IDENTIFICATION OF LOW MOLECULAR WEIGHT METABOLITES THAT CAN PREDICT LOW DOSE ASPIRIN INDUCED GASTRIC TOXICITY AND RESISTANCE IN RATS AND STABLE CORONARY ARTERY DISEASE PATIENTS USING NMR-BASED PHARMACOMETABONOMICS

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by

SHA'ABAN ABUBAKAR

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LIST OF ABBREVIATIONS

1D	One-Dimensional
^{1}H-NMR	Proton Nuclear Magnetic Resonance
2D	Two-Dimensional
AA	Arachidonic Acid
ACEI	Angiotensin Converting Enzyme Inhibitor
ACS	Acute Coronary Syndromes
ARB	Angiotensin II Receptor Blockers
ARU	Aspirin Reaction Units
AUROC	Area Under the Receiver Operating Characteristic
BMI	Body Mass Index
BMRB	Biological Magnetic Resonance Data Bank
CAD	Coronary Artery Disease
CADP	Collagen Together with Adenosine Diphosphate
CCBs	Calcium Channel Blockers
CE-MS	Capillary Electrophoresis-Mass Spectrometry
CEPI	Collagen Together with Epinephrine
COX	Cyclooxygenase
CT	Closure Time
CVD	Cardiovascular Diseases
CYP	Cytochrome P450 Enzyme
D_2O	Deuterium Oxide
DAPT	Dual Antiplatelet Therapy
DES	Drug-Eluting Stents
DESI	Desorption Electrospray Ionization

DM	Diabetes Mellitus
DNA	Deoxyribonucleic Acid
DP	Prostaglandin D2 Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
EP	Prostaglandin E2 Receptor
ESC	European Society of Cardiology
FDR	False Discovery Rate
FP	Prostaglandin F2α Receptor
GC-MS	Gas Chromatography-Mass Spectrometry
GIT	Gastro-Intestinal Tract
H_2RA	Histamine H_2-Receptor Antagonist
HCA	Hierarchical Cluster Analysis
HMDB	Human Metabolome Database
HOX	Hydroperoxidase
HRMAS	High-Resolution Magic Angle Spinning
IHD	Ischaemic Heart Disease
IP	Prostacyclin Receptor
JRES	J-Resolved
k-NN	K-Nearest Neighbours
L	Litre
LC-MS	Liquid Chromatography-Mass Spectrometry
LDA	Low Dose Aspirin
LDL	Low Density Lipoproteins
LTA	Light Transmittance Aggregometry
MEA	Multiple Electrode Aggregometry
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance

NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
OPLS-DA	Orthogonal Partial Least Squares -Discriminant Analysis
PC	Principal Component
PCA	Principal Component Analysis
PCI	Percutaneous Coronary Intervention
PFA	Platelet Function Analyzer
PG	Prostaglandins
PLA	Phospholipases
PLS	Partial Least Squares
PPI	Proton Pump Inhibitors
PRP	Platelet Rich Plasma
RF	Random Forests
RNA	Ribonucleic Acid
SD	Sprague-Dawley
SIMCA	Soft Independent Modelling of Class Analogy
$STxB_2$	Serum Thromboxane B_2
SVM	Support Vector Machine
TMAO	Trimethylamine-N-Oxide
TOCSY	Total Correlation Spectroscopy
TR	Thromboxane Receptor
TSP	Sodium 3- (Trimethylsilyl)-Propionate-2,2,3,3-D4
TXA_2	Thromboxane A_2
TXB_2	Thromboxane B_2
VIP	Variable Importance for The Projection
vWF	Von Willebrand Factor

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PENGENALAN METABOLIT BERAT MOLEKUL RENDAH YANG BOLEH MERAMAL TOKSISITI GASTRIK DAN KERINTANGAN TERARUH ASPIRIN DOS RENDAH DALAM TIKUS DAN DALAM KALANGAN PESAKIT KORONARI ARTERI YANG STABIL MENGGUNAKAN FARMAKOMETABONOMIK BERASASKAN NMR

ABSTRAK

Aspirin Dos Rendah (LDA) merupakan asas dalam pencegahan sekunder bagi penyakit koronari arteri (CAD). Walaupun terbukti keberkesanannya, ketoksikan gastro-usus menjadi kekurangan utama. Sebagai tambahan, sesetengah pesakit masih mengalami peristiwa atherotrombotik semasa mengambil profilaksis sekunder aspirin, istilah ini dikenali sebagai kerintangan aspirin. Punca sesetengah orang mengalami ketoksikan gastrik yang serius manakala yang lain tidak masih belum difahami dengan jelas. Begitu juga tiada penjelasan yang tepat bagi punca mengapa sesetengah pesakit terhindar daripada mengalami peristiwa sekunder manakala yang lain masih mengalaminya sekali lagi. Tujuan kajian ini adalah untuk menilai penggunaan farmakometabonomik dalam mencari metabolit baru yang boleh meramalkan ketoksikan gastrik aruhan aspirin dan kerintangan aspirin dalam tikus. Kajian ini juga bertujuan untuk mengesahkan metabolit tersebut dalam pesakit penyakit arteri koronari. Kajian ini melibatkan 2 fasa, iaitu fasa penemuan dalam tikus dan fasa pengesahan dalam manusia. Model pra-dos telah dibangunkan menggunakan data spektroskopi H-NMR dari cecair bio tikus Sprague Dawley (SD) dan identiti kelas masing-masing dalam tikus. Data pada awalnya menjalani analisis statistik multivariat termasuk analisis komponen utama dan analisis *orthogonal-partial least square discriminant*. Data kemudian dikelaskan kepada kumpulan yang mengalami kesan

toksik gastrik berbanding kumpulan yang tidak mengalami kesan toksik gastrik atau kumpulan yang mengalami kerintangan aspirin berbanding kumpulan yang sensitif aspirin untuk kajian ketoksikan gastrik dan seterusnya kajian kerintangan aspirin. Analisis statistik multivariat, daripada model-model yang telah dibangunkan, menunjukkan pemisahan yang signifikan antara 2 kelas dalam setiap kes. Hal ini juga membawa kepada pengenalpastian metabolit yang telah diskriminasi yang disahkan menggunakan pangkalan data metabolit. Analisa urin farmakometabonomik pra-dos telah mengenalpasti sitrat, metilamina, trimetilamina N-oksida dan hipurat sebagai biopenanda untuk toksisiti gastrik yang disebabkan oleh aspirin. Juga, analisis serum farmakometabonomik pra-dos mengenal pasti valina, laktat, asetoasetat dan piruvat sebagai biopenanda untuk toksisiti gastrik yang disebabkan oleh aspirin. Lactat, trimetilamina N-oksida dan 4-hidroksifenilasetat telah dikenalpasti sebagai penanda bio untuk kerintangan aspirin. Seterusnya, model yang dibangunkan dengan menggunakan data daripada tikus divalidasi dengan menggunakan model yang diperolehi daripada data pesakit manusia. Secara keseluruhan, daripada 6 model yang divalidasi (4 untuk ketoksikan gastrik dan 2 untuk rintangan aspirin), 5 model mempunyai kejituan yang sangat baik (nilai, >97%), semua model mempunyai ketepatan yang baik (nilai, >50%) dan 4 model mempunyai kesensitifan yang rendah (nilai, <5%). Kesensitifan yang rendah menunjukkan bahawa model yang dibangunkan menggunakan tikus tidak dapat diterjemahkan dengan sempurna untuk meramalkan ketoksikan gastrik dan / atau rintangan aspirin LDA dalam manusia. Oleh itu, kajian masa depan bagi membangunkan dan mengesahkan model perlu menggunakan spesies yang sama untuk mengelakkan masalah ini.

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ABSTRACT

Low Dose Aspirin (LDA) is the cornerstone of secondary prevention in coronary artery disease (CAD). Despite its established efficacy, it suffers a major setback of causing gastrointestinal toxicity. In addition, some patients still experience atherothrombotic events while on aspirin secondary prophylaxis, a term known as aspirin resistance. The reasons why some people experience its serious gastric toxicity while others do not is still poorly understood. Likewise, the reason why some patients are adequately protected from a secondary event while others experience another event is yet to be adequately understood. The aim of this project was to evaluate the use of pharmacometabonomics, in finding novel metabolites that can predict aspirin-induced gastric toxicity and aspirin resistance in rats. It also aimed at validating such metabolites in CAD patients. The study involved 2 phases, namely the discovery phase in rats and the validation phase in humans. Pre-dose models were developed using H-NMR spectroscopic data from the biofluids of Sprague Dawley (SD) rats and the respective class identities of the rats. The data were initially subjected to multivariate statistical analysis including principal component analysis and orthogonal-partial least square discriminant analysis. The class identities were either gastric toxic versus non-gastric toxic or aspirin resistant versus aspirin sensitive for the gastric toxicity and aspirin resistance studies respectively. The multivariate statistical analysis, from which

the models were developed, showed a significant separation between the 2 classes in each case. They also led to identification of discriminating metabolites which were confirmed using metabolite databases. Pre-dose pharmacometabonomic urine analysis identified citrate, methylamine, trimethylamine N-oxide and hippurate as biomarkers for aspirin-induced gastric toxicity. Also, pre-dose pharmacometabonomic serum analysis identified valine, lactate, acetoacetate and pyruvate as biomarkers for aspirin-induced gastric toxicity. Lactate, trimethylamine N-oxide and 4-hydroxyphenylacetate were identified as biomarkers for aspirin resistance. Finally, the models developed using the data from rats were validated with models developed using data from human patients. Overall, out of the 6 validated models (4 for gastric toxicity and 2 for aspirin resistance), 5 models had an excellent specificity (value, >97%), all models had a good accuracy (value, >50%) and 4 models had a poor sensitivity (value, <5%). The poor sensitivity indicates that models developed using rats could not perfectly translate to predict LDA-induced gastric toxicity and/or aspirin resistance in humans as they did in the rats. Future studies should therefore develop and validate a model using the same species to avoid this problem.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Research background

1.1.1 The burden of cardiovascular diseases (CVDs)

Cardiovascular diseases (CVD) are diseases that affect the heart, such as coronary heart disease, stroke, heart failure and hypertension (Benjamin et al., 2018). According to the Heart disease and statistics, 2018, CVDs are the principal cause of mortality worldwide (Benjamin et al., 2018). Just like other countries, the burden of CVD also greatly affects Malaysia. It remains the principal cause of death for more than a decade in Malaysia, accounting for 20-25% of all deaths in public hospitals (Ministry of Health, 2014). Among the CVDs, Coronary Artery Disease (CAD) is the leading cause of such mortalities. In 2017, CAD accounted for 13.9% of all recorded deaths (168,168 deaths) in Malaysia (Department of Statistics Malaysia, 2018).

1.1.2 Coronary artery disease

Coronary Artery Disease is a condition in which the vascular supply to the heart is obstructed by fat, occlusion or spasm of coronary arteries (Mcrobbie, 2011). This may impair the provision of aerated blood to cardiac tissue sufficiently to cause cardiac muscle ischemia that, if severe or prolonged, could cause the death of heart muscle cells (Mcrobbie, 2011). The terms, “Coronary Artery Disease, Coronary Heart Disease (CHD) and ischaemic heart disease (IHD)”, are often used interchangeably (Mcrobbie, 2011).

However, more specifically, CAD refers to atherosclerotic involvement of the coronary arteries, while IHD is usually used to refer to the presentation of clinical symptoms and CHD often includes other factors leading to insufficient blood flow to heart muscles, such as valvular heart disease. The term CAD will be used subsequently throughout this thesis and is used to refer to atherosclerotic heart disease affecting the coronary arteries of the heart.

1.1.3 Pathophysiology of coronary artery disease

Obstruction of blood flow by atheromatous plaques within the coronary arteries is the most typical reason behind stable coronary artery disease and low-risk unstable angina. Anginal symptoms are a clinical manifestation of ischemia. Risk factors to the advancement of CAD include diabetes, hyperlipidaemia, hypertension, obesity, and smoking (Abrams, 2005).

Exposure of the blood vessel epithelial tissue to the products of glycosylation associated with diabetes, Low Density Lipoproteins (LDLs), vasoconstrictor hormones associated with hypertension, excess adipose tissue or proinflammatory molecules from smoking results in the expression of adhesion molecules that permit leukocytes to stay to the blood vessel wall. Upon entry into the artery wall, blood monocytes begin to scavenge lipids and become foam cells. Macrophage foam cells unleash further cytokines and effector molecules that stimulate smooth muscle cell migration from the arterial intima into the media, as well as smooth muscle cell proliferation. During this process, the prime fatty accumulation of lipoprotein within the blood vessel tissue layer develops into atherosclerotic plaques. Ischemic symptoms may manifest resulting from impediment of

blood flow due to atherosclerotic plaques or when a clot or vasospasm is overlaid on less critical plaques (Libby & Theroux, 2005).

The focal point for management of CAD should therefore be modifiable risk factor reduction through healthy lifestyle, by underscoring weight monitoring and control, increasing physical activity, blood pressure control, lipid control, and smoking cessation (Fihn et al., 2014). Antiplatelet therapy is recommended for every patient while dual antiplatelet therapy may be considered in designated patients (Fihn et al., 2014). Patients with chronic symptoms of angina should be furnished systematically with an add-on of beta-blockers, calcium-channel antagonists, and/or long-acting nitrates (Fihn et al., 2014). Revascularization to diminish symptoms may be necessary in patients with sustained angina undeterred by lifestyle adjustments and guideline-directed medical care. Besides, simultaneous revascularization, lifestyle adjustments and medical therapy is advocated for patients with high possibility of ischemia (Boden et al., 2007).

1.1.4 Role of platelets in coronary artery disease

Platelets are non-nucleated, disc-shaped blood components with a lifespan of 7-10 days in blood circulation before being removed by the spleen (Patrono & Rocca, 2012). The concentration of platelets ranges from 150 - 400 x 10^9/l of blood. Though, their principal function is haemostasis, they provide additional functions such in angiogenesis and innate immunity (Dimitrios, Dimitrios, & Konstantinos, 2012; Patrono & Rocca, 2012).

Platelets are activated in cases of vessel wall injury to form a fine unitary layer over the injured endothelium to avert excessive haemorrhage and to additionally initiate the

procedures of vessel wall repair. Nonetheless, under certain diseased conditions, they form pathogenic, unrestrained aggregates that lead to thrombotic events (Ruggeri, 2002). In such scenarios, dysfunction of the endothelium or the presence of atherosclerotic lesions leads to platelet adhesion, activation and aggregation. Myocardial ischemia or infarction presenting as acute coronary syndromes (ACS) may arise due to thrombotic incidents in the coronary arteries subsequent to sudden breakage of an atherosclerotic plaque coupled with platelet-mediated coronary vessel constriction and micro-embolization (Dimitrios et al., 2012). Inhibition of platelet function is therefore essential in the management of CAD. Several mechanisms of inhibition have been postulated, but the Cyclooxygenase (COX) inhibition is the most established pathway.

1.1.5 The cyclooxygenase-1 pathway

Arachidonic acid is a polyunsaturated fatty acid. It occurs in the phospholipids of membranes of the body's cells, and is ample in the brain, muscles, and liver. Arachidonic acid is also available in the phospholipids of platelet membrane and it is the major precursor of eicosanoids. Eicosanoids are a group of molecules comprising leukotrienes and prostanoids (prostacyclins, prostaglandins, and thromboxanes). They are signalling molecules made by the enzymatic or non-enzymatic oxidation of arachidonic acid (Smith, 1987).

Prostanoids are a sub-type of eicosanoids comprising of the prostaglandins (mediators of anaphylactic and inflammatory reactions), thromboxanes (mediators of blood vessel constriction), and prostacyclins (functional in the resolution stage of inflammation). Prostaglandins (PG) and thromboxane A_2 (TXA_2), are produced after arachidonic acid

(AA) is released from the plasma membrane by phospholipases (PLAs) and metabolized by the successive actions of prostaglandin G/H synthase (also known as Cyclooxygenases), and other synthases (Ricciotti & FitzGerald, 2011).

Arachidonic acid is released from the membrane phospholipids by numerous forms of PLA, which are activated by various stimuli. Arachidonic acid is converted to the unstable intermediate prostaglandin H_2 by cytosolic prostaglandin H synthases, which have both COX and hydroperoxidase (HOX) activity (Ricciotti & FitzGerald, 2011). Prostaglandin production depends on the activity of COX, which occurs as peculiar isoforms referred to as COX-1 and COX-2.

COX-1, which is constitutively found in most cells, is the major source of prostanoids that aids homeostasis functions, such as gastric epithelial cyto-protection (Ricciotti & FitzGerald, 2011). Meanwhile, COX-2, which is promoted by inflammatory stimuli, hormones and growth factors, is the principal source of prostanoids synthesis during inflammation and in proliferative diseases, like cancer. Nevertheless, the two enzymes contribute to both homeostatic and inflammatory conditions (Ricciotti & FitzGerald, 2011).

1.1.6 Thromboxane A_2 and B_2

TXA_2 plays a distinctively vital role in platelet aggregation. TXA_2 is produced by activated platelets and acts as a potent vasoconstrictor and stimulator of platelet aggregation mainly by increasing platelet expression of glycoprotein IIb/IIIa fibrinogen receptors (Würtz, 2015). It further propagates the activation signal to adjacent platelets

contributing to additional platelet activation and TXA_2 release, thereby initiating an amplification circle (Patrono, 2013; Würtz, 2015). TXA_2 exerts its effect during primary haemostasis and large amounts are released during platelet aggregation. TXA_2 (half-life = 30-40 seconds) is instantly hydrolysed non-enzymatically to its biologically inert metabolite TXB_2 (half-life = 5-7 minutes), which is then rapidly metabolized to form urinary metabolites for renal clearance. Given the short-lived nature of TXA_2, measurement of serum TXB_2 or the urinary metabolites, 11-dehydro TXB_2 and 2,3- dinor TXB_2, reflects endogenous TXA_2 synthesis with a greater degree of certainty than measurement of the parent compound (Patrono, 2013).

1.2 Antiplatelet Therapy

1.2.1 Acetylsalicylic acid (Aspirin) and dual antiplatelet therapy (DAPT)

Low Dose Aspirin (LDA) is the anchor of secondary prevention in CAD. Aspirin therapy is virtually taken for life in coronary artery disease patients (Levine et al., 2016). Aspirin is the backbone of dual antiplatelet therapy (DAPT); a term used precisely to refer to the concurrent use of aspirin and a P_2Y_{12} receptor inhibitor (ticagrelor, prasugrel or clopidogrel) (Levine et al., 2016). It is the standard of care for prevention of cardiovascular outcomes in patients with CAD undergoing percutaneous coronary intervention (Lewis et al., 2013).

The recommended dose of aspirin ranges from a once daily dose of 75 mg to 100 mg per orally in patients treated with DAPT (Levine et al., 2016). For non-DAPT, aspirin is usually taken at a dose of 75-325mg according to different guidelines but the optimal risk–benefit ratio appears to be achieved with aspirin in a dose range of 75–150 mg/day (Montalescot et al., 2013). The latest version of "the Malaysian clinical practice guideline on management of Stable Coronary Artery Disease" (Ministry of Health, 2018) also adapts this dose.

The two main goals of DAPT is prevention of stent thrombosis and reduction in systemic atherothrombotic events (Bittl, Baber, Bradley, & Wijeysundera, 2016). Generally, the length of DAPT after PCI with implantation of a newer-generation drug-eluting stents (DES) involves a simultaneous assessment between a decrease in stent thrombosis/MI and an increase in bleeding. Previously (Levine et al., 2011), a minimum duration of at least

12-month DAPT was endorsed irrespective of the clinical presentation. Recently (Bittl et al., 2016), with the advent of safer newer-generation DES, there has been a little paradigm shift, recommending a minimum of 3-6 month and a maximum of 12-month DAPT. Twelve months of DAPT was the most recommended duration based on trade-off between lower risk of complications related to bleeding and greater benefit of ischemic protection (Levine et al., 2016). Prescription beyond 12 month requires a critical appraisal of the risk/benefit and may only be warranted in peculiar patient scenarios (Bittl et al., 2016; Levine et al., 2016).

1.2.2 Pharmacology of aspirin

Aspirin inhibits platelet reactivity by irreversibly inactivating the vital platelet protein, COX-1 which leads to persistent suppression of platelet TXA_2 production and TXA_2-mediated platelet activation and aggregation (Patrono, 2015). This effect explains aspirin's distinct (from other COX-1 inhibitors) efficacy in preventing atherothrombosis, as well as its common (with other antiplatelet agents) bleeding glitches (Patrono, 2015). Unlike with aspirin, COX-1 inhibition by other non-steroidal anti-inflammatory drugs (NSAIDs) is reversible.

Aspirin attains a peak plasma level about 30 min after intake due to its prompt absorption in the stomach and upper small intestine. It has an oral bioavailability of 45 - 50%. COX-1 enzyme is in control of the conversion of arachidonic acid to TXA_2 (Eikelboom, Hirsh, Spencer, Baglin, & Weitz, 2012; Pettersen, Arnesen, & Seljeflot, 2015). Because aspirins' inhibition of the COX-1 enzyme is irreversible, its antiplatelet effect lasts for the 7 to 10-

day lifetime period of the platelets. The non-nucleated nature of platelets does not permit them to regenerate COX-1, making the platelet inhibitory effect to be renewed only through the generation of new platelets (Pettersen et al., 2015). This justifies the use of once daily dosing of aspirin despite its short half-life (15-20 mins).

Aspirins' mechanism of action is clearly depicted in Figure 1-1. A low-dose aspirin selectively inhibits COX-1, whereas a high-dose inhibits both COX-1 and COX-2. Prostaglandin H_2 is converted to multiple prostanoids by tissue-specific isomerases. These bioactive lipids activate specific cell membrane receptors of the superfamily of G-protein-coupled receptors.

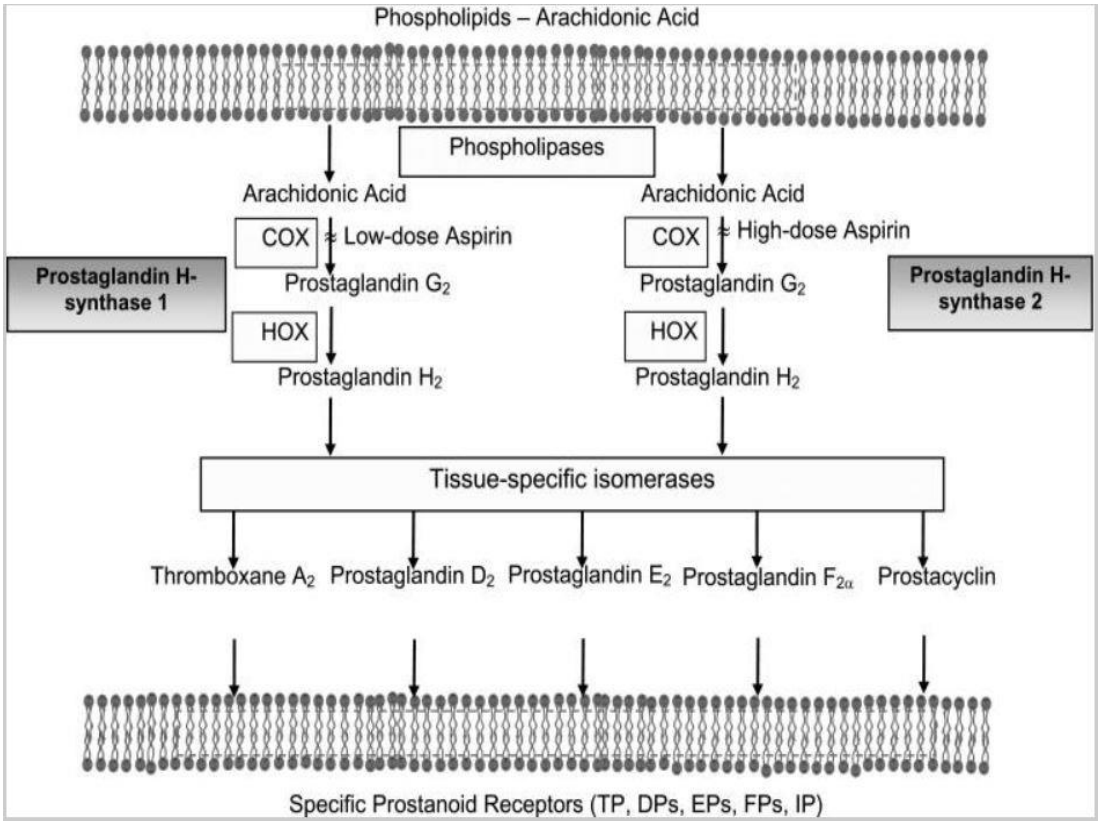

Figure 1-1 Arachidonic Acid Metabolism and Mechanism of Action of Aspirin

Adapted from (Eikelboom *et al*., 2012) COX=cyclooxygenase; DP=prostaglandin D2 receptor; EP=prostaglandin E2 receptor; FP=prostaglandinF2α receptor; HOX=hydroperoxidase; IP=prostacyclin receptor; TP=thromboxane receptor.

1.2.3 Side effects of aspirin

Despite the established efficacy, treatment with low-dose aspirin is accompanied by upper gastrointestinal (GI) side effects. These effects range from dyspepsia (point prevalence of 31%), gastro-duodenal erosions (point prevalence of 60%), endoscopic peptic ulcer (3-month incidence of 7%) to symptomatic or complicated ulcers (annual incidence of upper GI bleeding: 0.6%; relative risk of upper GI bleeding: 2.6) (Hsu & Tsai, 2015). The prevalence of upper GI ulcers is 10-40% amidst patients taking low-dose aspirin and aspirin heightens the risk of upper GI bleeding by up to 2-fold (Cryer & Mahaffey, 2014; Yamagata & Hiraishi, 2007).

The adverse events related to LDA in the upper gastrointestinal tract are highly distinct and include esophagitis, petechiae, gastroduodenal ulcers and recurring peptic ulcers (Sostres & Lanas, 2011).

1.2.4 Mechanism of gastrointestinal damage induced by aspirin

Local Effects: Prostaglandin depletion through COX inhibition seems to be the main mechanism responsible for the development of aspirin-induced gastroduodenal ulcers, however its direct contact with the gastric and duodenal mucosa can also induce injury by influencing the gastric epithelial cell barrier (Sostres & Lanas, 2011). Enteric coated formulations were designed to decrease the incidence of gastroduodenal injury. Meanwhile, clinical experience and some meta-analysis show no pertinent distinction in

the incidence of mucosal damage between enteric coated and non-coated aspirin formulations (Derry & Loke, 2000; Sostres & Lanas, 2011).

Systemic effects: The affinity of aspirin to COX-1 is more than 10-folds compared to COX-2 (Simmons, Botting, & Hla, 2004). COX-1 produces prostaglandins with cytoprotective effects while COX-2 produces PGs that mediate inflammation. Therefore, inhibition of COX-1 mediated PG synthesis is the primary mechanism through which aspirin results to injury to the upper GI mucosa (Lavie, Howden, Scheiman, & Tursi, 2017; Sostres & Lanas, 2011).

1.2.5 Gastric toxicity as a reason for low dose aspirin therapy discontinuation

Gastric toxicity is the major reason of non-adherence to aspirin therapy (Martín-Merino, Johansson, Bueno, & Rodríguez, 2012; Rodríguez, Cea-Soriano, Martín-Merino, & Johansson, 2011). Non-adherence on the other hand leads to treatment failure. A systematic review revealed a 10-50% poor compliance in patients taking low dose aspirin for prophylaxis against CVD (Herlitz, Tóth, & Næsdal, 2010). A study that investigated the reasons for discontinuation of LDA in CVD prophylaxis reported "stomach problems" among the major causes for therapy disruption or termination (Lavie et al., 2017).

1.2.6 Prevention and treatment of gastro-intestinal side effects of aspirin

Current strategies to ameliorate the GI toxicity of LDA include prescription of alternative antiplatelet therapy, co-therapy with gastro-protective agents, eradication of H. *pylori* and endoscopic therapy (Cryer & Mahaffey, 2014; Sostres & Lanas, 2011). Limitations from

all these, ranges from increase in cost burden on the patient (associated with all the strategies) to invasiveness (in the case of endoscopy). Other strategies employed to specifically mitigate against LDA-induced GI toxicities include development of risk stratification tools to evaluate cardiovascular and GI risks to ease the clinical assessment (Abu-Assi et al., 2012; Lanas, Polo-Tomás, & Casado-Arroyo, 2013).

It is important to point out once again that the assumption that enteric coated (EC) aspirin will give less gastric toxicity is unlikely as confirmed by previously large observational studies (de Abajo & García Rodríguez, 2001; Patrono & Rocca, 2017) and meta-analysis (Sostres & Gargallo, 2012), therefore EC aspirin is not superior to conventional formulation.

The best available strategy for the prevention of LDA-induced GIT toxicity is the concomitant use of aspirin with medications that reduce gastric acid secretion. Proton pump inhibitors (PPIs) are preferred options than H_2-receptor antagonists in this regard (Lavie et al., 2017). This may be explained by the PPIs, being acid-activated prodrugs, which bind covalently to cysteine residues on the luminal surface of the gastric H^+/K^+-ATPase, thereby inhibiting acid secretion (Lavie et al., 2017). Also, a meta-analysis of randomized controlled trials that compared the preventive abilities of PPIs to H_2-receptor antagonists in terms of LDA-induced GIT toxicities showed that the PPIs were superior to H_2-receptor antagonists in this regard (Lavie et al., 2017).

However, the option of concurrent usage with PPIs is also faced with some major challenges viz; LDA is taken for lifetime and therefore the economic burden on the patient is usually high and must be considered. Though researches on the pharmacodynamic drug-

drug interaction of PPI and clopidogrel (used as DAPT with aspirin) showed mixed results, it is still worthy of consideration (Agewall et al., 2013; Scott, Owusu, & Hulot, 2014; Sherwood et al., 2015). Moreover, the evidence that patient compliance to medication decreases with increase in the pill burden cannot be overemphasised (Farrell, French Merkley, & Ingar, 2013), coupled with the fact that most CAD patients have other co-morbidities requiring other medications.

1.2.7 Aspirin resistance or high on-aspirin residual platelet reactivity (HRPR)

The wide inter-individual variation witnessed in aspirin response despite an adequate decrease in thromboxane A_2 indicates the possible existence of unknown alternative, biochemical and metabolic pathways that augments aspirin's mechanism of action (Du, Lin, & Wang, 2016). Persistent platelet reactivity identified through a platelet function test is termed laboratory aspirin resistance (Hovens et al., 2007) whereas the occurrence of atherothrombotic events while still on aspirin therapy is termed clinical aspirin resistance (Hankey & Eikelboom, 2006). However, some researchers argue that, it is more appropriate to refer to the latter as "aspirin treatment failure" (Hankey & Eikelboom, 2006) or "high on-aspirin residual platelet reactivity" (Pettersen et al., 2015). The term aspirin resistance will be used in this research and it refers to persistence of platelet reactivity identified through a laboratory test.

1.2.8 Mechanism of aspirin resistance

Many factors have been implicated in the phenomenon known as aspirin resistance. Many studies have found patient non-adherence to therapy as one of the reasons for the presumed

aspirin resistance after a witnessed aspirin consumption (Homoródi et al., 2016; Schwartz, Schwartz, Barber, Reeves, & De Franco, 2008). Other causes of presumed resistance include drug-drug interactions (e.g. with NSAIDs or PPIs) and the use of enteric coated aspirin (due to decreased absorption). Potential causes of true aspirin resistance include heightened platelet turnover such as that seen in diabetes and essential thrombocythemia (Grove, Hvas, Mortensen, Larsen, & Kristensen, 2011; Pascale et al., 2012; Rocca et al., 2012), single nucleotide polymorphisms, inflammation, metabolic syndrome and miRNAs (Du et al., 2016; Floyd & Ferro, 2014). These have been summarized in Figure 1-2 below.

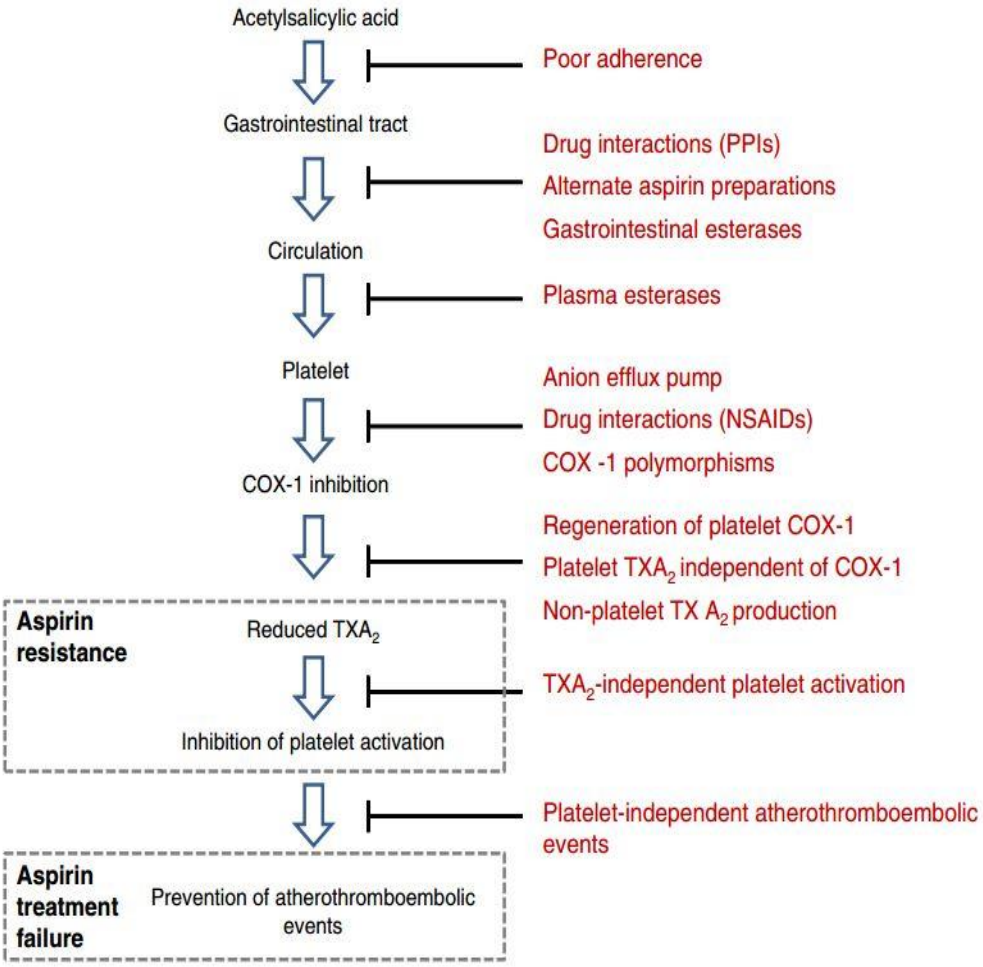

Figure 1-2 Mechanism of Aspirin Resistance

Adapted from (Floyd & Ferro, 2014). The boxes labelled 'aspirin resistance' and 'aspirin treatment failure' highlight the methodology to define the terms. [PPIs = proton pump inhibitors; NSAIDs = non-steroidal anti-inflammatory drugs; COX-1 = cyclooxygenase-1; TXA_2 = thromboxane A_2] (Floyd & Ferro, 2014)

1.2.9 Detection of aspirin resistance through platelet function tests

The effects of aspirin on platelets have been monitored through several laboratory tests. The light transmittance aggregometry (LTA) was regarded as the gold standard (Pettersen et al., 2015). This is an *ex vivo* platelet aggregation test done with platelet rich plasma (PRP). This involves addition of one of the different agonists of platelet (arachidonic acid, collagen, serotonin, ristocetin or adenosine-diphosphate, depending on the study rationale) and measuring the increase in light transmittance through the optically dense sample. The PRP should ideally become clearer after the addition of an agonist due to precipitation of platelet aggregates. A photometer then measures the change in light transmittance as a percentage from 0-100%. Zero percent implies no aggregation at all while 100% indicates total aggregation of platelets. The technique is however too laborious, time consuming and requires a highly skilled and experienced operator. Other non-point of care tests includes; lumiaggregometry and impedance aggregometry on whole blood. Point of care analysis frequently used include Multiple Electrode Aggregometry (MEA), Platelet Function Analyzer-100 (PFA-100; Dade Behring), Plateletworks (Helena Laboratories, Beaumont, Texas) and the VerifyNow aspirin assay (Accumetrics, San Diego, California).

The PFA-100 computes the duration it takes platelets to block the orifice of its test cartridges when whole blood is passed through under shear stress. The time taken by platelets to halt the citrated whole blood flow due to blockage of the orifice is referred to as closure time (CT). The cartridges either contain collagen together with epinephrine (CEPI) or collagen together with ADP (CADP). The CEPI is primarily used to measure

platelet inhibition due to aspirin therapy while the CADP is used to identify other intrinsic platelet defects like Von Willebrand disease. The technique is very sensitive but its dependence on Von Willebrand factor (vWF) and platelet haematocrit, and its expensive price are its major limitations (Gaoyu et al., 2016).

The VerifyNow aspirin Test uses arachidonic acid as agonist to trigger off platelets. It functions by quantifying platelet function based upon the capacity of activated platelets to bind fibrinogen. Fibrinogen-coated microparticles clump together in whole blood proportionate to the number of unblocked platelet GP IIb/IIIa receptors. Transmission of light increases as activated platelets bind and agglutinate fibrinogen-coated beads. This device computes the change in optical signal caused by clumping together of platelets and records the result as aspirin reaction units (ARU). A value < 550 ARU is consistent with aspirin-induced platelet inhibition while a value ≥550 ARU implies non-aspirin induced platelet dysfunction. The reference range value for pre-aspirin samples is 620-672 ARU (Accumetrics Inc., 2006).

1.2.10 Detection of aspirin resistance through thromboxane metabolites

Thromboxane metabolites are also used to assess the effect of aspirin on platelets. They have the advantage of directly measuring the effect of aspirin on COX-1. Thromboxane A_2 is the major product of the platelet arachidonic Acid metabolism. However, its transient nature ($t_{1/2}$=30-40s) due to rapid hydrolysis into TXB_2 makes its measurement non-practical. TXB_2 is biologically inactive but a stable product. It's metabolised and excreted into urine as 2,3-dinor-TXB_2 and in 11-dehydro-TXB_2. The latter is abundantly more than the former in urine. This makes it a target for the measurement of aspirin's effect on

platelet COX-1. In inflammatory diseases and other pathologic conditions, up to 30% of urinary metabolites may be derived from non-platelet sources. This makes the measurement of urinary thromboxanes to be non-specific for monitoring the effects of aspirin. Due to the ease and non-invasiveness of obtaining urine samples, urinary 11-dehydro-TXB_2 is still determined with the aid of the FDA approved AspirinWorks® test (Corgenix, Broomfield, Colorado) or other available kits, but it is usually normalised to creatinine to increase its predictive value.

On the contrary, serum thromboxane B_2 ($STxB_2$), exhibits the overall ability of platelet to synthesize TXA_2. This renders the contribution of other haematocytes in its synthesis to be negligible and hence making $STxB_2$ the most precise method to test the pharmacologic effect of aspirin on platelet (Cattaneo, 2013; Frelinger et al., 2008; Grove et al., 2010; Halvorsen et al., 2014; Rozalski, Watala, & Golanski, 2014). These reasons guided in the choice of $STxB_2$ enzyme linked immunosorbent assay (ELISA) to measure aspirin response in this research.

Serum thromboxane B_2 has been used in so many researches to measure the effect of aspirin on platelets. However, the major limitation of its use is the absence of a universally accepted cut-off value to define aspirin non-responsiveness (Fontana et al., 2010; Frelinger et al., 2009; Reny et al., 2012). The values are highly kit specific. Previous studies have found the use of receiver operator characteristic analysis of serum TxB_2 levels in relation to major adverse cardiovascular (Fontana et al., 2010; Frelinger et al., 2009; Reny et al., 2012) and the selection of at least 95% inhibition (Patrono & Rocca, 2007) of $STxB_2$ (compared to the pre-aspirin $STxB_2$ value) to increase its predictive value.

1.3 Systems biology and the "omics" sciences

"Systems biology is the study of the molecular, biochemical, and supramolecular networks, along with their connections as well as interactions with environmental factors in order to determine an intricate biological perturbation of the living organism"(Louridas, Kanonidis, & Lourida, 2010). This is an integrative approach that investigates biological "Omics" and the environmental factors that interfere with their systematic connections. "Omics" is a somewhat new terminology utilised in referring to the fields of genomics, epigenomics, transcriptomics, proteomics, metabolomics, microbiomics and a host of others. Unlike conventional biology, systems biology takes into account the connections and interactions between the biological processes in living system and their environment.

Genomics centres on determining genetic variants associated with a disease, response to a treatment, or future patient prognosis (Hasin, Seldin, & Lusis, 2017). Genomics is the most prominent of all the omics fields. However, epigenomics deals with genome-wide characterization of reversible alterations of DNA or its associated proteins, like DNA methylation or histone acetylation (Romanoski, Glass, Stunnenberg, Wilson, & Almouzni, 2015). Whereas, transcriptomics investigate RNA levels genome-wide, both qualitatively (which transcripts are present, identification of novel splice sites, RNA editing sites) and quantitatively (how much of each transcript is expressed) (Lowe, Shirley, Bleackley, Dolan, & Shafee, 2017). Proteomics is used to measure peptide abundance, modification, and interaction. While, microbiomics is a fast-growing field in which all the microorganisms of a given community are studied together (Hasin et al., 2017). When any of the omics is studied in relation to the effect of drugs, the prefix

"pharmaco" is attached, e.g. pharmaco-genomics. The relationship between the omics is highlighted in the Figure 1-3

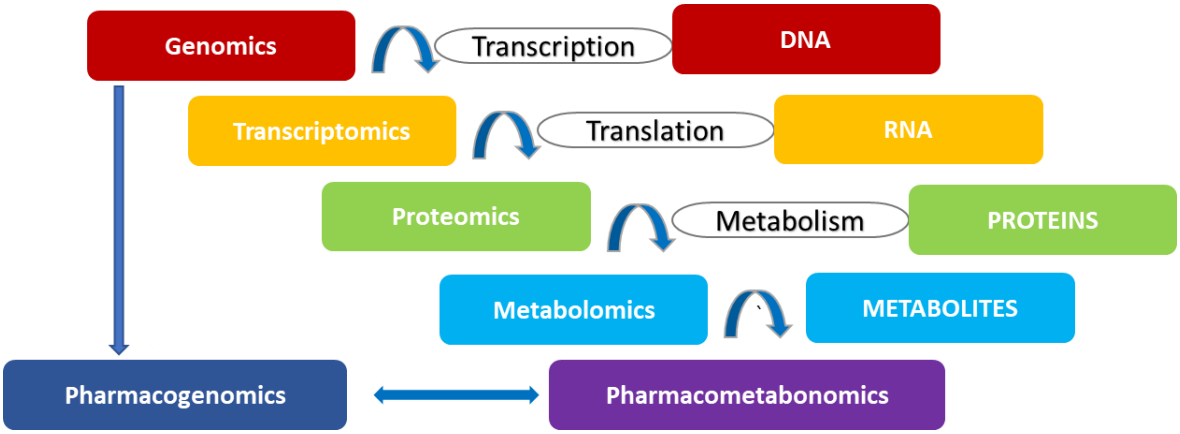

Figure 1-3 Relationship Between the Omics Sciences

1.4 Metabonomics and metabolomics

Metabonomics is defined as ‘‘The study of the metabolic response of organisms to disease, environmental change or genetic modification” (Lindon, Nicholson, Holmes, & Everett, 2000). Metabolomics on the other hand is defined as “The holistic and quantitative measurement of all the metabolites in a biological system”(Fiehn, 2002). The definition of metabolomics seems impractical, considering the complexity and unexhaustive number of metabolites in a biological system. Although the terms are sometimes used interchangeably in the literature, it is more prudent to stick to the term, metabonomics.

1.5 Personalised medicine

Personalised medicine is defined as “the use of the collective knowledge (genetic or otherwise) about an individual to predict disease susceptibility, disease prognosis, or treatment response and as such improve that individual’s health”(Redekop & Mladsi, 2013). Personalised medicine is also referred to as precision medicine or stratified medicine (Pokorska-Bocci et al., 2014). The present-day idea of personalised medicine is built upon pharmacogenomics to predict the effect of patient gene mutations on treatment outcomes. However, the fact that the effects of drugs on humans and vice versa is influenced by other environmental factors like the microbiome of the patient, asides their genetic profiles has constrained this concept (Everett, 2017). This made the clinical utility of pharmacogenomics less than earlier envisaged.

Pharmacometabonomics, however, can account for both genetic and environmental factors that affects the efficacy, safety, metabolism, transport, and pharmacokinetics of

drugs. Combination of both omics is therefore hoped to be able to provide more sensitive and specific predictions of drug safety and efficacy (Everett, 2017).

1.6 Pharmacometabonomics

In the year 2000, a new methodology that is interested in the prediction of drug effects using pre-dose metabolic profiles of biofluids was found and the outcomes published in 2006 (Everett, 2015). This was termed *Pharmacometabonomics*. It is defined as "the prediction of the outcome (e.g., toxicity or efficacy) of a drug or xenobiotic in an individual based on a mathematical model of pre-intervention metabolite signatures" (Clayton et al., 2006).

It is pertinent to highlight that Pharmacometabonomics is precisely an example of a broader group of experiments labelled as predictive metabonomics, where pre-event metabolite profiles may be utilised to predict post event outcomes; in this case, the event is drug dosing. The advantage of this approach is it that, it does not only reveal the variation in the genetic and metabolic profiles, it also expresses the environmental interaction with them (Guţiu et al., 2010). Several studies have shown the success of this approach in predicting efficacy and toxicity of drugs in animals and humans (Clayton, Baker, Lindon, Everett, & Nicholson, 2009; Everett, 2015; Everett, Loo, & Pullen, 2013; Park et al., 2013; Zhu et al., 2013). Few of these studies were related to response variability/resistance to aspirin (Ellero-Simatos et al., 2014; Ellero-Simatos et al., 2015; Lewis et al., 2013), but none was related to the gastric toxicity of aspirin. This can be a comprehensive, fast, economical, and less invasive approach to predict the gastric toxicity

of aspirin and thus help in the personalization of its therapy. The findings of some pharmacometabonomic studies are summarised in Table 1-1 below.