increase in serum cholesterol and with increase in the age.
These results may suggest a role of platelets in increased incidence of thrombotic events and diseases in post-menopausal women.

FUTURE RECOMMENDATIONS

Further studies are needed to be undertaken to investigate the effect of hormone replacement therapy in post-menopausal women on platelet activation markers. As we have shown in our study that there is negative correlation between estradiol level and CD62P and PAC-1. This can be achieved by double blinded cross over interventional study. Besides long term prospective looking at incidence of thrombotic events and platelet activation among post menopausal women. Such studies shall give more conclusive evidence about the possible effect of platelet activation markers.

CLINICAL IMPLICATIONS OF THE STUDY:

Demonstration of increased activation markers may provide valuable marker for evaluating patients with high risk for clinical complications and also to evaluate efficacy of anti-platelet drugs or effect of hormone replacement therapy in this susceptible group of population.

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Determination of Platelet Activation Markers in post and pre-menopausal women by Flow cytometer.

(Presented in Malaysian Society of Hematology Meeting 2002, NCMS USM 2002 & ISEH) (Best oral presentation and travel grant award)

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Abstract

Introduction. Pre-menopausal women have lower risk of cardiovascular disease compared to post menopausal women which is more age dependent in women than in men. Increased and disturbed platelet activation plays a major role in the pathophysiology of thrombosis and has been noted in different thrombotic disorders. Temporal association between cardiovascular disease and increased platelet activation is well established. The role of platelet activation needs to be evaluated in this population.

Methods. Evaluation of Platelet activation markers was done in 49 post-menopausal (56.16 ± 0.83 years; mean ± SEM) and 42 pre menopausal women (39.38years ± 1.09). Subjects were recruited after informed verbal consent. The technique used for in vivo evaluation of Platelet Activation Markers by Flow cytometer using 3-color analysis (CD61 PerCP, CD62P and PAC1) was previously developed and optimized. Blood samples were drawn in a standardized manner to minimize in vitro activation of platelets. Samples were processed and analyzed in duplicate.

Results. There was a significant increase in CD62P in post-menopausal women as compared to the control (pre-menopausal) group (7.28 ± 1.63 vs 0.8887 ± 0.1922; mean ± SEM, $p=0.001$). Similarly PAC1 were significantly increased in post-menopausal group (29.45 ± 3.04 vs 5.22 ± 0.710; $p<0.001$). However there was no significant correlation between CD62P or PAC1 markers with serum triglycerides, estradiol and body mass index in both groups.

Conclusion. Platelet Activation markers CD62P and PAC1 are increased in post-menopausal women as compared to premenopausal women. These results may suggest a role in high incidence of cardiovascular disease in this group.

Key words. Platelet activation markers, Post-menopausal women, Flow cytometry.

Methodological development for in vivo determination of Platelet Activation Markers by Flow cytometry

(Presented in Malaysian Society of Hematology Meeting 2002)

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Abstract

Background: Platelet activation either disturbed and / or increased plays a major role in the pathophysiology of thrombosis. Different techniques show changes in platelet reactivity but cannot establish its association with the particular condition. Some techniques on the other hand determine this association but cannot measure changes in platelet reactivity and / or the extent of activation of individual platelets or detect distinct subpopulation of platelets. Diagnostic value of the flow-cytometric analysis of platelets activation in prothrombotic syndromes (diabetes, anti-phospholipid syndrome or secondary to drug induced platelet activation) is being established. Platelet aggregation studies are semi-quantitative and subject to standardization problems. Plasma separation is required for radioimmunoassays of plasma b-thromboglobulin and platelet factor 4 concentration are vulnerable to artifactual in vitro platelet activation. Soluble P-selectin in plasma may be of endothelial origin. Whole blood flow-cytometry assay has none of these limitations. Our aim was to develop and optimize the later technique.

Methods: Blood was drawn using standardized technique to minimize artifactual activation of platelets. And staining of platelets was performed within 10 minutes of sample collection. Sodium citrate (3.8%) was used as anticoagulant. Using butterfly cannulae (20 gauge) first 6mls of the sample was used for biochemical and hematological investigations and then 2mls were collected in a final dilution with anticoagulant of 1:9. Use of tourniquet was kept minimal only for puncture, where necessary. Three colors analysis of platelet activation was performed using CD61 PerCP, PAC-1 and CD62-P. Tests were performed in duplicate. Data acquisition and analysis were done using Cell Quest software and Becton Dickson flow cytometer.

Conclusion: Flow cytometery is a very sensitive and accurate technique for activation analysis of platelets and the use of this technique has good clinical implication. It has been shown to have high diagnostic sensitivity in ongoing thrombogenesis in subclinical progression of peripheral vascular disease. It does not have limitations seen by other techniques.