Faculty of Pharmacy, Nursing and Health Professions

Master's Program in Clinical Laboratory Science

Prevalence of Aspirin Resistance among Hemodialysis Patients: A Pilot Study in Palestine

By

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Birzeit-Palestine

February 2022

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Declaration

I certify that this thesis submitted for the degree of Master in Clinical Laboratory Sciences, is the result of my research, except where otherwise acknowledged, and that this study has not been submitted for a higher degree to any other university or institution.

Signed:

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Date:

Dedication

This research is passionately dedicated to my parents Mousa and Ibtisam Manasrah, whose inspiration was my motive to complete the whole journey and taught me the value of patience and hard work, whose support was endless on the spiritual, moral, and emotional levels.

It is also dedicated to my dear brothers (Mohammad, Nasha’t, Abdullah, and Mahmoud) and sisters (Anwar and Manar), all by their value in my heart, to my beloved wife Basima and adored son Nasha’t and my sincere friends whose value mean so much to me. I dedicate it to their kind and encouraging words, to their advice, and also for their energy to bear me during the hard time in the past period until the last day of this work.

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List of abbreviations

Abbreviations	Full name
11dhTxB2	11-Dehydrothromboxane B2
AA	Arachidonic acid
AC	Alternating current
ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AR	Aspirin resistance
ASA	Acetylsalicylic acid (aspirin)
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BUN	Blood urea nitrogen
cAMP	Cyclic adenosine monophosphate
CKD	Chronic kidney disease
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase 2
CRF	Chronic renal failure
CVD	Cardiovascular diseases
DTS	Dense tubular system
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

ESAs	Erythropoiesis stimulating agents
ESRD	End-stage renal disease
GFR	Glomerular filtration rate
GI	Gastrointestinal
GPaIIb, β3	Glycoprotein aIIb, β3
GPIa	Glycoproteins Ia
GPIb	Glycoprotein Ib
GPIb-IX-V	Glycoprotein Ib-IX-V
GPIIB/GPIIIa	Glycoprotein IIb/IIIa
GPVI	Glycoprotein VI
HD	Hemodialysis
HDL	High-density lipoprotein
HPR	High platelet reactivity
KD	Kidney disease
LDL	Low-density lipoproteins
LTA	Light transmission aggregometry
MCV	Mean corpuscular volume
MOH	Ministry of Health
MPV	Mean platelet volume
NKF/KDOQI	National Kidney Foundation and the Kidney Disease Outcomes Quality Initiative
NSAIDs	Non-steroidal anti-inflammatory drugs
PAR1	Protease-activated receptor 1
PCI	Percutaneous coronary intervention

PFA	Platelet Function Analyzer
PGE2	Prostaglandin E2
PGG2	Prostaglandin G2
PGH2	Prostaglandin H2
PGI2	Prostaglandin I2 (prostacyclins)
PLA2	Phospholipase A2
PLT	Platelets
PTFE	Polytetrafluoroethylene
PTGS2	Prostaglandin-endoperoxide synthase
PTH	Parathyroid hormone
RPFA	Ultra-rapid platelet function assay
RPM	Round per minute
SCCS	Surface connected canalicular system
SHPT	Secondary hyperparathyroidism
SPSS	Statistical Package for the Social Sciences
TIBC	Total iron-binding capacity
TP	Thromboxane-prostanoid
TXA2	Thromboxane A2
TXB2	Thromboxane B2
VLDL	Very low-density lipoprotein
VWF	Von willebrand factor
WBA	Whole blood aggregometry
WBC	White blood cells

Prevalence of Aspirin Resistance among Hemodialysis Patients: A Pilot Study in Palestine

Abstract

Keywords: Hemodialysis patients, Aspirin resistance, Whole blood/Lumi-Aggregometry.

Background: Palestinian Ministry of Health's (MOH) annual reports indicate elevated numbers of hemodialysis cases and chronic renal failure patients in Palestine. Moreover, the limited numbers of dialysis units in West Bank in proportion with the increased number of patients, receiving regular dialysis. This is a significant indicator for providing these patients with prophylactic medications to reduce their higher risk of developing cardiovascular events and to save their lives. Aspirin is the most prescribed antiplatelet agent for preventing cardiovascular disease (CVD) events among hemodialysis (HD) patients. However, studies have shown limited evidence on aspirin efficacy on some patients which puts them at high risk and could be fatal. This aspirin resistance (AR) may be due to various possible causes such as genetic polymorphism, factors related to compliance and absorption of aspirin, inadequate dose, and up-regulation of alternative pathways for thromboxane synthesis.

Objectives: To the best of our knowledge, the problem of AR among HD patients has not been addressed in Palestine. Therefore, this study will be the first to determine the prevalence of AR among HD patients and compare it with other countries. Consequently,

both physicians and HD patients will be familiar with this phenomenon. Physicians will be encouraged to perform platelet aggregation to make sure the drug is working properly in these patients.

Methodology: The study included 40 patients (55 % males and 45%females) undergoing hemodialysis at An-Najah National University Hospital who were taking regular low-dose aspirin and met the study inclusion criteria. Blood samples were drawn following aseptic technique in three tubes (3.2% sodium citrate, EDTA, and Lithium heparin). The sodium citrate whole blood tubes were used to determine platelet aggregation using CHRONO-LOG Model 700 whole blood/Lumi-Aggregometry in a two-channel configuration. Platelet aggregation was induced with 0.5 mM arachidonic acid, along with other characteristics and laboratory tests to observe their health status.

Results: For the assessment of AR, the study results showed that 90% of the study population were aspirin-sensitive while the remaining 10% showed AR with increased ohms values (10 and 12 Ohms). This Ohm elevation and/or AR were investigated with the related HD patients included in the study, who appeared to stop the low dose aspirin intake for almost two weeks before conducting the test. Few studies have shown that patients using aspirin irregularly (non-compliance) are prone to develop aspirin resistance. On the other hand, the majority of the study population have hypertension (82.5%) and 57% of them have diabetes. The group's hemoglobin and hematocrit values (10.3 ± 1.4g/dl and 30.9 ± 4.2%, respectively) were below the normal range. The liver and kidney functions tests showed elevated values of BUN (58.6 ± 11.8 mg/dl) and

creatinine (8.1 ± 1.9 mg/dl). The e.GFR mean value was 6.2 ± 1.8 ml/min/1.73 m^2, as they are all end-stage renal disease (ESRD) patients. Although the average values for liver function tests were normal, the ALP values (130.0 ± 121.9 U/L) were slightly higher than the normal range. The lipid profile average values of cholesterol and HDL were within the normal range for the study population. In contrast to triglyceride values (156 ± 92 mg/dl) that were higher and the LDL values (73.5 ± 31 mg/dl) were lower than the normal range. The iron status for these HD patients was illustrated by the values of iron (46 ug/dL) and TIBC (216 mcg/dL) that were below the normal range. Meanwhile, the ferritin values (389 ng/mL) were high. Finally, the plasma glucose (random test) and glycosylated hemoglobin (Hb A1C) were above their normal range (157 mg/dl and 5.9 %, respectively).

Conclusions: So far, the mechanism of aspirin resistance has not been elucidated, as there are large variations between studies and the methods of testing. In Palestine, further studies with a larger population should be conducted to better assess AR and to explain clinically relevant issues. In addition, HD patients should do this test before aspirin dose treatment to find out if they are AR and to administer proper therapy.

مدى انتشار مقاومة الأسبرين بين مرضى غسيل الكلى: دراسة تجريبية في فلسطين

الطالب

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ملخص الدراسة

الكلمات المفتاحة: مرضى غسيل الكلى، مقاومة الأسبرين.

الخلفية: تشير التقارير السنوية لوزارة الصحة الفلسطينية إلى ارتفاع عدد مرضى غسيل الكلى والفشل الكلوي المزمن في فلسطين. علاوة على ذلك فإن العدد المحدود لوحدات غسيل الكلى في الضفة الغربية مقارنة مع العدد المتزايد من المرضى الذين يتلقون غسيل الكلى بانتظام يعد مؤشرًا مهمًا وهامًا لزيادة الوعي وتزويد هؤلاء المرضى بالأدوية الوقائية من أجل تقليل مخاطر الإصابة بأمراض القلب والأوعية الدموية وبالتالي إنقاذ حياتهم. الأسبرين هو الدواء (antiplatelet agent) الأكثر وصفًا للوقاية من أمراض القلب والأوعية الدموية بين مرضى غسيل الكلى، إلا أن بعض الدراسات قد أظهرت أدلة محدودة على فعالية الأسبرين على بعض المرضى مما يعرضهم لخطر كبير وقد يكون قاتلاً. قد تكون مقاومة الأسبرين ناتجة عن أسباب مختلفة مثل تعدد الأشكال الجيني، والعوامل المتعلقة بامتصاص الأسبرين، أو الجرعة الغير الكافية والتنظيم الإضافي للمسارات البديلة لتصنيع (thromboxane).

الأهداف: لم يتم دراسة او معالجة مشكلة مقاومة الأسبرين بين مرضى مرضى غسيل الكلى في فلسطين. لذلك، ستكون هذه الدراسة الأولى من نوعها لتحديد مدى انتشار مقاومة الأسبرين بين مرضى غسيل الكلى ومقارنتها مع البلدان الأخرى. وبالتالي ، سيكون كل من الأطباء ومرضى غسيل الكلى على دراية بهذه الظاهرة وسيتم تشجيع الأطباء على إجراء فحص تجميع الصفائح الدموية للتأكد من أن الدواء يعمل بالشكل الصحيح عند هؤلاء المرضى.

طريقة العمل: اشتملت الدراسة على 40 مريضاً (55% ذكور و 45% إناث) يخضعون لغسيل الكلى في مستشفى جامعة النجاح الوطني والذين يعتمدون على الأسبرين ويستوفون معايير الدراسة التضمينية. حيث تم سحب عينات الدم باستخدام تقنية التعقيم في ثلاثة أنابيب (3.2% sodium citrate، EDTA، Lithium heparin). وبعد ذلك تم استخدام أنابيب الدم الكاملة لسيترات الصوديوم وجهاز CHRONO-LOG Model 700 whole blood/Lumi-Aggregometry (المكون من قناتين) لاجراء فحص التجميع الناتج عن الصفائح الدموية مع اضافة (0.5 mM arachidonic acid)، جنبًا إلى جنب مع الخصائص الأخرى التي تم ذكرها والنتائج المختبرية لمراقبة حالة المرضى الصحية.

النتائج: لتقييم عدم استجابة حمض (ASA) ، أظهرت نتائج الدراسة أن 90٪ من المرضى استجابوا للأسبرين بينما النسبة المتبقية (10٪) أظهرت مقاومة للأسبرين حيث ارتفعت قيم المقاومة ل 10 و 12 أوم. لذلك تم فحص اسباب ارتفاع مقاومة الأسبرين لدى هؤلاء المرضى ، حيث تبين أنهم توقفوا عن تناول الجرعة المنخفضة من الأسبرين لمدة أسبوعين تقريبًا. وفي هذا السياق، فقد أظهرت دراسات قليلة أن المرضى الذين يستخدمون الأسبرين بشكل غير منتظم معرضون لتطوير مقاومة الأسبرين لديهم. من ناحية أخرى ، يعاني غالبية مرضى هذه الدراسة من ارتفاع ضغط الدم (82.5٪) وكذلك فإن 57٪ منهم مصابون بمرض السكري. كانت قيم الهيموغلوبين والهيماتوكريت للمجموعة (10.3 ± 1.4 غم / ديسيلتر و 30.9 ± 4.2٪، على التوالي) وكانت أقل من المعدل الطبيعي. كما وأظهرت نتائج اختبارات وظائف الكبد والكلى ارتفاع قيم BUN (58.6 ± 11.8 مغم / ديسيلتر) والكرياتينين (8.1

± 1.9 مغم / ديسيلتر). وكان متوسط قيمة e.GFR (6.2 ± 1.8 مل / دقيقة / 1.73 م^2) ، لأنهم جميعًا مرضى الكلى في المرحلة النهائية (ESRD). على الرغم من أن متوسط قيم اختبارات وظائف الكبد كانت طبيعية ، إلا أن قيم ALP (130.0± 121.9 وحدة/لتر) كانت أعلى قليلاً من النطاق الطبيعي. وكانت القيم المتوسطة للكوليسترول و HDL ضمن المعدل الطبيعي لمرضى الدراسة. على عكس قيم الدهون الثلاثية (156 ± 92 مغم / ديسيلتر) التي كانت عالية وكانت قيم LDL (73.5 ± 31 مغم/ديسيلتر) أقل من المعدل الطبيعي. ولقد تم توضيح حالة الحديد لهؤلاء المرضى من خلال قيم الحديد (46 ميكروغرام / ديسيلتر) و TIBC (216 ميكروغرام/ديسيلتر) التي كانت أقل من المعدل الطبيعي. بينما كانت قيم الفيريتين عالية (389 نانوغرام / مل). وأخيرًا ، كان السكر العشوائي ووالسكر التراكمي (Hb A1C) أعلى من المعدل الطبيعي (157 مغم / ديسيلتر و 5.9 ٪، على التوالي).

الملخص: في فلسطين ، من الضروري إجراء المزيد من الدراسات بتواجد عدد كبير من المرضى، حيث يجب إجراؤها لنكون أكثر دراية بهذه الظاهرة وبالتالي حل المشكلات السريرية ذات الصلةبهذه الظاهرة. ويجب على مرضى غسيل الكلى إجراء هذا الاختبار قبل تناول جرعة الأسبرين من أجل تشخيص ما إذا كانوا مقاومين للأسبرين وبالتالي تخصيص علاجهم بفاعلية.

Chapter 1

Introduction

1.1 Background

Kidneys are vital organs that perform many important functions such as filtering and purifying blood, cleaning the body from excess substances generated from metabolic processes (*i.e.* water, salts, and wastes). In addition, to regulate acid-base balance to prevent excess acidity, and produce three essential substances (activate vitamin D, renin, and erythropoietin) which help in many important processes (Brown *et al.*, 2016). Any deterioration in the kidney function indicates diseases such as persistently impaired kidney function, which can develop very slowly and ultimately lead to kidney failure; leading to chronic kidney disease (CKD). Otherwise, it can appear suddenly as loss in kidney function within hours or days; where is known as acute kidney disease (AKD) (Chaturvedi *et al.*, 2017; Collister *et al.*, 2016).

Kidney damage or chronic kidney disease (CKD) describes having insufficient kidney glomerular filtration rate (GFR) to less than 60 ml/min/1.73 m^2 (Chen *et al.*, 2014; Polzin *et al.*, 2016). The GFR level can classify CKD into five stages. Stage five is described as kidney failure or end-stage renal disease (ESRD), where patients' GFR is less than 15 ml/min/1.73 m^2 and need immediate treatment by dialysis or transplantation (Baber *et al.*, 2015; Lim *et al.*, 2015; Gremmel *et al.*, 2013).

The risk of death is increased due to several factors including CKD which may increase cardiovascular disease (CVD) complications, because of dialysis traditional risk factors (*i.e.* hyperlipidemia, diabetes mellitus, hypertension, and others) (Cantaluppi *et al.*, 2017; Wang *et al.*, 2016). Platelet dysfunction as a result of the hemodialysis (HD) process may also increase the risk of high hemorrhagic and thrombotic complications' particularly among patients with ESRD. Therefore, these patients need antithrombotic drugs including antiplatelet and anticoagulant agents to prevent these complications (Jeong *et al.*, 2015; Migliori *et al.*, 2015).

The platelet cyclooxygenase 1 (COX-1) enzyme is inhibited irreversibly by effective antiplatelet drugs such as aspirin, resulting in the decrease of thromboxane A2 (TXA2) synthesis (an effective vasoconstrictor and platelet cofactor activator) (Abaci & Kilickesmez, 2013; Liu *et al.*, 2016). Aspirin reaches its peak level in blood circulation in about one hour; meanwhile, its availability within the circulation occurs after 30 min of drug intake. The metabolism of aspirin occurs mainly by the liver and intestine where most platelet inhibition occurs in pre-hepatic circulation (Herrington *et al.*, 2015; Grinstein & Cannon, 2012).

Despite the demonstrated benefit of aspirin in primary and secondary prevention of CVD, recent studies showed that HD patients develop a phenomenon of aspirin resistance (AR). In which these patients don’t derive the anticipated anti-platelet response from the low dose of aspirin leading to an increase in the risk of CVD events (Kim *et al.*, 2014; Liu *et al.*, 2016). Many reports showed that several risk factors may contribute to

AR including, diabetes, metabolism, age, genetic factors, and hypercholesterolemia. In addition, smoking has pro-coagulative properties that may contribute to this phenomenon (Faraday *et al.*, 2007; Friend *et al.*, 2003; Gum *et al.*, 2001). Drug interactions have also been implicated to play a significant role in causing AR, such as statins and non-steroidal anti-inflammatory drugs (Catella-Lawson *et al.*, 2001; Feher *et al.*, 2006). However, the mechanism of AR has not been elucidated, where few studies have shown that patients using aspirin irregularly (non-compliance) are more prone to develop aspirin resistance (Grinstein & Cannon, 2012; Mayer *et al.*, 2014; Schwartz, 2011). This study aims to evaluate the prevalence of AR in HD patients at An-Najah National University Hospital using a platelet aggregometry device.

1.2 Statement of the problem

Although aspirin is an antiplatelet agent that is prescribed for preventing CVD events among HD patients, studies have shown evidence of limited aspirin efficacy in some cases, aspirin resistance among hemodialysis patients, which will affect their therapeutic process and may lead to death in some cases.

1.3 Significance of the study

Palestinian Ministry of Health's (MOH) annual reports indicated elevated number of dialysis patients in Palestine up to 14.9% in 2011 (about 670 patients) compared with the same period in 2010. Moreover, records indicated increased number of patients with

chronic renal failure up to 31.1% in 2010 (about 583 patients) compared with half the period in 2009. (http://www.site.moh.ps/index/Books/BookType/2/Language/ar)

The recent annual health report (2018) issued by the ministry of health, indicates the presence of 12 dialysis units in the West Bank. Eleven units belong to the MOH and one to An-Najah National University Hospital in Nablus. The total number of patients receiving regular dialysis in the West Bank is 2,071 patients. This shows a clear and significant increase in the number of dialysis patients annually. (http://www.site.moh.ps/index/Books/BookType/2/Language/ar)

Dialysis patients are at higher risk to develop cardiovascular events, which makes it essential to provide these patients with prophylactic medications to decrease the risk of CVD. Aspirin is listed among the most prescribed medications. This study will be the first in Palestine to evaluate AR prevalence among HD patients using Whole blood aggregometry (WBA) for that purpose

Unfortunately, some HD patients do not respond properly to aspirin, which may increase the risk of cardiovascular problems and death. Aspirin resistance may be attributed to various possible causes such as genetic polymorphism, factors related to compliance and absorption of aspirin, inadequate dose, and up-regulation of alternative pathways for thromboxane synthesis.

The problem of aspirin resistance (AR) among HD patients has not been addressed in Palestine. Therefore, this study will be the first to determine the prevalence of AR

among HD Palestinian patients and compare it to other countries. Consequently, both physicians and HD patients will be aware of this phenomenon. Physicians will be encouraged to request platelet aggregation regularly to make sure the drug is working properly.

1.4 Main objective:

To identify the prevalence of aspirin resistance among hemodialysis patients: a pilot study in Palestine.

1.5 Specific objectives:

1- To assess the hemogram test (CBC) of the study population.

2- To evaluate lipid profile tests (HDL, LDL, cholesterol, and triglyceride) for the participants in the study.

3- To analyze the biochemistry tests (such as liver and kidney functions, iron, and electrolytes) for the participants.

4- To evaluate aspirin efficiency for HD patients using Whole blood aggregometry (WBA).

Chapter 2

Literature review

2.1 Platelets

2.1.1 Platelet physiology

Thrombocytes (or platelets) are tiny nucleus-free cells having (2-4 μm) diameters. Their manufacturing process is achieved by the cytoplasmic division of megakaryocytes in bone marrow, where each megakaryocyte produces nearly 1000-5000 platelets having a shelf life of eight to ten days in the human body (Santos-Gallego & Badimon, 2021; van der Meijden & Heemskerk, 2019). Additionally, the normal platelets' concentration in the circulation of a healthy individual range from 150 to 400 x 10^9 cells/L, where the inactive platelets are cleared from the body by the spleen (Quach *et al.*, 2018).

Platelets have an essential physiological role in bleeding inhibition (hemostasis), thrombus formation, and other pathophysiological routes (*i.e.* angiogenesis, inflammation, cancer, infection, and innate immunity). Platelets are currently considered to be involved in atherothrombosis and acute coronary syndromes (ACS) (Holinstat, 2017; Wojtukiewicz *et al.*, 2017; Xu *et al.*, 2016). Platelet function is due to their structure and the presence of several organelles (*i.e.* Golgi apparatus, mitochondria, dense tubular system, granules, and lysosomes) that have a major role in protein secretion. In addition, a membrane that is rich in receptors facilitates external and internal signaling (Gremmel *et al.*, 2016).

In hemostasis, platelets reactivation from their normal inactive state, adhering to the damaged wall in the blood vessel, their aggregation, and molecule secretion are all supported by complex biochemical systems (Gupta *et al.*, 2020). The process starts with the expression of glycocalyx (a membrane coating protein secreted from lipids and proteins on platelets membrane) that coats platelets surface for aggregation and extracellular signaling. This coating protein includes glycoproteins Ia (GPIa), glycoprotein Ib (GPIb), and glycoprotein IIb/IIIa (αIIbβ3 integrin) which bind to collagen, Von Willebrand factor (VWF), and a specific receptor for fibrinogen, respectively (Leung, 2016; Periayah *et al.*, 2017). Moreover, platelets' middle membrane layer is composed of phospholipid for structure support during activation with arachidonic acid. Meanwhile, the inner layer has the surface-connected canalicular system (SCCS) that plays a role in translating signals from the platelet's surface to its substructure, allowing their activation followed with appropriate stimuli (Gremmel *et al.*, 2016; Wang *et al.*, 2014). The activation process also involves the dense tubular system (DTS) which works side to side with SCCS by segregating Ca^{+2} and enzymes such as Cyclooxygenase, phospholipase A2, and TXA2 synthase (Gremmel *et al.*, 2016; Hamilos *et al.*, 2018).

Although the platelets activation process is firmly synchronized, any changes or functional disorders in the activation steps may lead to hyperactivity or hypoactivity state causing an inappropriate thrombus formation or extreme bleeding tendency, respectively. Therefore, stroke and myocardial infarction are the most two common contributions of

platelet hyperactivation leading to morbidity (Koupenova *et al.*, 2017; Rao, 2003; Xu *et al.*, 2016).

In recent years, clinical practices have focused on controlling platelet reactivity and reducing thrombus formation. This reactivity is irregular within individuals either with or without antiplatelet drugs, for example, cardiovascular patients receiving high treatment platelet reactivation drugs have been linked with ischemia (Alhazzani *et al.*, 2021; Eikelboom *et al.*, 2012; Zhu *et al.*, 2019). In contrast, those receiving low treatment drugs have been linked with hemorrhagic events. Besides, family-established researches have pointed out that platelet reactivity is mostly an inherited phenotype (Mavrakanas *et al.*, 2018; Mingant *et al.*, 2018).

2.1.2 Platelets activation

In healthy people, platelet activation is a multistep process involving adhesion, secretion, and aggregation. The primary mechanism for indirect platelets-collagen-binding starts just after an injury, caused by trauma or a cut, where platelets respond to collagen release from sub-endothelium to the blood (Hosseinzadegan & Tafti, 2017). However, under excessive release of collagen, a complex of VWF-collagen is created by the binding of VWF to collagen (Golebiewska & Poole, 2015; Peyvandi *et al.*, 2011), a specific platelet receptor (GPIb-IX-V) cooperates and slows down platelet's activation letting other platelet's receptors to interact. For more platelet activation, another two collagen receptors become essential on the platelet's membrane (Glycoprotein VI (GPVI) and α2β1 integrin); α2β1 integrin permits the direct adhesion to collagen and

immobilizes platelets at the injury site, while GPVI binds with collagen leading to final interactions. After that, platelets are activated and the platelet plug is then initiated through interchanging and binding of the three main receptors (Colvin, 2004, Santos-Gallego & Badimon, 2021; van der Wal & Becker, 1999). Subsequently, platelets change their shape radically by extending their pseudopodia and eventually lamellipodia through rearrangement of myosin and actin within the cytoskeleton and the exposure of aggregation mediators from granules (dense and alpha granules) within platelets (van der Meijden & Heemskerk, 2019). These mediators aid in the recruitment of additional platelets to the injury site. On one hand, mediators from dense granules are adenosine triphosphate (ATP), adenosine diphosphate (ADP), serotonin, calcium, and others, that bind to their receptors such as P2Y12 receptors for ADP (Hou *et al.*, 2015; Santos-Gallego & Badimon, 2021). This receptor-mediator binding on platelets membrane and their neighboring commands a cascade of intracellular events that increase the binding affinity through the transformation of integrin αIIbβ3 receptor, a receptor for VWF and fibrinogen (Hvas & Favaloro, 2017). After these steps, the platelet plug expands creating a monolayer bridge between αIIbβ3 on neighboring activated platelets. This platelet plug stops the leakage of blood from the injured vessel. Furthermore, receptors and mediators from alpha granules (*i.e.* P-Selectin, VWF, and fibrinogen) are released to the surface of the platelet membrane and extracellular space (Leung, 2016; McMichael, 2005).

2.1.3 Production of Thromboxane A2 (TXA2)

Thromboxane A2 (TXA2) is a type of lipid mediator thromboxane that is chemically unstable and involved in several pathophysiological processes, such as primary hemostasis, atherothrombosis, inflammation, and cancer (Jing *et al.*, 2020). It is produced by activated platelets and has prothrombotic properties; stimulates the activation of new platelets and increases platelet aggregation. In addition, it acts as a positive feedback mediator during hemostatic plug formation and vasodilators released from platelets after activation (Jing *et al.*, 2020; Zhu *et al.*, 2019).

Thromboxane-prostanoid (TP) is the TXA2 receptor located on cells of the heart, kidney, and spleen. In hemostasis, TXA2 binds to receptors on the vasculature and platelet cell membranes leading to platelet shape change, granule release, platelet activation, as well as vasodilation of the vessel (Davi *et al.*, 2012). TXA2 fabrication starts with the activation of phospholipase A2 (PLA2) (phospholipids cleaving enzyme) leading to the release of arachidonic acid (AA) into the cytoplasm where it is bound with the aid of cyclooxygenase enzyme isoform 1 or 2 (COX-1/2) (Jing *et al.*, 2020). The latter is then numerously expressed within platelets and is the primary target of the drug acetylsalicylic acid/aspirin (ASA). However, within a normal circulation, the COX-2 enzyme is only found in platelets in an inducible form and low concentrations (Hart *et al.*, 2003; Limongelli *et al.*, 2010). The attachment of COX-1/2 to AA transforms it into prostaglandin G2 (PGG2) that is then, with the aid of peroxidase, converted into prostaglandin H2 (PGH2). Thromboxane synthase then converts PGH2 into TXA2 and

exocytose from the platelet. As TXA2 is produced in the body, it is rapidly hydrolyzed to its inactive form thromboxane B2 (TXB2) within 30 sec (Marvin *et al.*, 2007; Nakahata, 2008). TXA2 concentrations assessment can be determined ex vivo through measurement of serum TXB2 by using enzyme-linked immunosorbent assay (ELISA). Moreover, a non-invasive method for determining platelet activation (platelet COX-1 activity) is by testing urinary enzymatic metabolites (Liu *et al.*, 2015) as shown in Figure 1.

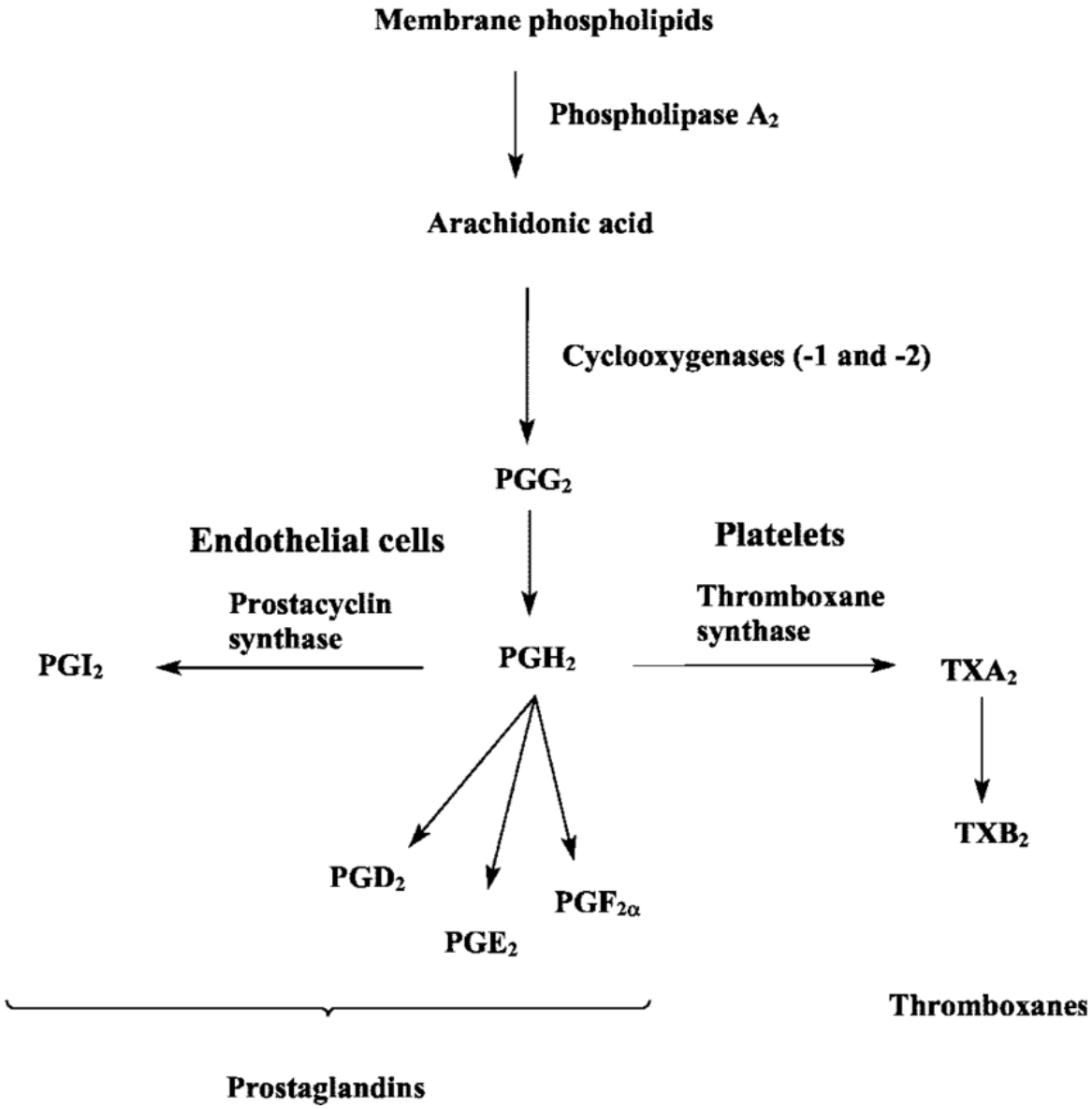

Figure 1: Pathway cascade of Thromboxane A2 production (Dogne *et al*., 2004).

2.1.4 Antiplatelet therapy

Although thromboxane A2 (TxA2) production and its action (in hemostasis, vasoconstriction, and wound healing) are essential and lifesaving. It can be lethal to susceptible patients, those with cardiovascular disease (CVD) and acute coronary syndrome (ACS), patients with stable coronary artery disease, and those undergoing revascularization procedures (such as percutaneous coronary intervention (PCI)) (Weber *et al.*, 2002). Currently, non-steroidal anti-inflammatory drugs (NSAIDs) act as first-line therapy to these cases to inhibit platelets activation. Four main categories of drugs are currently used clinically, either alone or in combination including cyclooxygenase 1 (COX1; also known as PTGS1) inhibitors (aspirin or ASA), inhibitors of the ADP P2Y12 receptor (cangrelor, clopidogrel, prasugrel, and ticagrelor), protease-activated receptor 1 (PAR1) antagonists (vorapaxar), and glycoprotein GPIIb/IIIa antagonists (abciximab, eptifibatide, and tirofiban). All these drugs are used for inhibiting processes important for both thrombosis and hemostasis (Figure 2) (Fokunang *et al.*, 2018; Schjerning *et al.*, 2020). For example, ASA targets the COX-1 enzyme by acetylating, and irreversibly inhibiting or even blocking the conversion of AA into PGH2 (Alegbeleye *et al.*, 2020). Also, an antiplatelet agent used currently to inhibit phosphodiesterase is now used only for the treatment of peripheral vascular disease (Eikelboom *et al.*, 2012; Hong *et al.*, 2020). However, patients taking the treatment suffer from side effects such as severe reduced or shutting down the whole prostanoid cascade, increased risk of bleeding, and others (Hiremath *et al.*, 2009).

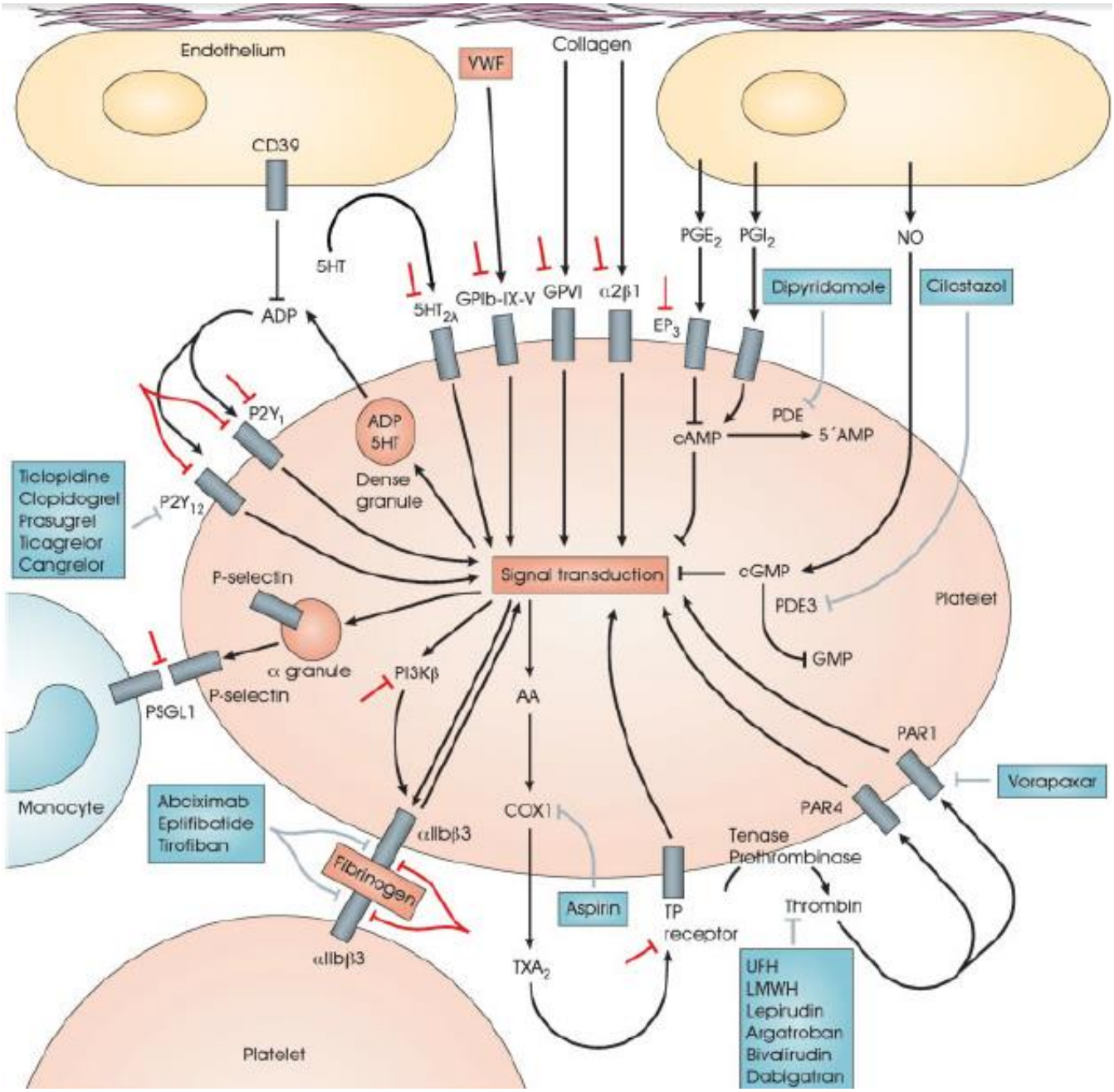

Figure 2: Platelet function and molecular targets of antiplatelet agents. Initial platelet adhesion to exposed collagen from damaged vessel walls to GPVI and integrin α2β1 and VWF to GPIb-IX-V complex. Thrombin is a potent activator of human platelets that binds to PAR-1 and PAR-4 receptors. Positive feedback loops for platelet activation are P2Y1 and P2Y12 stimulated by ADP released; 5HT 2A receptors are stimulated by 5HT, and the thromboxane prostanoid (TP) receptor is stimulated by TXA2 generated by the platelet COX1. Platelet-to-platelet aggregation is mediated by fibrinogen. Platelet-monocyte adhesion is mediated by the binding of P-selectin to PSGL1. Approved antiplatelet agents and their molecular targets are shown in boxes. Thrombin inhibitors are UFH, LMWH, lepirudin, argatroban, bivalirudin, and dabigatran. Investigational strategies for novel antiplatelet agents are shown by the symbols adjacent to GPIb-IX-V, GPVI, α2β1, EP3, 5HT2A, P2Y1, P2Y12, PSGL1, PI3Kβ, αIIbβ3, and TP receptor. AA, arachidonic acid; COX1, cyclooxygenase 1; EP3, prostaglandin E2 receptor EP3 subtype; GP, glycoprotein; NO, nitric oxide; PAR, protease-activated receptor; PDE, phosphodiesterase; PG, prostaglandin; PI3Kβ, phosphoinositide 3-kinase β-isoform; PSGL1, P-selectin glycoprotein ligand-1; TXA2, thromboxane A2; UFH, unfractionated heparin; VWF, von Willebrand factor; 5HT, 5-hydroxytryptamine (Michelson, 2011).

2.2 Chronic kidney diseases (CKD)

2.2.1 Chronic kidney diseases (CKD) definition

When kidneys start to perform less efficiently this condition is called kidney disease (KD). Chronic kidney disease (CKD) is the case when their function declines over time. Diabetes and high blood pressure are considered two of the most common causes of CKD (De Lima *et al.*, 2021; Webster *et al.*, 2017). Although there is no cure for CKD; follow-up of patients helps to keep kidneys functioning as long as possible. Dialysis or a kidney transplant is required in the late stages of renal disease. A patient can be diagnosed with CKD, if his glomerular filtration rate (GFR) is less than 60 ml/min/1.73 m^2, or has albumin in the urine for more than three months. Moreover, the National Kidney Foundation and Kidney Disease Outcomes Quality Initiative (NKF/KDOQI) have produced a classification of chronic kidney disease (CKD) as shown in Table 1 (Thurlow *et al.*, 2021; Webster *et al.*, 2017). The classification is based on GFR, and describes 5 stages, with subdividing stage 3 into 3a and 3b.

Table 1: Chronic kidney disease stages according to GFR level and the related dysfunctions.

Stage	GFR	Kidney damage	dysfunction
1	≥90	+	Normal renal function with signs of kidney damage
2	60–89	+	Mildly impaired renal function
3a	45–59	+	Mildly to moderately impaired renal function
3b	30–44	+	Moderately to severely impaired renal function
4	15–29	+	Severely impaired renal function
5	<15	+	Kidney failure (End-stage renal disease)

During the last decades, the CKD prevalence has increased significantly due to the aging population worldwide and the increasing incidence of diabetes and hypertension (Thurlow *et al.*, 2021). According to the Palestinian Ministry of Health's annual reports, the number of dialysis patients in the West Bank of Palestine increased by 14.9% in 2011 (about 670 patients) as compared to the same period in 2010. Furthermore, the number of patients with chronic renal failure (approximately 583 people) increased by 31.1% in 2010 compared to half of the same period in 2009. Globally, 697.5 million cases of CKD (all stages) were reported in 2017, with a global prevalence of 9.1%. Between 1990 and 2017, the global prevalence of CKD in people of all ages increased by 29.3%, but the age-standardized prevalence remained steady. Universally, 1.2 million people died from CKD in 2017, between 1990 and 2017, the global death rate from CKD increased by 41.5 percent for all ages (Bikbov *et al.*, 2020).

2.2.2 Platelets (PLT) and cardiovascular diseases (CVD) events in CKD

CKD patients experience complex haemostatic disorders. Meanwhile, uremic patients have a bleeding diathesis caused by primary hemostasis disorders (Lutz & Jurk, 2017; Lutz *et al.*, 2014). Hemostasis is essentially composed of platelets; they adhere and accumulate to injured blood vessels at the sites of injury, and also by overhauling of covered atherosclerotic plaque, which is well known as the atherogenic process, (Jeong *et al.*, 2015). On the other hand, the uncontrolled platelet evolution may lead to many temporary or constant pathological conditions such as ischemia or necrosis, stroke, and myocardial infarction (MI), through thrombus formation and vascular obstruction (Baber

et al., 2015; Herrington *et al.*, 2015). Some studies illustrated lower platelet activation within CKD patients, while others report increased activation cases. Yet, platelet activation is a debated topic in these patients (Lutz & Jurk, 2017). Although ESRD has a high prevalence of cardiovascular and thrombotic complications, they exhibit decreased platelet function according to Boccardo et al 2004 findings (Boccardo *et al.*, 2004). Even though bleeding diathesis or uremic bleeding, which occurs due to primary hemostasis abnormalities and is associated with CKD patients, is multifactorial, platelet-platelet and platelet-vessel wall interactions are crucial in this case (Ozkan & Ulusoy, 2013). In uremic patients, platelets deactivation occurred as a result of the presence of uremic toxins in the circulating blood. Additionally, abnormalities in platelet alpha-granules, involving storage pool deficiency, can cause the reduction in ADP and serotonin levels and increase cAMP levels in patients with terminal renal insufficiency; which is considered as another reason for platelet dysfunction, imperfect platelet aggregation, and adhesion to injured vessels (Liang *et al.*, 2021; Shin *et al.*, 2021; Zhang *et al.*, 2021). Platelets with vessel interaction have a major role in hemostasis at the lesion site, so patients with the defective function of the α2β3 complex would have decreased binding of both fibrinogen and VWF to stimulated uremic platelets (Daugirdas & Bernardo, 2012; Rios *et al.*, 2010). Particularly, the reduced binding activity is triggered by dialyzable toxic substances or the occupying of fibrinogen fragments to α2β3 receptor in uremic plasma, which prevents aggregation by reducing cross-linking between adjacent platelet. Moreover, fibrinogen fragments have been confirmed to be the cause of

diminishing the expression of activated GP IIb/IIIa. This gives a clear image of the role of fibrinogen fragments in causing uremic platelet dysfunction (Lutz *et al.*, 2014; Moal *et al.*, 2003). On the other hand, platelet activation is marked by the expression of CD62P (P-selectin) and increased soluble P-selectin, thus, patients with the greatest degree of platelet activation are combined with worse GFR rate (Palmer *et al.*, 2013; Yu *et al.*, 2019).

Studies have revealed that the major cause of death in chronic renal failure (CRF) cases is strongly related to hemodialysis and CVD events (Masson *et al.*, 2015; Webster *et al.*, 2017), where, cardiovascular events and diseases risk (*i.e.* ischemic and hemorrhagic stroke) are more common in HD patients (Herrington *et al.*, 2015). While the traditional vascular disease risk factors such as diabetes and hypertension, increase stroke risks through these patients (Holzmann *et al.*, 2014; Sonmez *et al.*, 2015).

CVD patients are treated either by anti-platelet agents or by anticoagulant drugs (such as aspirin), which are considered as a cornerstone in atherosclerotic disease, stroke cases, atrial fibrillation management, and coronary peripheral vascular disease (Lim *et al.*, 2015; Sonmez *et al.*, 2015). Despite being on aspirin, researchers revealed that some patients recorded increased platelet activity compared to healthy controls, by the expression of CD62P, soluble P-selectin, and continuing alpha granule degranulation on platelets, as illustrated before (Yu *et al.*, 2019). Therefore, this alone cannot justify the ongoing risk of thrombosis. At the end, it can be emphasized that successful treatment of

coronary artery disease and cerebrovascular disease is disturbed by aspirin insufficiency or aspirin resistance (Goicoechea *et al.*, 2018).

2.2.3 Anti-platelet therapy and hemodialysis patients

Currently, the available anti-platelets are categorized according to their ability to interfere within PLT accumulation process steps (Migliori *et al.*, 2017). The roles of anti-platelet drug treatment in preventing vascular events within HD patients who are diagnosed with CKD have been reviewed within a published data by Palmer *et al.* (2013), in which, they reported anti-platelet effectiveness in reducing the risk of vascular events. Furthermore, the extensive use of aspirin and clopidogrel as the most commonly used anti-platelet drugs for vascular events in CKD patients has been discussed by other studies (Capodanno & Angiolillo, 2012). The risk of vascular events is reduced to approximately 32% by receiving a low dose of aspirin in high-risk patients. Aspirin acetylates the serine residue of cyclooxygenase's (COX) catalytic site and causes enzyme inhibition. This event is followed by preventing the access of arachidonic acid to the catalytic site of the enzyme (Wurtz & Lerkevang Grove, 2012). However, there is an insufficient observed response from 2% to 57%through patients having 300 mg daily aspirin doses (Pregowski *et al.*, 2007). These insufficient responses were identified using PLT in vitro function test and those patients are diagnosed to have biochemical resistance or high platelet reactivity (HPR), which is considered as a primary ex vivo testing event (Baber *et al.*, 2015). Although HPR is not definitively approved to be the cause of clinical thrombosis, the least is considered to be the very late sign of HPR. Moreover, the

results of several studies have demonstrated that patients whose antiplatelet dosing was based on the ex vivo PLT function test showed different thrombotic outcomes (Breet *et al.*, 2014; Jeong *et al.*, 2015). Results have also shown that inflammatory response, increased PLT turnover, generation of aspirin-sensitive COX and PLT glycoprotein receptor's genetic polymorphisms of COX-1 and COX-2 alleles are identified as possible mechanisms for HPR (Baber *et al.*, 2015; Cho *et al.*, 2018). Additionally, bleeding tendency and the increase in thrombotic complications in HD patients are induced by PLT abnormalities (Palmer *et al.*, 2013; Polzin *et al.*, 2016).

Recent studies have reported that an irregular activation of platelet receptors causes frequent access obstruction, a decreased membrane expression, or an increase in platelet receptor number (Chen *et al.*, 2021). Still, PLT is activated by adhering to the extracorporeal circuit shearing high stress and turbulence in the vascular access and the artificial surface of the polytetrafluoroethylene (PTFE) graft. Finally, PLT may also be activated by the native arteriovenous fistula through fibrinogen involving mechanism, which is increased in HD patients and also create a pro-thrombotic microenvironment (Algahtani & Heptinstall, 2017; Bartels *et al.*, 2015; Lim *et al.*, 2015).

Antiplatelet responsiveness varies among CKD patients, so the inadequate antiplatelet effects of these agents are associated with the increased risks of vascular events (Palmer *et al.*, 2013; Snoep *et al.*, 2007). Aspirin has a lower platelet response within certain patients, whereas preventive patients with a two-year follow-up of aspirin therapy have a risk of 8% to 18% recurrent vascular events (Wurtz *et al.*, 2010). For

example, Polzin et al., (2016), at the Heinrich Heine University Medical Center in Düsseldorf, Germany showed that impaired antiplatelet effects risk of aspirin is increased among patients with CKD. In another study, Breet et al., 2014 reported that there was no impairment of antiplatelet (aspirin) effects in CKD patients after adjustment for covariates. This multifactorial phenomenon of reduced aspirin response may be attributed to pharmacodynamics, clinical, genetic, and biological elements. For example, an increased platelet turnover based on a large fraction of immature platelets is suggested as a biological mechanism for the reduced response. Where, on the contrary, to mature platelets, newly formed platelets may influence their hemostatic potential by expressing cyclooxygenase-2 and containing ribonucleic acid (RNA) enabling protein synthesis (Aksu *et al.*, 2015; Polzin *et al.*, 2016; Staszewski *et al.*, 2018).

Hemodialysis patients have indistinct benefits and risks of using anti-platelets according to the Dialysis Outcomes and Practice Patterns Study (DOPPS), DOPPS is an observational study that was performed over 28,320 HD patients worldwide on the safety and secondary prevention efficacy of aspirin within these patients, which revealed the varied use of anti-platelet in HD patient (Kumar *et al.*, 2018). Also, others investigated the effect of anti-platelet among HD patients after placement in a dialysis unit. The results of the study showed a 41% proportional reduction in serious vascular events, such as vascular death, nonfatal stroke, and nonfatal MI, after antiplatelet therapy. However, this result was based on only 99 vascular events among such patients and the difference was not statistically significant. Even though this study showed a decreased risk of

bleeding with a low aspirin dose, it failed to determine aspirin benefit in the morbidity and mortality of cardiovascular (Behera *et al.*, 2021; Lim *et al.*, 2020).

Aspirin resistance among HD patients has been tackled by several studies, where the authors investigated the effect of hemodialysis process in increasing or decreasing AR. These studies have indicated the presence of aspirin resistance within patients undergoing hemodialysis, showing as well a significant reduction of this resistance following treatment with hemodialysis (Aksu *et al.*, 2015; Geara *et al.*, 2012). Additionally, the antiplatelet drugs (*i.e.* aspirin and clopidogrel) were investigated in other studies for their role on platelet activation, and the effect of hemodialysis mainly on these drugs within ESRD patients. The results of these studies showed an increase in platelet activation due to hemodialysis leading to attenuation in antiplatelet drug response in patients with renal disease than patients without renal insufficiency. Indeed, also it these studies showed the superior effect of aspirin over clopidogrel for preventing recurrent ischemic stroke in patients with ESRD patients undergoing dialysis (Chen *et al.*, 2014; Htun *et al.*, 2014; Jeong *et al.*, 2015). Furthermore, measuring aspirin activity varies according to the evaluating and analyzing instrument. For example, various papers showed insufficient aspirin effects when measured by the verified platelet analyzer, while, showed no effect when measured by the accumulation of light transmission (Gremmel *et al.*, 2015).

2.3 Aspirin

2.3.1 Aspirin history

Acetylsalicylic acid (commercially known as Aspirin) is a type of non-steroidal anti-inflammatory drug (NSAIDs), considered to be the oldest antiplatelet and still used worldwide (Paez Espinosa *et al.*, 2012). In the USA for example, more than 50 million patients regularly take ASA tablets, and over 40% of people, above the age of 50 years, all over the world take aspirin (Johnston *et al.*, 2016). It is massively produced globally with an estimated consumption of 44,000 tons (50-120 billion tablets) each year and is included in the World Health Organization's List of Essential Medicines according to its antiplatelet, antipyretic, and anti-inflammatory properties (Cai *et al.*, 2016).

The journey began when Reverend Edward Stone used the powder of Willow tree bark for treating fever, taking this idea from 4000 years ago when its tea was used to treat pain (Santos-Gallego & Badimon, 2021). Later, a pharmacy professor Joseph Buchner was the first discoverer of aspirin from the bark of the tree; he isolated and modified yellow crystals calling them salicin or commercially Salix, the Latin name for Willow (Wick, 2012). Then in 1829 these crystals were improved and purified into salicylic acid (Figure 3) by Raffaele Piria, which didn’t spread widely because of its side effects on the gastric lining in its formal state (Fuster & Sweeny, 2011). Therefore, in 1852 Charles Gerhart was able to discover the chemical structure and acetylate its hydroxyl group to create acetylsalicylic acid (Figure 4). However, it had purity and stability problems. Later on, Felix Hoffman, who was inspired by his father’s rheumatic disease, was able to create

salicylic acid with reduced unwanted side effects in 1887 (Fuster & Sweeny, 2011; Santos-Gallego & Badimon, 2021). Finally, ASA was successfully purified and clinically tested for its beneficial therapeutic properties to be registered and also stamped into tablet form for mass production and use in 1899(Wick, 2012).

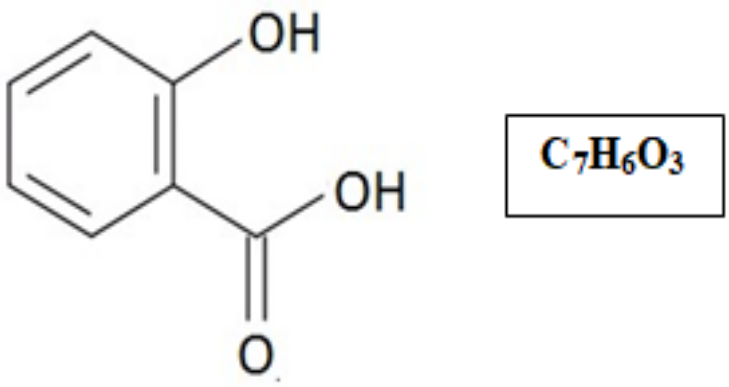

Figure 3: Chemical and linear formulae of salicylic acid (Huremovic *et al.* 2017).

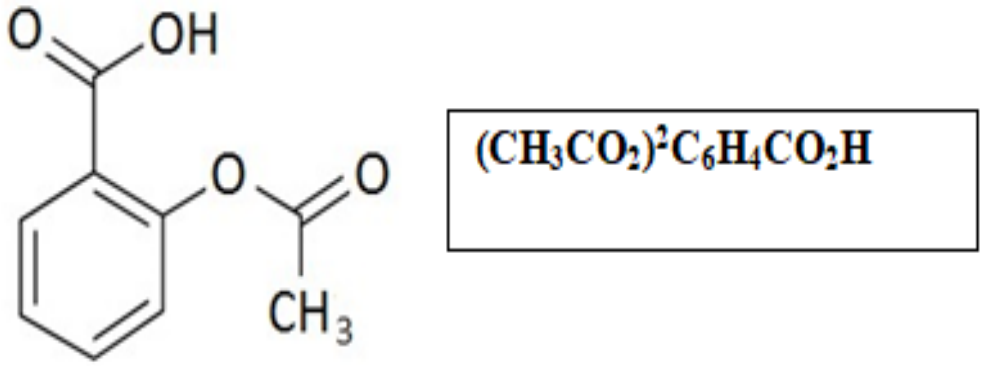

Figure 4: Chemical and linear formulae of acetylsalicylic acid (Huremovic *et al.* 2017).

2.3.2 Aspirin pharmacology and mechanism of action

As mentioned previously, during the activation process of platelets by their contact with collagen in the subendothelial surface, phospholipase A2 release AA from the plasma membrane of platelets, afterward COX-1/2 convert it to PGG2. The latter is then altered to PGH2 by Peroxidase, and finally, by thromboxane synthase, transformed to TXA2, which promotes secondary activation of activated platelets by releasing and

binding the TP receptor on the platelet surface (Lee *et al.*, 2019; Paez Espinosa *et al.*, 2012). The two isoforms of the cyclooxygenase enzymes (COX-1/2) are the core for prostanoid production within cells, where, COX-1 is present in most cells and constitutively expressed, and COX-2 is expressed at low levels in platelets then its level starts to increase after platelets activation (Chandrasekharan & Simmons, 2004; Mitchell *et al.*, 2021). Consequently, aspirin work of action appears by covalently and irreversibly acetylating the serine group (serine 529 in COX-1 and serine 516 in COX-2), which prevents the conversion of arachidonic acids to PGG2. Hence, subsequently preventing the upstream prostanoids biosynthesis (including thromboxanes TXA2, prostaglandins, and the prostacyclins PGI2), leading to platelets' inability to produce TXA2 during the activation and then blocking the activation of neighboring platelets and hemostatic plug formation (Alegbeleye *et al.*, 2020; Parker *et al.*, 2019). In response to a variety of stimuli, mature platelets express only COX-1 and produce TXA2 while the main site of PGI2 production in vascular endothelial cells, which express both COX-1 and COX-2 (Zacharias-Millward *et al.*, 2017).

ASA is considered a COX-1 inhibitor and a modifier of the enzymatic activity of COX-2, where the binding of ASA to this enzyme is irreversible unlike other NSAIDs (*i.e.* ibuprofen/naproxen) which bind reversibly (Saxena *et al.*, 2013). Therefore, as a result of blocking the COX pathway, scientists hypothesized that the released AA are transferred to the lipooxygenase pathway. Lipoxins (anti-inflammatory) production rises from the modification of prostaglandin-endoperoxide synthase (PTGS2). These

compounds are called aspirin-triggered resolvins, aspirin-triggered maresins, and aspirin-triggered Lipoxins (Ramkumar & Sharma, 2017; Schjerning *et al.*, 2020). Moreover, aspirin is rapidly absorbed from the intestine into the blood after being ingested reaching its peak levels within one hour, and after two hrs following ingestion, aspirin is metabolized by the liver and cleared from the blood circulation (Alegbeleye *et al.*, 2020; Zuniga-Ceron *et al.*, 2016). Platelets are non-nuclear, so they limit the ability to generate new COX-1 after the action of aspirin. Therefore, its inhibitory action extends for the platelet's lifetime (7-12 days) (Parker, 2020). However, in humans, around 10% of the circulating platelets are been released from the bone marrow in the means of normal thrombopoietic turnover, which in cardiovascular patients can be considered as a limiting factor. Thus, Drug dosage is essential to continue drug effects (Gasparyan *et al.*, 2008; Saxena *et al.*, 2013). Moreover, ASA treatment side effects are dose-dependent, at a high dose of ASA or long-term therapy few side effects appear including, gastric ulcers, bleeding, nausea, and rarely renal toxicity. At lower doses, it can cause gastrointestinal bleeding (Hsu & Tsai, 2015; Li *et al.*, 2020). These actions are due to platelet inhibition, PGE2-mediated cytoprotection impairment in the gastrointestinal (GI) mucosa, and ulcerogenic effect by direct contact of this drug with the gastric mucosa. However, ASA is still considered the first antiplatelet treatment line for patients with ACS, MI, and unstable angina (Bhatt & Pollack Jr, 2021).

The most common forms of aspirin are immediate-release and enteric-coated. After oral administration, immediate-release of ASA is quickly and completely absorbed

by a passive diffusion mechanism in the acidic conditions of the stomach and upper small intestine, resulting in a rapid peak concentration (15-20 minutes), despite the presence of gastric pH and the presence of food which can slow down the rate of absorption (Alegbeleye *et al.*, 2020; Santos-Gallego & Badimon, 2021). Due to the high pH in the small intestine, the intestinal mucosa absorbs the enteric-coated form, which leads to a decrease in bioavailability and a later peak (3-4 hr). Salicylate binds to albumin by 60% to 80%, where the volume of distribution is 0.1 to 0.2 L/kg. Acidosis increases the amount of distribution by allowing the salicylate to penetrate deeper into the tissues (Santos-Gallego & Badimon, 2021). As aspirin is metabolized (inactivated) in the liver for up to 80% of therapeutic doses, it acts on its targets in the portal circulation, where platelets are exposed to a higher level of the drug than in the systemic circulation (Alegbeleye *et al.*, 2020; Paez Espinosa *et al.*, 2012). Salicylates are mostly eliminated as salicyluric acid by the kidneys (75 %) (Figure 5).

The use of urine alkalization to enhance salicylate clearance in excessive doses of ASA is based on the fact that renal excretion of salicylic acid is particularly sensitive to changes in urine pH. When the urine pH changes from 5 to 8, the renal clearance increases 10-fold (Alegbeleye et al., 2020). Acetylsalicylic acid is considered an antipyretic agent due to its ability to interfere with the production of prostaglandin E1 in the brain, besides its antiplatelet, analgesic, and anti-inflammatory properties (Awtry & Loscalzo, 2000).

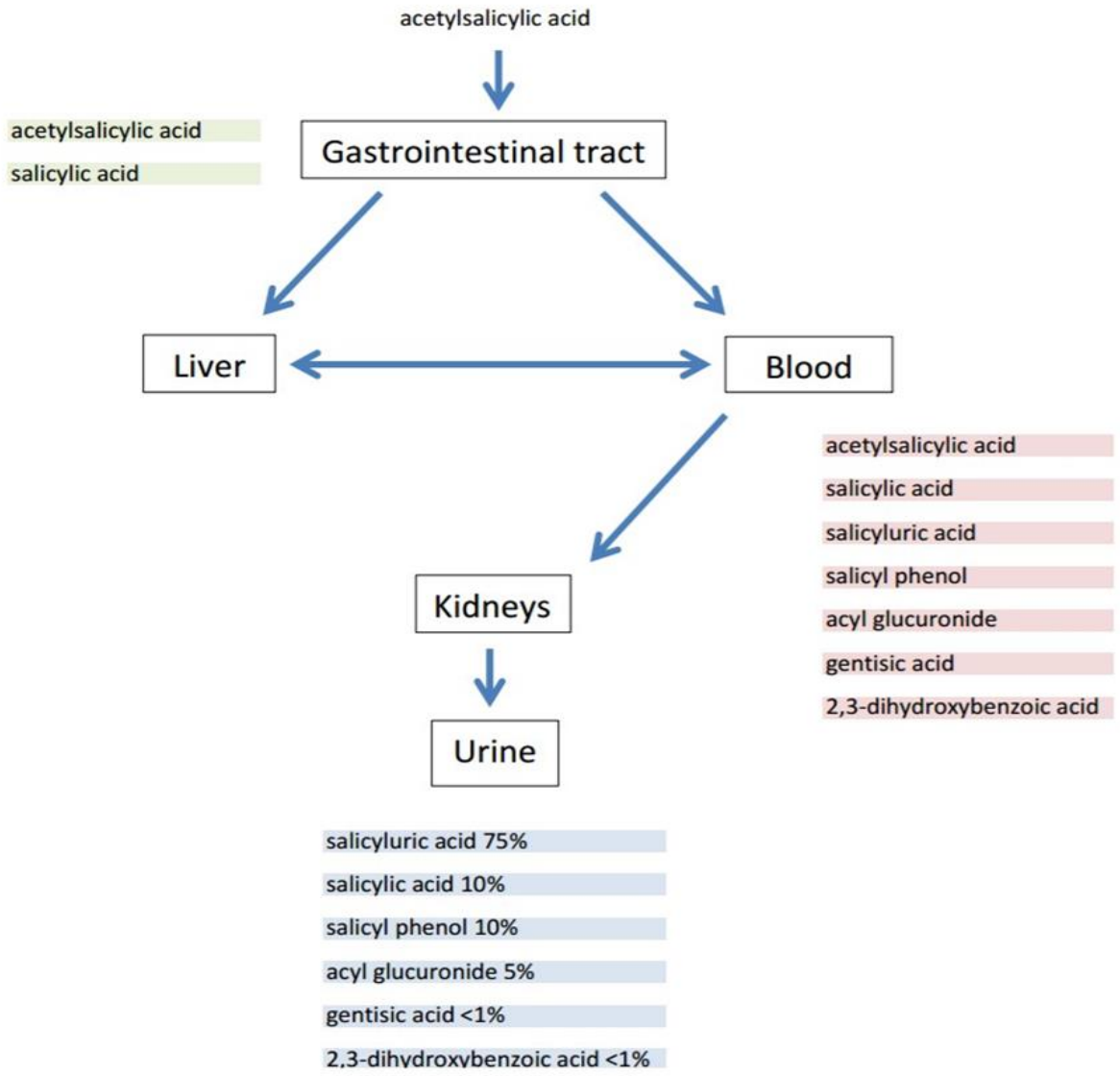

Figure 5: Overview of the generation of aspirin's metabolites (.Navaratnam, *et al*. 2017).

2.3.3 Aspirin resistance

As illustrated previously, approximately 25% of thrombotic and cardiovascular events are minimized by the therapeutic action of aspirin. However, recent reports have shown that not all ASA-treated patients obtain the complete platelets inhibition after stander dose (Biondi-Zoccai & Lotrionte, 2008; Good *et al.*, 2015). Moreover, these studies have shown the remaining platelet activation even after ingesting 81 mg ASA.

This incomplete action has been illustrated as aspirin treatment failure, ASA non-response, ASA non- sensitivity, and ASA resistance (Good *et al.*, 2015; Mykhalojko, 2018). There is no clear definition or conclusion of AR; however, it can be expressed as the inability of ASA dose to prevent adverse events of thrombosis, the increase of bleeding time, the TXA2 synthesis inhibition, and the anticipated effect test on the respective platelet functions (Macchi *et al.*, 2006; Vaduganathan & Lev, 2014). Indeed, the absence of regular testing of aspirin sensitivity or non-responsiveness although of the wide available platelet function testing variety. However, either the pharmacokinetic or pharmacodynamic disorder could explain this phenomenon (Cattaneo *et al.*, 2013; Santos-Gallego & Badimon, 2021). On one hand, if aspirin didn't reach platelets and it continued to aggregate without complete inhibition, this can be described as pharmacokinetic non-sensitivity. Patients can be linked to high platelet turnover, low absorption within the gut, high platelet count, and other reasons (Du *et al.*, 2016; Floyd & Ferro, 2014). On the other hand, when there are enough aspirin concentrations in the plasma for a complete platelet aggregation inhibition and it doesn't occur. This is considered as pharmacodynamic non-sensitivity, which can be due to COX-1 enzyme genetic polymorphism, or in other proteins within the aspirin pathway that prevent ASA efficient acetylation and/or inhibit the COX-1 enzyme (Du *et al.*, 2016; Xu *et al.*, 2012).

In recent years, various researches have been conducted to study this important fact. The findings have documented that 20-30% of patients (taking 81mg ASA) are AR, others documented a rate of 60% in their study population (Clavijo *et al.*, 2018;

Krasopoulos *et al.*, 2008). Krasopoulos et al in 2008 illustrated, after 20 studies with a total of 2930 patients that AR patients were with a greater threat of death, vascular intervention failure, and adverse cardiovascular events. They also find that patients with renal impairment were expected to have AR. Moreover, other studies have considered the associated risk factors combined with AR, where age, race, and even gender are excluded from AR association, in contrast to obesity and increased glycosylated Haemoglobin A1c (HbA1c) that have a substantial association (*i.e.* though the sample size was small) (Geara *et al.*, 2012; Tasdemir *et al.*, 2014). However, one study by Shen et al (2009) on over 745 patients, studied the association of ASA with different parameters including age, gender, high total cholesterol, and low-density lipoprotein C (LDL-C), high platelet counts, and lower hematocrit excluding race and smoking from the association. Yet, few small studies illustrated the connotation between smoking and AR by reducing the effect of aspirin on platelet aggregation (Cai *et al.*, 2020; Krekels *et al.*, 2019; Shen *et al.*, 2009; Weber *et al.*, 2000).

Furthermore, other studies have considered the antagonism between aspirin antiplatelet effects and the regular consumption of certain NSAIDs (*i.e.* ibuprofen and indomethacin). These NSAIDs blocks aspirin access to the COX-1 binding site preventing aspirin from gaining access to its target. Still, there are no specific studies to find the relationship between patients having vascular events who are treated with aspirin as well as NSAIDs (Saxena *et al.*, 2013; Shibata *et al.*, 2017). So far, having clear

evidence of risk factors associated with AR is still needed, as there are variations between studies and methods of testing (Guirgis *et al.*, 2017).

2.4 Aspirin resistance testing

The assessments of COX-1 activity have been used to determine residual platelet function in aspirin-treated patients. Ex vivo platelet aggregation can be detected by light transmission (turbidimetric) aggregometry in platelet-rich plasma or electrical impedance on whole blood after agonists, like collagen and arachidonic acid, have been added (Feinman *et al.*, 1977; Paniccia *et al.*, 2015). In addition to functional assays, there are biochemical tests that assess thromboxane metabolites in serum or urine, where TxA2 is rapidly transformed to the stable inactive (11-dehydroTxB2 (11dhTxB2)) metabolites, with a half-life measured in seconds, for example, an enzyme-linked immunosorbent assay test (ELISA) can be performed on a random urine or serum sample to measure the presence of 11dhTxB2 or TxB2 in the sample, respectively (Paniccia *et al.*, 2014). However, since these tests are labor-intensive and time-consuming, they are rarely employed in clinical practice. Platelet function testing has become more accessible to clinicians since the emergence of automated and semi-automatic tests. Numerous clinical trials have used whole blood aggregation analyses (Platelet Function Analyzer (PFA) and Ultegra rapid platelet function assay (RPFA)) as well as whole blood aggregation as determined by electrical impedance (Multiplate analyzer) (Lenk & Spannagl, 2014; van Oosterom *et al.*, 2021).

Aspirin efficiently decreases platelet TXA2 production by inhibiting platelet COX-1. TXB2 is a persistent metabolite of TXA2, and its concentrations in serum reflect platelet capacity for TXA2 synthesis, in which, abnormally in aspirin-treated patients the TXB2 levels are frequently low. To achieve significant platelet inhibition, TX production must be blocked by more than 95%, and even a tiny residual generation of platelet TXA2 is enough to activate platelets (Patrono & Rocca, 2019). Although measuring the urinary excretion of the TX metabolite (11-dehydro-TXB2) reflects actual systemic production of platelet TX and thus allows a better estimate of the system, a variable and non-negligible component of extra-platelet production of this metabolite implies a non-ideal low specificity of the test for aspirin's antiplatelet effects (Liu *et al.*, 2015). At the low doses used for antiplatelet therapy, aspirin inhibition of COX-2 enzyme is less effective and does not occur to a considerable level. TxA2 is a powerful platelet agonist that can contribute to platelet activation regardless of the source of thromboxane (platelet versus extra-platelet). As a result, even if aspirin inhibits the platelet COX-1 pathway, some patients may continue to produce thromboxane and have prolonged platelet reactivity (Maleki *et al.*, 2016; Shin *et al.*, 2021).

2.4.1 Platelets aggregometry

Platelet aggregometry is one of the instruments used to assess platelet function. It applies one of three methods: electrical impedance in whole blood, optical density in plasma, or luminescence to detect ATP release (Cardinal & Flower, 1980; Chrono-log corporation, 2012). Born initially described light transmission aggregometry in 1962,

when he characterized the aggregation of platelets by ADP and designed a colorimeter to continually monitor this aggregation in platelet-rich plasma. This method includes incubation at 37°C, stirring, and using a pen recorder to track the change in light transmission over time. Later in 1977, Feinman et al designed a Lumi-Aggregometer instrument to measure simultaneously platelet aggregation and ATP secretion. This equipment used infrared light to assess aggregation and a sensitive photomultiplier to quantify ATP release by firefly illumination at right angles to the aggregation light path. Following Born in 1980, Cardinal and Flower described impedance aggregometry to assess aggregation in whole blood by passing a very modest electric current between two electrodes. When the electrodes come into touch with blood, they are covered in a monolayer of platelets. Platelets clump on the monolayer when an aggregating agent is introduced, increasing the impedance. A pen recorder is used to record the rise in impedance. The Whole Blood Lumi-Aggregometer was produced by adding impedance measurement to the Lumi-Aggregometer. Platelets can be studied in a more physiologic whole blood environment using the impedance approach for evaluating platelet aggregation. Because sample preparation is considerably decreased, labile modulators such as prostacyclin and thromboxane A2 are preserved, resulting in a testing environment that is more sensitive to the effects of several anti-platelet medications (e.g., Aspirin, Dipyridamole) (Alessi *et al.*, 2020; Flower, 2018; Hechler *et al.*, 2019; Packham & Rand, 2011).

2.4.2 Impedance whole blood aggregometry (WBA)

The impedance (or electrical resistance) method of aggregation is non-optical. An electrode assembly is inserted into a cuvette containing a whole blood test sample. The electrode assembly consists basically of two precious metal wires that are immersed in the sample. An AC voltage in the millivolt range is applied to the probe circuit. The instrument measures the electrical resistance or impedance between the two immersed wires. During a brief period of equilibration, a monolayer of platelets forms on the exposed portions of the wires, resulting in a stable impedance value. This stable baseline of impedance is assigned a value of zero ohms of resistance. An aggregating agent is added to the cuvette and then stimulated platelets aggregation to the platelet monolayer on the immersed wires. This accumulation of platelets adds electrical resistance to the circuit, which is measured and quantified in ohms (the measurement of electrical resistance). The change in impedance is displayed as a function of time on a computer with the Chrono-Log Aggro/Link8 Software. The changes in resistance are measured and quantified in ohms (the measurement of electrical resistance). Tests are generally run for four to six minutes after the addition of an agonist (Chrono-log corporation, 2012; *et al.*, 2017).

The increase in impedance is directly proportional to the mass of the platelet aggregate. Sensitivity is increased to hyper aggregation and drugs such as aspirin and dipyridamole when compared to light transmission in platelet-rich plasma. Impedance aggregation in the blood is not dependent on the optical characteristics of the sample, so

tests can be performed on lipemic and thrombocytopenic samples. As centrifugation is not required, impedance aggregation is especially useful in conditions where megathrombocyte count is increased (Fritsma & McGlasson, 2017).

Impedance whole blood aggregometry (WBA) allows one to assess platelet function by using anti-coagulated whole blood, without the need to isolate them from other components of blood. As there is no need to centrifuge the specimen to produce an optically transparent suspension of cells, the entire platelet population is tested. The process of testing consumes less technical time, and labile factors in the blood that may influence platelet function are preserved. The Chrono-Log Whole Blood Aggregometer consists of sample receptacles heated to 37°C. There is a provision made for stirring the samples utilizing magnetic stir bars or non-magnetic disposable stir bars. Cuvettes containing the test sample and a stir bar are placed in the receptacles (Chrono-log corporation, 2012).

There are two types of agonists, the agonists that directly induce platelet aggregation, TxA2 synthesis, and platelet granule secretion are known as strong agonists (such as thrombin, collagen, TXA2, and arachidonic acid). Meanwhile, those that induce platelet aggregation without inducing secretion are considered weak agonists (such as epinephrine and ADP) (Davi *et al.*, 2012). Weak agonists induce platelet secretion and aggregation that triggers the secretion of endogenous TXA2 with platelet-to-platelet closeness during platelet aggregation. When the strong agonists are utilized at low

concentrations will act like weak ones. In contrast, weak agonists wouldn't act as strong agonists even if they are taken at high concentrations.

When some weak agonists (*i.e.* adrenaline and ADP) are taken at specific concentrations to create a biphasic appearance of platelet aggregation, a primary wave (initial) appears followed by a secondary wave of aggregation, which usually is irreversible. The aggregation response to an agonist is amplified by the production of TXA2 from membrane phospholipids and by the secretion of ADP from the dense granules. ADP and TXA2 are agonists, which, by interacting with their specific receptors, amplify the aggregation response of the platelet.

The Chrono-log Model 700 aggregometer measures platelet function on patient samples using electrical impedance in whole blood or optical density in plasma. The Model 700 Aggregometer (Chrono-log cooperation. Havertown, PA, US) can simultaneously measure ATP release with either method using luminescence. It is also used to run the Ristocetin cofactor assay which is used to diagnose patients with von Willebrands disease. The instrument works with kits consisting of collagen, epinephrine, ADP, arachidonic acid, thrombin, ristocetin, cuvettes, stir bars, and pipettes. The output of the Model 700 can be connected to either a strip chart recorder or to a computer. Software is provided for the computer interface option. The computer interface option is used to collect data only as shown in Figure 6 (Chrono-log corporation, 2012).

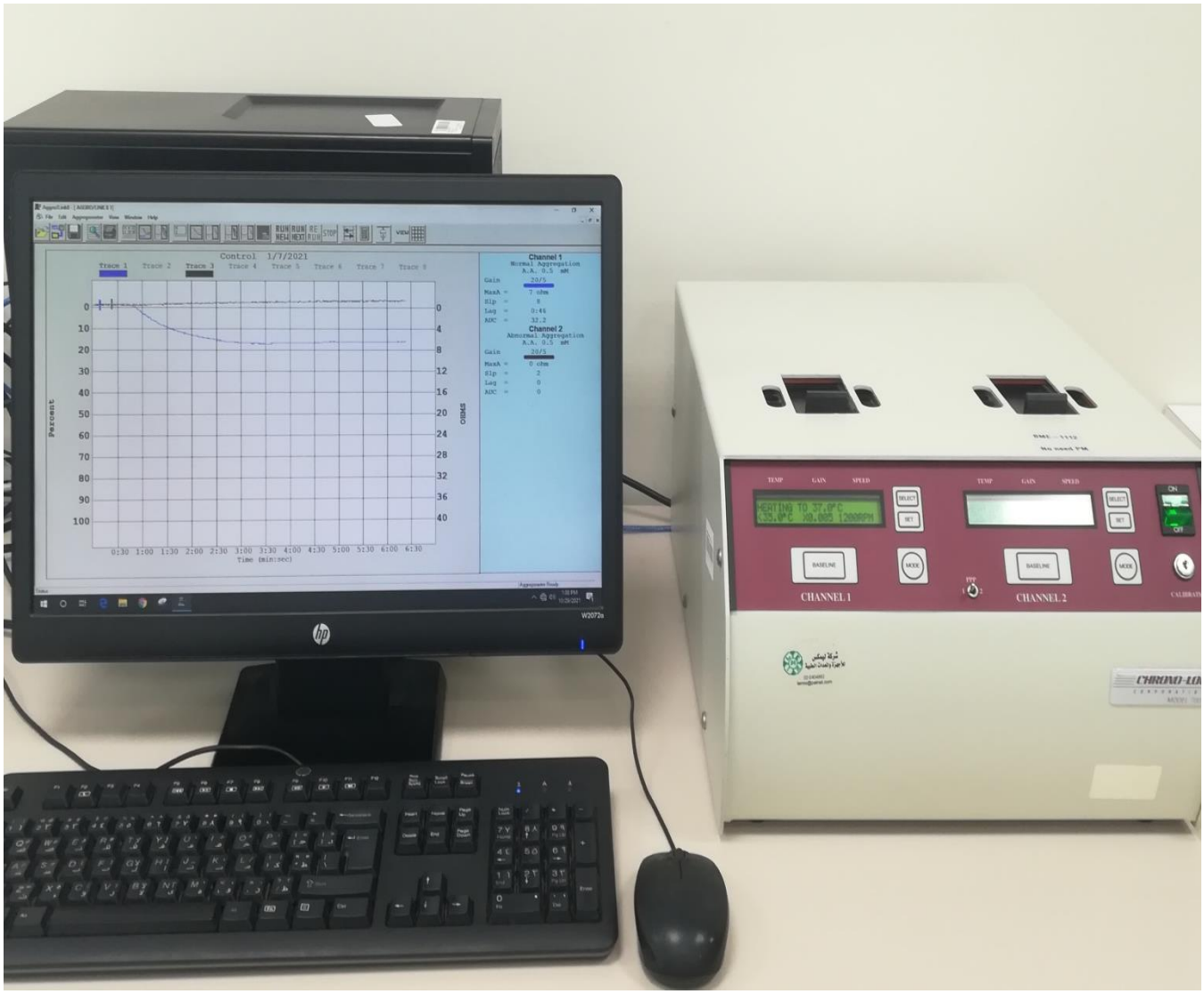

Figure 6: Chrono-log Model 700 aggregometer (Chrono-log cooperation. Havertown, PA, US).

Chapter 3

Materials and method

3.1 Study setting and population

The study population included all patients undergoing hemodialysis at An-Najah National University Hospital who were taking aspirin and met the study inclusion criteria from both genders, the study was conducted from May to July 2021.

3.2 Participants

In this pilot study, patients undergoing dialysis at An-Najah National University Hospital were enrolled to participate during the period May to June 2021. Information was collected from patients regarding their medical history and the intake of low-dose aspirin for at least 4 weeks. Patients were then selected according to the inclusion and exclusion criteria set for the study. A total of 40 patients were enrolled, all patients provided written informed consent before they participate in the study (See Appendix A).

3.3 Inclusion criteria

Only dialysis patients taking regular aspirin therapy are included in the study.

3.4 Exclusion criteria

Patients were excluded if they had one or more of the following conditions:

- Patients taking antiplatelet therapy other than aspirin such as ticlopidine, clopidogrel, dipyridamole, nonsteroidal anti-inflammatory drug, pentoxifylline, and cilostazol.

- Patients treated with glycoprotein IIb/IIIa inhibitors within the previous 10 days.
- Patients with a diagnosis of acute coronary syndrome, active malignancy, or hemorrhagic diathesis.
- Patients taking thrombolytic treatment within the last month.
- Patients with liver disease.
- Patients with platelet counts of less than 100, 000/uL.
- Children were not included in the study.

3.5 Variables included in this study

Age, gender, smoking, hypertension, diabetes mellitus, hyperlipidemia, coronary artery disease, and laboratory tests related to cardiovascular disease.

3.6 Data collection and clinical parameters

The demographic, medical history, and the results of some tests conducted regularly were obtained from the medical files of the patients at An-Najah National University Hospital for all the study population.

The following tests were performed for lipid profile including (Triglycerides, Total Cholesterol, HDL, and LDL) as well as hemoglobin A1C and platelet aggregation tests were performed on the study population (see Appendix B).

Venous blood was collected in EDTA tubes and used for the determination of hematological indices including red blood cell count (RBC), hemoglobin concentration,

and mean cell volume (MCV) using Nihon Kohden (MEK-9100K) cell counter (Diamond Diagnostics, Japan), and it was also used to determine hemoglobin A1C level using Cobas 6000 chemistry analyzer (Hoffmann-La Roche Ltd). Another blood sample was collected using a lithium heparin tube (green cap) and it was used for determining plasma lipids. The tube was centrifuged for 5 min at 4000 rpm, then the plasma sample was analyzed. Hemoglobin A1C, total cholesterol (TC), triglyceride (TG), and high-density lipoprotein cholesterol (HDL-C) were measured enzymatically using Cobas 6000 chemistry analyzer (Hoffmann-La Roche Ltd). Low-density lipoprotein cholesterol (LDL-C) levels were estimated from the Friedewald formula (FF): (LDL-C = TC – [HDL-C + (TG/5)].

3.7 Preparation of Arachidonic Acid

The Arachidonic acid in the vial is a lyophilized oily drop, which was shaken and tapped to the bottom. A cap cracker was used to break the vial tip, and 100μL of reconstituted albumin was pipetted into both the tip and body of the vial until reaching a total volume of 700 μL. While albumin was added, the vial and the tip were rotated to mix in the Arachidonic Acid on their sides. The Arachidonic acid was vigorously mixed with albumin using a plastic transfer pipette, followed by combining the suspensions from the tip with that in the body of the vial while continuing mixing until the suspension reached the maximum turbidity. The reagent was transferred to a micro-centrifuge tube and vortexed at the highest speed for 4-5 minutes tell it appeared very milky with

numerous small bubbles. The reagent as well was vortexed for 2 minutes immediately before running each test.

3.8 Blood sample collection and whole blood aggregometry

Blood samples were drawn from all patients following the aseptic technique in 3.2% sodium citrate with no harm to patients as blood drawing was done from an existing central line by well-trained staff (nurses). Blood samples were mixed by inverting five times and placed at room temperature until being analyzed. All analyses were conducted within 1-2 hr of blood collection. Platelet aggregation was performed by CHRONO-LOG Model 700 whole blood/Lumi-Aggregometry in a two-channel configuration.

Samples were diluted with a 1:1 ratio of 450 µl of irrigation saline and 450 µl of a whole blood sample from the sodium citrate tube. The process was prepared in a pre-wormed cuvette with stirring bars and placed in the incubation well at 37 °C for five min. Following incubation, the cuvette was placed in the reaction well and an electrode was inserted into the cuvette sample and incubated for two min with a stirring speed set at 1200 RPM. At this moment, platelets in the blood sample adhere and coat two fine wires on the electrode, forming uniform monolayers. A small voltage difference was applied across the two wires, and the impedance caused by the platelets coating the wires was measured. In the absence of an aggregating agent or agonist, the interactions between the platelets and the electrodes stabilize, and the impedance between the two electrodes becomes constant, producing a baseline. When an agonist was added, platelets in the specimen were activated and began to aggregate. The platelet coating on the wires

thickened overpassed minutes with a corresponding increase in electrical impedance between the electrode wires. This change in impedance was directly proportional to the extent of platelet aggregation and was indicated on the digital display in ohms after six min. Platelets were activated using 0.5 mM lyophilized arachidonic acid reconstituted in 100 mg bovine albumin, fraction V powder 96% to 99% purity.

Platelet aggregation susceptibility was expressed as a change in electrical impedance and is expressed in ohms. Aggregation curves were recorded for six min and analyzed using AGGROLINK_ software.

ASA resistance was defined as an increase in electrical impedance after the addition of arachidonic acid agonist, as described in the literature (Harrison *et al.*, 2005; Kottke- Archant *et al.*, 2001; Lev *et al.*, 2006; Lordkipanidzé *et al.*, 2007; Maree *et al.*, 2005; Pedersen *et al.*, 2009; Tantry *et al.*, 2005). The normal range of platelet aggregation in whole blood using 0.5 mM arachidonic acid agonist is from 5-17 ohms.

3.9 Quality control

A control sample was also tested with each run to ensure the validity of the results.

Positive controls were provided by collecting samples from aspirin volunteers, the results of these samples showed no platelet aggregation.

Negative controls were provided by collecting samples from healthy volunteers, who do not suffer from platelet disorder or who take aspirin, the results of these samples showed platelet aggregation.

3.10 Ethical consideration

The study was approved by the ethical review committee of Birzeit University and An-Najah National University Hospital Institutional Review Board where the principles of the Helsinki Declaration were implemented. The potential risks were explained to each participant and all patients provided written informed consent before they participated in the study (See Appendix A).

3.11 Statistical analysis

Data were entered and analyzed using the Statistical Package for Social Sciences program (SPSS) ver. 21. Data were expressed as means ± SD for continuous variables and as frequencies and percentages for categorical variables. Variables that are not normally distributed will be expressed as medians (lower-upper quartiles).

Chapter 4

Results

4.1 Identification of study patients

The first objective of this study was to identify the subpopulation of patients undergoing hemodialysis who were treated with low aspirin dose regularly. Forty (40) participants were recruited for this estimated study that met the study inclusion criteria from both genders and would make clinical conclusions of detecting ASA resistance using Whole blood aggregometry. Healthy control and aspirin control subjects were used for the optimization of the WBA analysis.

4.2 Characteristics and laboratory values of the study group

For the 40 HD patients receiving daily low dose ASA, the medical history and most laboratory tests were retrieved from An-Najah National University Hospital database. Table 2 shows the general characteristics of these patients. The study population was composed of 22 males (55 %) and 18 females (45%) with a mean age average of 57.9 ± 15.1 years.

Table 2: The study group's general characteristics.

Study Group (n = 40)	
General characteristics	
Age, years (mean ± SD)	57.9 ± 15.1
Male (n, %)	22 (55)
Female (n, %)	18 (45)

The existence of cardiovascular disease risk factors among these patients. The majority of the study population have hypertension (82.5%) and 57% of them have diabetes. Meanwhile, smoking frequency and hyperlipidemia was 6.15% and 35%, respectively (Table 3).

Table 3: The risk factors for cardiovascular disease of the study group.

Risk factor for cardiovascular disease	
Smoking (n, %)	6 (15)
Hypertension (n, %)	33 (82.5)
Diabetes mellitus (n, %)	23 (57)
Hyperlipidemia (n, %)	14 (35)

The hematology section shows that all mean ± SD values of WBC, MCV, PLT, and MPV (6.7 ± 1.9 K/µL, 86.9 ± 5.7fL, 228 ± 60.4 K/µL, and 9 ± 0.745fL, respectively) are in the normal range. Whereas, hemoglobin and hematocrit values (10.3 ± 1.4g/dl and 30.9 ± 4.2%, respectively) were below the normal range as it is used to be in this group of patients (Table 4).

Table 4: Hemogram tests of the study population.

Hematology section		
Test	**Mean ± SD**	**Normal range**
WBC (K/µL)	6.7 ± 1.9	4 – 9
Hemoglobin (g/dl)	10.3 ± 1.4	12 – 18
Hematocrit (%)	30.9 ± 4.2	33.5 – 52
MCV (fL)	86.9 ± 5.7	80 – 100
PLT (K/µL)	228 ± 60.4	140 – 400
MPV (fL)	9.0± 0.8	7 – 11

For the liver and kidney functions tests, the average values of BUN (58.6 ± 11.8 mg/dl), creatinine (8.1 ± 1.9 mg/dl) are above their normal range, and e.GFR (6.2 ± 1.8 ml/min/1.73 m2) is within the normal range for stage 5. This is predictable since they are end-stage kidney disease patients. Meanwhile, the average values for liver function tests were normal such as Total bilirubin, AST, ALT, and Albumin where 0.3 ± 0.1 mg/dl, 13.5 ± 5.5 U/L, 9.4 ± 3.8 U/L, and 3.5 ± 0.4 g/dl, respectively. However, the ALP values (130.0 ± 121.9 U/L) were slightly higher than the normal range (Table 5).

Table 5: The liver and kidney function tests of the study group.

Liver and kidney functions tests:		
Test	**Mean ± SD**	**Normal range**
BUN (mg/dl)	58.6 ± 11.8	5 - 22
Creatinine (mg/dl)	8.1 ± 1.9	0.7 - 1.2
e.GFR (ml/min/1.73 m2)	6.2 ± 1.8	Stage 1 (GFR >90) Stage 2 (GFR 60-80) Stage 3 (GFR 30-59) Stage 4 (GFR 15-29) Stage 5 (GFR <15)
Total bilirubin (mg/dl)	0.3 ± 0.1	0.2 - 1.2
AST (U/L)	13.5 ± 5.5	1 - 40
ALT (U/L)	9.4 ± 3.8	1 - 41
ALP (U/L)	130.0 ± 121.9	40 - 129
Albumin (g/dL)	3.5 ± 0.4	3.5 - 5.2

Table 6 shows the lipid profile tests, where the average values of both cholesterol and HDL (142 ± 37mg/dl and 37 ± 13 mg/dl) were within the normal range. In contrast to triglyceride values (156 ± 92 mg/dl) that were higher than the normal range and LDL values (73.5 ± 31 mg/dl) that were lower than the normal range.

Table 6: The lipid profile tests of the study group.

Lipid profile tests:		
Test	**Mean ± SD**	**Normal range**
Cholesterol (mg/dl)	141.7 ± 37.0	0 - 200
Triglyceride (mg/dl)	156.0 ± 92.0	0 - 150
HDL (mg/dl)	37.0 ± 12.7	35 - 55
LDL (mg/dl)	73.5 ± 31.0	100 - 129

Moreover, the serum electrolyte profile values were all within the normal range. This profile includes Sodium, Potassium, Chloride, and Calcium tests that have resulted in an average of 138 ± 2.9 mmol/L, 4.9 ± 0.65mmol/L, 98.4 ± 3.5 mmol/L, and 8.6 ± 0.68 mg/dl, respectively (Table 7).

Table 7: The electrolyte tests of the study group.

Electrolytes tests:		
Test	**Mean ± SD**	**Normal range**
Sodium (mmol/L)	138 ± 2.9	135 - 155
Potassium (mmol/L)	4.9 ± 0.7	3.5 - 5.2
Chloride (mmol/L)	98.4 ± 3.5	98 - 107
Calcium (mg/dl)	8.6 ± 0.7	8.6 - 10

The iron status for these HD patients was illustrated by the values of both iron (46 ug/dL) and TIBC (216 mcg/dL) that were below the normal range. Meanwhile, the Ferritin values (389 ng/mL) were high. This is expected because of hemoglobin and hematocrit low levels, while the ferritin elevation is due to their current treatment as well as it being an acute phase reactant in this group of patients (Table 8).

Table 8: The iron status tests of the study group.

Iron status tests:		
Test	**Mean ± SD**	**Normal range**
Iron (ug/dL)	46.0 ± 17.8	50 - 160
TIBC (mcg/dL)	216.0 ± 50.1	250 - 410
Ferritin (ng/mL)	389.0 ± 279.0	20 - 300

Finally, Table 9 shows plasma glucose (random test) and glycosylated hemoglobin (Hb A1C), both were above their normal range (157 mg/dl and 5.9 %, respectively).

Table 9: The diabetes control tests of the study group.

Diabetes Control tests:		
Test	**Mean ± SD**	**Normal range**
Glucose random (mg/dl)	157.0 ± 73.7	Less than 140
Glycosylated hemoglobin (%)	5.9 ± 1.6	4 – 5.6

4.3 Assessment of ASA non-responsiveness

Whole blood impedance aggregometry was performed on the study population (40 HD patients), as well as the quality control procedures in each run. The control also included samples collected from healthy volunteers (not using aspirin or other medication) to create normal aggregation (normal control), as well as samples collected from healthy volunteers who had taken aspirin daily for prophylaxis to create abnormal or no platelet aggregation (abnormal control) as shown in Figure 7.

As shown in Figure 9, the resulted ohms readings were 10 ohms for the normal control sample (normal aggregation) and it was zero ohms for the abnormal control

samples (no aggregation), after inducing platelet aggregation by using 0.5 mM arachidonic acid.

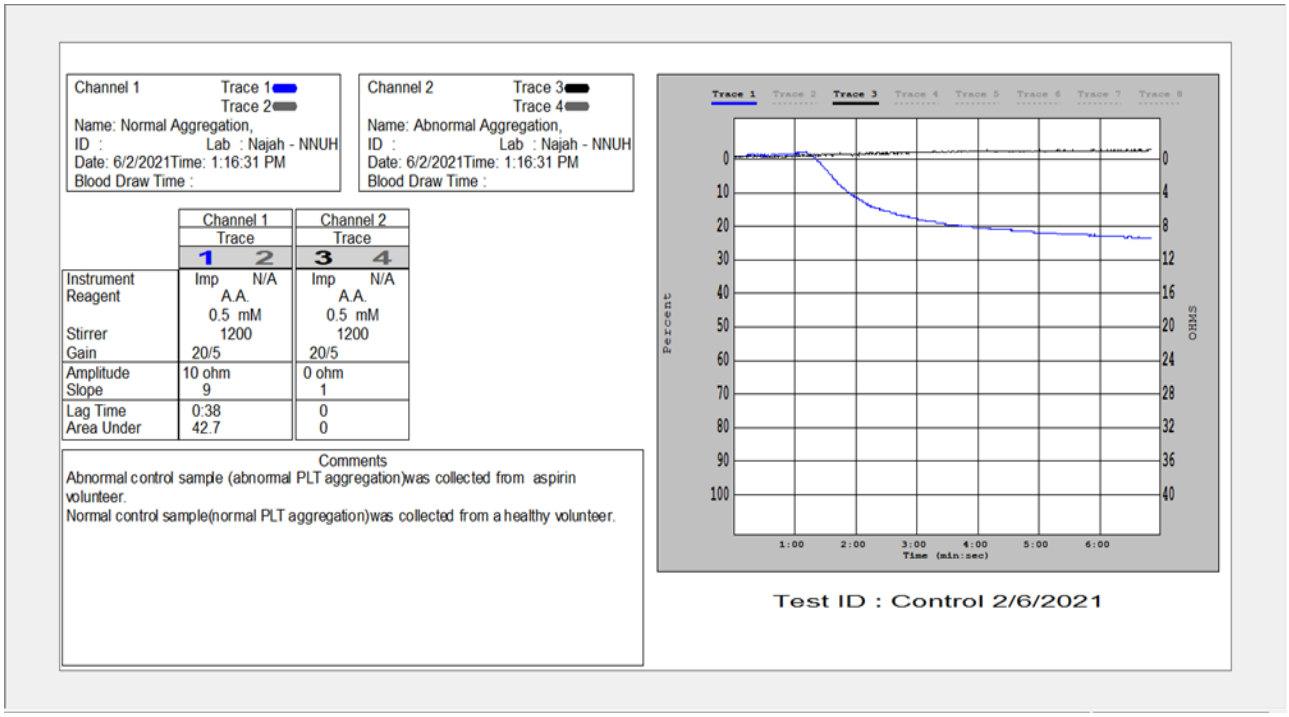

Figure 7: A Chronolog 700 Impedance aggregometer reading for control samples: channel 1 (Trace 1, blue) normal control sample; channel 2 (Trace 3, black) abnormal control sample, after inducing platelet aggregation by using 0.5 mM arachidonic acid.

WBA demonstrated that 62.5% of the population (25 patients) had no aggregation (0 Ohms), where these patients are considered sensitive to the effect of daily low dose ASA as shown in **Error! Reference source not found.**, Figure 8. Moreover, the study showed that 27.5% of the patients had slightly aggregation (1 Ohm, 2 Ohms, 3 Ohms, and 4 Ohms) that also are considered sensitive to ASA according to the standard normal range (5-17 Ohms) of arachidonic acid-induced platelet aggregation in whole blood (Table 10, Figure 9). The remaining 10% of the population (4 HD patients) have increased ohms values (10 Ohms and 12 Ohms) as they are considered as aspirin non-sensitive or non-responding to the daily ASA low dose as shown in Table 10, Figure 10.

Table 10: Platelet aggregation values of the study group.

Normal Range to A.A 0.5 mM (5-17 Ohms)			Study Group (n = 40)	
Aggregation in Ohms	**Frequency (n = 40)**	**Percent %**	**Cumulative Percent**	**Response to aspirin**
0	25	62.5	62.5	Responding
1	2	5.0	67.5	Responding
2	3	7.5	75.0	Responding
3	2	5.0	80.0	Responding
4	4	10.0	90.0	Responding
10	2	5.0	95.0	Non-responding
12	2	5.0	100.0	Non-responding
Total	40	100.0		

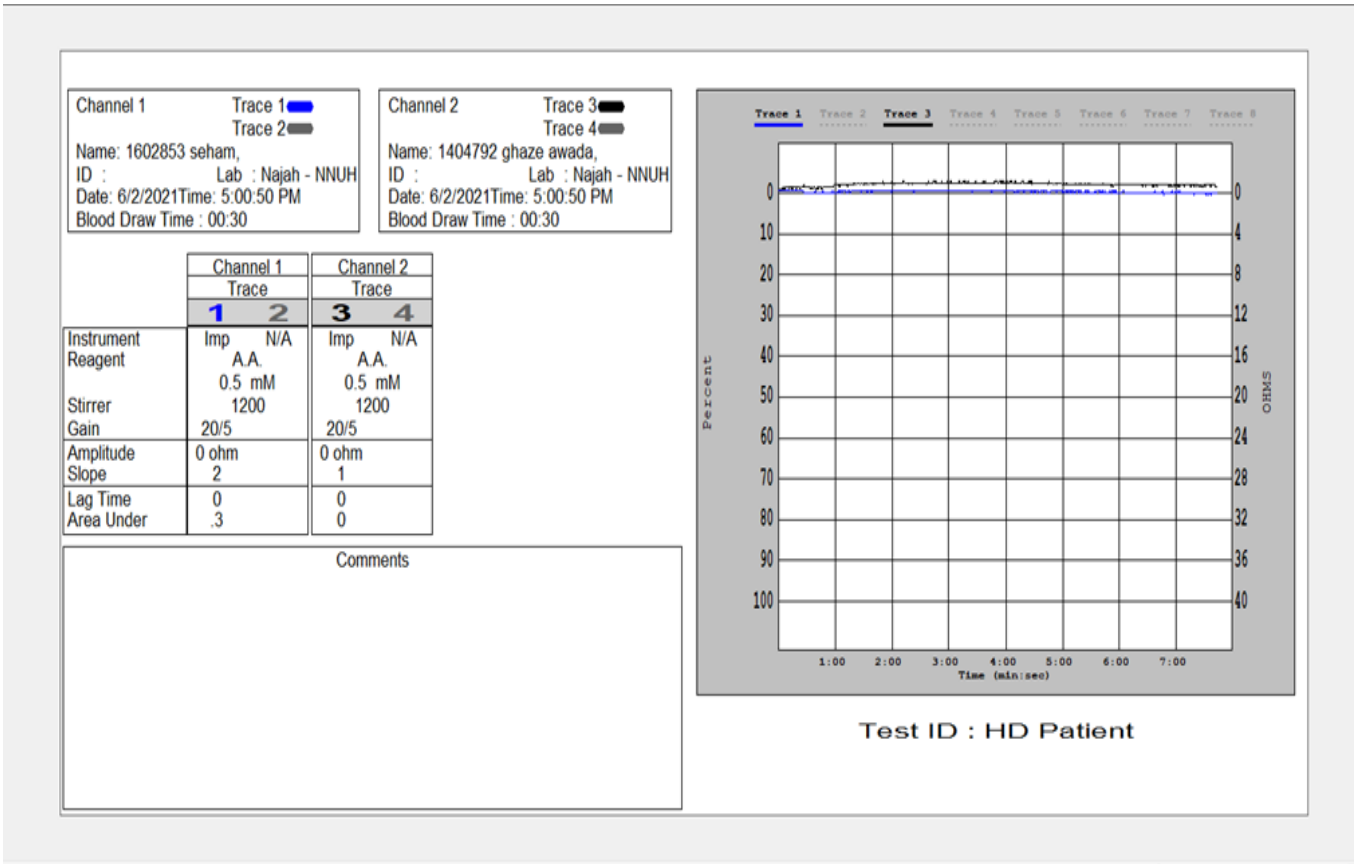

Figure 8: A Chronolog 700 Impedance aggregometer reading zero Ohms for HD patients responding to daily low dose ASA, after inducing platelet aggregation with 0.5 mM arachidonic acid.

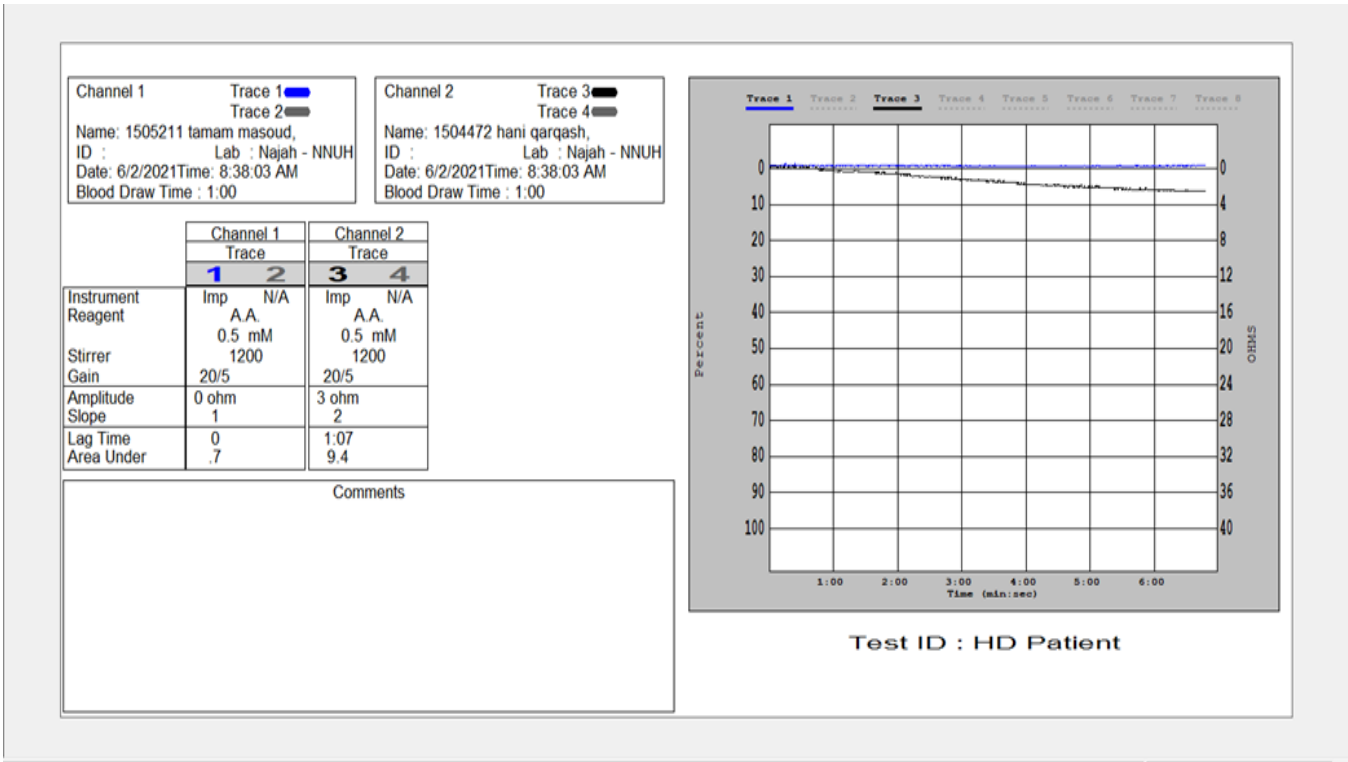

Figure 9: A Chronolog 700 Impedance aggregometer reading 0 and 3 Ohms for HD patients responding to daily low dose ASA, after inducing platelet aggregation with 0.5 mM arachidonic acid.

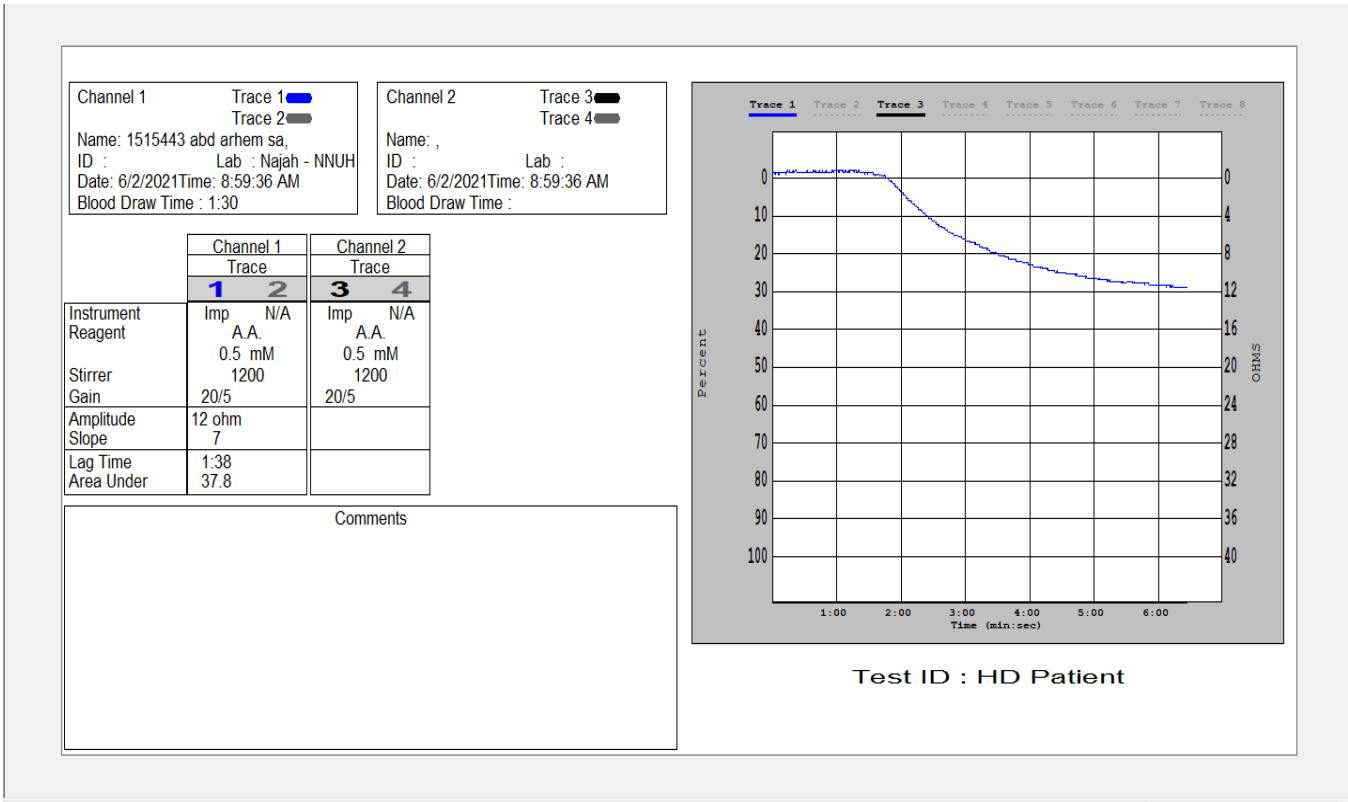

Figure 10: A Chronolog 700 Impedance aggregometer reading 12 Ohms for HD patients none responding to daily low dose ASA, after inducing platelet aggregation with 0.5 mM arachidonic acid.

Chapter 5

Discussion

The low levels of RBCs indices including hemoglobin, hematocrit, as well as iron status for the study population, are observed since the majority of CKD patients suffer from anemia, which can be illustrated by deficiencies in iron and erythropoietin. Moreover, CKD patients with end-stage kidney disease suffer from very low levels of hemoglobin because of uremic intoxication that inhibits the erythropoiesis process, insufficiency of pre-ESKD nephrology precautions, and deficiency of adequate anemia cure (Karaboyas *et al.*, 2020; Amjad *et al.*, 2021). However, the elevated ferritin levels in those patients are due to their treatment with regular intravenous (IV) iron and erythropoiesis-stimulating agents (ESAs) (Kuragano *et al.*, 2020; Fishbane *et al.*, 2018).

Patients with CKD are regularly tested for BUN, Creatinine, and GFR, where these tests are considered as indicators for the development of CKD. Kidneys are responsible for removing urea nitrogen (it is a waste product of body metabolism and protein consumption) from the body. Although urea-nitrogen levels rise by eating more protein, in this study urea-nitrogen elevation is due to Kidney failure. Moreover, Creatinine is another muscles waste product that is removed from the body through the kidneys. Creatinine elevation in serum is an indicator for calculating the GFR of the patient to observe kidneys conditions. Hence, if the GFR is below 30, a nephrologist is consulted, and if it is below 15, direct treatment (either dialysis or kidney transplant) should be initiated. This explains the elevation of serum creatinine levels in the study population

and the GFR levels that were below 15 in which they are all under dialysis treatment (Bjornstad *et al.*, 2015; Omar *et al.*, 2016).

Moreover, elevated levels of alkaline phosphatase (ALP) are associated with end-stage renal disease patients on hemodialysis therapy. In which screening and monitoring patients with liver disease is accomplished by observing ALP levels in serum (Blayney *et al.*, 2008). This elevation is due to renal osteodystrophy, secondary hyperparathyroidism (SHPT), cardiovascular disease (CVD), cardiac failure, and diastolic dysfunction (Guo *et al.*, 2020; Natikar *et al.*, 2020). Additionally, the total alkaline phosphatase in the serum generally includes isoenzymes from the liver, bones, intestines, kidneys, and/or leukocytes. Although the documented elevation of ALP isoenzyme is correlated to the elevated level of parathyroid hormone (PTH), few studies have inspected the possibilities associated with this levitation (Fan *et al.*, 2017). For example, Kovesdy *et al.* (2010) had verified that in HD patients the higher baseline and time-varying ALP levels are associated with increased risk of all-cause of mortality. Their study was held without including the elevated levels of phosphorus and calcium in the serum that have a strong association with higher mortality risk. In another study by Soohoo *et al.* (2016), they confirmed also the association of high calcium, phosphorus, alkaline phosphatase (ALP), and intact parathyroid hormone (iPTH) concentrations within the serum of patients with advanced chronic kidney disease (CKD) and end-stage renal disease (ESRD), those patients are linked with increased cardiovascular disease and mortality. They revealed that changes in the previously mentioned minerals could lead to structural and functional

abnormalities in the bone and cardiovascular system due to CKD-mineral and bone disease (CKD-MBD) along with higher morbidity and mortality in these patients.

Cardiovascular events are the main cause of death within the majority of HD patients. This, hyperlipidemia (lipoprotein abnormalities) is the main risk factor for these events. Hyperlipidemia, which is characterized by increased cholesterol and/or low-density lipoprotein (LDL) levels in plasma, and dyslipidemia are related to progressive renal failure. Hypertriglyceridemia is considered a common lipid abnormality characteristic in HD patients. This is caused by Apo B protein increased production and decreased levels in the metabolism of very-low-density lipoprotein VLDL, which is primarily a result of decreased endothelial cell debilitation of VLDL. And this emphasizes the elevated triglyceride levels and the depletion of LDL levels in the tested study population (Mikolasevic *et al.*, 2017; Saini *et al.*, 2021).

The electrolytes were within the normal range but the calcium was observed to be at the lower limit and this is due to the fact that vitamin D and parathyroid hormone (PTH) help regulate how much calcium is absorbed and how much calcium is the kidneys eliminate. In hemodialysis (HD) patients, the regulation of calcium homeostasis is very complex, due to an imbalance of PTH and calcitriol levels (Timofte *et al.*, 2021; Xu *et al.*, 2021).

Glycemic state is a risk factor for sudden cardiac death in dialysis patients. The glycemic control tests were carried out in our study population to observe their status.

The results showed a slight elevation in their average value which indicates that these patients are under control for their glucose levels (Hill *et al.*, 2014; Williams *et al.*, 2014).

According to whole blood impedance aggregometry results for this small study population, the device was able to follow aspirin sensitivity and non-sensitivity and to be adjusted with the normal and abnormal controls. After investigating with the 10% non-sensitive HD patients, they appeared to stop low-dose aspirin intake for almost two weeks. Few studies have shown that patients using aspirin irregularly (non-compliance) are prone to develop aspirin resistance. However, so far having clear evidence of risk factors associated with AR is still needed, as there are variations between studies and the methods of testing (Ghorbani-Shirkouhi *et al.*, 2021; Mayer *et al.*, 2014; Osinska *et al.*, 2017). Cattaneo *et al* (2013) also declared that the absence of regular testing of aspirin sensitivity or non-responsiveness wouldn't help in explaining this phenomenon by understanding both pharmacokinetic and pharmacodynamic disorder.

Moreover, other studies have considered the associated risk factors combined with AR, where age, race, and even gender are excluded from AR association, in contrast to obesity and increased glycosylated Haemoglobin A1c (HbA1c) that have a substantial association (Geara et al., 2012; Tasdemir *et al.*, 2014). Their results were quite compatible with this research. Within the 10% AR HD patients the gender was equally distributed (5% male and 5% female). Hence, this cancels the gender association with AR in this study group. Showing also the glycemic state (a risk factor for sudden cardiac

death in dialysis patients) association for AR, in which the results showed a slight glucose level elevation in the average value which indicates that these patients are under control for their glucose levels.

In contrast to Cai *et al* (2020) that illustrated the connotation between smoking and AR through reducing the effect of aspirin on platelet aggregation, this study showed that 10% of AR HD patients are all nonsmokers, joining the foundation of Shen *et al.* (2009) that excluded the gender and smoking from ASA sensitivity association. Furthermore, the antagonism between aspirin antiplatelet effects and the regular consumption of certain NSAIDs, as an AR-associated factor, was excluded from this study in which all the study populations were non-NSAID taking HD patients (Kilickesmez *et al.*, 2013; Scharf., 2012).

Chapter 6

Limitations

Our study has several important limitations. The small size number of subjects was a major limitation of this study due to the study criteria and also to the provided test volume. Larger sample size is necessary for a more accurate prediction of ASA non-sensitivity and to draw a concrete conclusion. Moreover, the ex vivo tests which may not necessarily reflect the in vivo response of patients to these agonists are considered as another study limitation.

The main outcome of our study was to identify the prevalence of aspirin resistance among hemodialysis patients, in a cross-sectional way, to better understand the AR in our HD patients. Firmly, a longitudinal study design and follow-up of patients will provide more information.

The present study will be more valuable if two tests were performed, TXB2 and whole blood impedance aggregometry on a total sample of 100 HD patients. This could help to obtain more accurate test results and to compare the results of the two tests on a larger representative sample. Due to financial problems and lack of enough budgets, we were forced to reduce the sample size and perform only the whole blood resistance test.

Chapter 7

Conclusion

Whole blood impedance aggregometry was able to detect platelet sensitivity and non-sensitivity to ASA. This is with great impact on both physicians and HD patients to be familiar with this phenomenon. The results of the present study will encourage physicians to perform platelet aggregation to make sure the drug is working properly in these patients.

In Palestine, further studies with a large scale population should be conducted to be more familiar with this phenomenon for resolving the clinical relevant issue and comprehensive point of care tests for diagnosing ASA non-sensitivity and personalizing therapy, where this test should be before aspirin dose intake.

Chapter 8

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Chapter 9

Appendix

9.1 Appendix A

Consent form:

نموذج طلب موافقة على المشاركة في بحث علمي

عنوان الدراسه: دراسة مدى إنتشار ظاهرة مقاومة الأسبرين لدى المرضى الذين يقومون بغسيل الكلى في مستشفى النجاح الوطني الجامعي .

إسم الباحث الرئيسي: خالد مناصره.

المشرفون على البحث: د. محمد فراج (جامعة بيرزيت - مشرف داخلي)، د. أدهم أبو طه (جامعة النجاح الوطنية -مشرف خارجي).

ملخص البحث:

يقوم هذا البحث على دراسة مدى فعالية دواء الأسبرين في حماية مرضى غسيل الكلى من الدوبات القلبية، والسكتاتالدماغية، والذبحة الصدرية أم يوجد هناك مقاومة لهذا الدواء لديهم. حيث سيتم سحب عينة دم من كل مريض يوافق علىالمشاركة بهذه الدراسة لعمل فحص مقاومة الأسبرين للتأكد من وجود هذه الظاهرة أو عدم وجودها لديهم. وتقام هذهالدراسة استيفاءاً لمتطلبات التخرج من برنامج ماجستير العلوم الطبية المخبرية في جامعة بيرزيت.

المخاطر المتوقعه والخصوصيه:

ليست هنالك اى مخاطر للدراسة سواء نفسية أم جسديةعلى المشاركين في هذه الدراسة. وسيتم حفظ خصوصيتك كمشارك/ة بالدراسة وسوف يتم التكتم على هويتك/ى وسيبقى اسمك/ى على الكتمان وسيتم التعامل مع المعلومات الخاصة بكبطريقة الترميز. لك حق الانسحاب من المشاركة في البحث في اى وقت دون وجود اى تبعات قد تاثر عليك او على الرعايةالطبية التي سوف تتلقها.

المنافع المتوقعة:

تتطلع هذه الدراسة إلى التعرف على ظاهرة مقاومة الأسبرين ومدى انتشارها بين مرضى غسيل الكلى وبالتالي سيكون كل من الطبيب والمريض على معرفه بمدى استفادة المريض من اخذ دواء الأسبرين، وفي حال ظهور مقاومه لهذا الدواء عند المرضى سيتم اختيار دواء أخر للمريض بنفس فعاليه الأسبرين، وبالتالي سيتم تشجيع الأطباء على عمل هذهالفحوصات قبل وصف الأسبرين كدواء لهؤلاء المرضى.

طريقه التواصل مع الباحث:

إذا كانت لديك اى سؤال او إستفسار عن الدراسة يمكنك التواصل مع الباحث (خالد مناصره) بكل رحابة وفي اى وقت عن طريق (الهاتف-0598369993) أو البريدالإلكتروني(khaled52729@yahoo.Com).

توقيع لمشاركه في البحث:

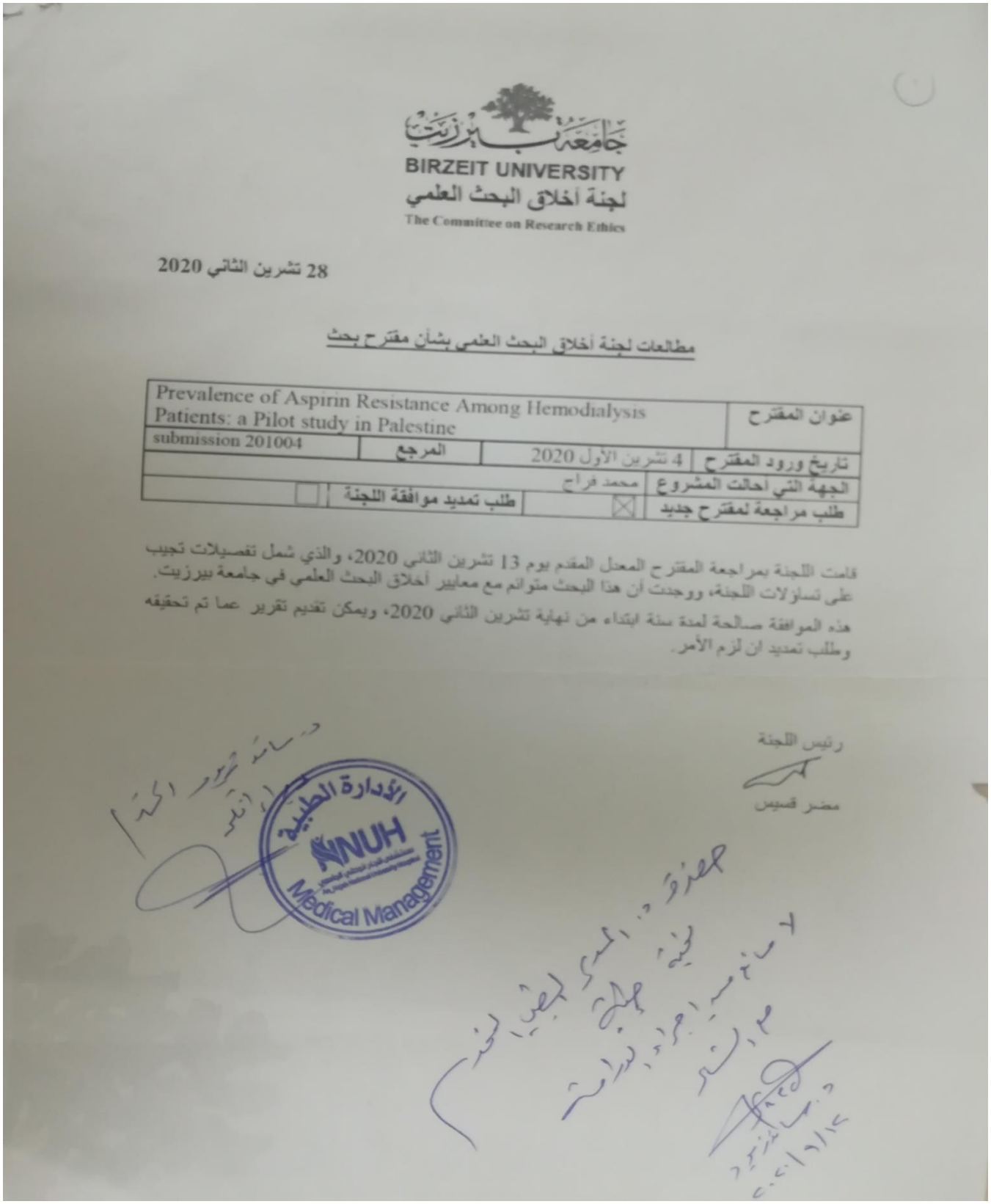
لقد حصلت على شرح مفصل عن الدراسة وأهدافها وإجراءاتها، ومدافعها، والمخاطر المحتملة. ولقد فهمت كافة المعلوماتالتي قدمت لي وتمت الإجابة عن كل اسئلتي. لذا فإنني أوافق وبمحض إرادتي على المشاركة في هذه الدراسة.

الاسم:.....

التوقيع:.....

التاريخ:.....

Figure 11: Consent form that was signed by the study population.



جامعة بيرزيت
BIRZEIT UNIVERSITY
لجنة أخلاق البحث العلمي
The Committee on Research Ethics

28 تشرين الثاني 2020

مطالعات لجنة أخلاق البحث العلمي بشأن مقترح بحث

عنوان المقترح	Prevalence of Aspirin Resistance Among Hemodialysis Patients: a Pilot study in Palestine		
تاريخ ورود المقترح	4 تشرين الأول 2020	المرجع	submission 201004
الجهة التي احالت المشروع	محمد فراج		
طلب مراجعة لمقترح جديد	☒	طلب تمديد موافقة اللجنة	☐

قامت اللجنة بمراجعة المقترح المعدل المقدم يوم 13 تشرين الثاني 2020، والذي شمل تفصيلات تجيب على تساؤلات اللجنة، ووجدت أن هذا البحث متوائم مع معايير أخلاق البحث العلمي في جامعة بيرزيت.

هذه الموافقة صالحة لمدة سنة ابتداء من نهاية تشرين الثاني 2020، ويمكن تقديم تقرير عما تم تحقيقه وطلب تمديد ان لزم الأمر.

رئيس اللجنة

مصر قسيس

الإدارة الطبية
NUH
Medical Management

Figure 12: Ethical consideration from the ethical review committee of Birzeit University and An-Najah National University Hospital Institutional Review Board.

9.2Appendix B

Table 11: The data collection and clinical parameters for HD patients.

1-General information about the patient:				
Patient name: MRD:	Serial #:	Date:		
Gender: Male ☐ Female ☐	Age:	Weight:	Height:	
Do you take aspirin: Yes ☐ No ☐ Frequency of Aspirin: Daily ☐ Other ☐ Do you smoke: Yes ☐ No ☐	Dose:			
2-Medical History:				
Do you have:	Yes	NO	Note	
Coronary artery disease				
Hypertension				
Diabetes mellitus				
Hypercholesterolemia				
Liver disease				
Hemorrhagic diathesis				
Active malignancy				
3-Medication history of the patient:				
Are you taking:	Yes	No	Note	
Thrombolytic treatment within the last month				
Glycoprotein IIb/IIIa inhibitors within the previous 10 days.				
Ticlopidine				
Clopidogrel				
Dipyridamole				
Nonsteroidal anti-inflammatory drug				
Pentoxifylline				
Cilostazol				
4- Lab tests that will be performed:				
Lab test:	Result	Unit	Normal Rang	Note
WBC count		$10^3/\mu l$	4-9	
Hb level		g/dl	11.6-15.6	

Hct		%	34-46	
MCV		fL	80-97.6	
Platelet count		$10^3/\mu l$	150-350	
Mean Platelet Volume (MPV)		fL	7-11	
BUN		mg/dl	5-22	
Cholesterol		mg/dl	< 200	
Triglyceride		mg/dl	< 150	
HDL		mg/dl	35-55	
LDL		mg/dl	100-129	
Creatinine		mg/dl	0.7-1.2	
Glomerular filtration rate (eGFR)		ml/min	Stage 1 (GFR >90) Stage 2 (GFR 60-80) Stage 3 (GFR 30-59) Stage 4 (GFR 15-29) Stage 5 (GFR <15)	
Total Bilirubin		mg/dl	0.2-1.2	
AST		U/L	1-40	
ALT		U/L	1-41	
Alkaline Phosphatase		U/L	35-104	
Albumin level		g/dl	3.5-5.2	
Sodium		mmol/L	135-145	
Potassium		mmol/L	3.5-5	
Chloride		mmol/L	98-107	
Iron		µg/dl	50-160	
TIBC		mcg/dl	250-410	
Ferritin		g/ml	20-300	
Calcium		mg/dl	8.6-10	
Blood Glucose		mg/dl	74-110	
HbA1c		%	4.8-5.8	
Platelet aggregation		Ohms (Ω)	5-17	
Serum thromboxane B2				