DOCTOR OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES

Association of cardiovascular occlusive events with BCR-ABL tyrosine kinase therapy Identification, understanding and prediction

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Award date:
2022

Awarding institution:
University of Namur

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Identification, understanding and prediction.

Submitted by Hélène Haguet
for the PhD degree in Biomedical and Pharmaceutical Sciences

2022

JURY

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Association of cardiovascular occlusive events with BCR-ABL tyrosine kinase therapy - Identification, understanding and prediction.

The identification of the BCR-ABL tyrosine kinase as the main culprit in the pathology of chronic myeloid leukemia led to the development of one of the most successful cancer therapies, imatinib, an inhibitor designed to specifically target BCR-ABL. Imatinib reduces durably the number of leukemic cells, providing a life span of patients with chronic myeloid leukemia in chronic phase close to that of the general population. However, some patients are intolerant or resistant, inducing the development of three additional BCR-ABL tyrosine kinase inhibitors (i.e. dasatinib, nilotinib and bosutinib). Ponatinib, a BCR-ABL tyrosine kinase inhibitor classified as a third generation was finally designed to overcome a bad prognosis mutation in the *bcr-abl* gene which provides resistance to all the other BCR-ABL tyrosine kinase inhibitors. However, during the ponatinib clinical development, numerous patients had a vascular occlusion.

The aim of this thesis is to investigate the vascular toxicity associated with BCR-ABL tyrosine kinase inhibitors in the treatment of chronic myeloid leukemia.

Through meta-analyses we highlighted that in addition to ponatinib, dasatinib and nilotinib also induce vascular toxicity, and more specifically arterial occlusion. Secondarily, through a review of the literature and *in vitro* experiments, we determined that these three treatments affect the vasculature differently. Ponatinib is highly toxic to endothelial cells, and this toxicity could be responsible for the arterial occlusion. Dasatinib impairs endothelial cells in a lesser extent, whereas nilotinib possesses a different vascular profile. It deregulates glucose and lipid metabolism and impacts platelet functions.

The identification of the precise mechanisms underlying tyrosine kinase inhibitor-induced arterial occlusions is a central element as it influences the management of the vascular toxicity by guiding treatment selection, vascular monitoring and anticipating the vascular toxicity. Future research should focus on the validation of predictive biomarkers of vascular toxicity.

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Occlusion cardiovasculaire associée aux inhibiteurs de tyrosine kinase ciblant BCR-ABL – Identification, compréhension et prédiction.

L'identification de BCR-ABL comme étant la tyrosine kinase responsable de la pathologie de la leucémie myéloïde chronique a été une grande avancée qui a permis le développement d'une thérapie anticancéreuse parmi les plus révolutionnaires, l'imatinib, un inhibiteur conçu pour cibler spécifiquement BCR-ABL. L'imatinib réduit durablement le nombre de cellules leucémiques, permettant d'obtenir une espérance de vie proche de celle de la population générale pour les patients atteints de leucémie myéloïde chronique en phase chronique. Cependant, certains patients développent une intolérance ou une résistance à l'imatinib. Pour cette raison, trois autres inhibiteurs de tyrosine kinase ciblant BCR-ABL ont été développés (dasatinib, nilotinib et bosutinib). Finalement, le ponatinib, un cinquième inhibiteur de tyrosine kinase ciblant BCR-ABL a finalement été commercialisé. Cet inhibiteur est efficace contre une mutation de mauvais pronostic dans le gène *bcr-abl* qui confère une résistance à l'ensemble des autres inhibiteurs de tyrosine kinase. Cependant, durant le développement clinique du ponatinib, de nombreux patients ont développé une occlusion vasculaire.

L'objectif de cette thèse est d'investiguer la toxicité vasculaire associée aux inhibiteurs de tyrosine kinase ciblant BCR-ABL dans le traitement de la leucémie myéloïde chronique.

Nous avons démontré par le biais de métaanalyses que le ponatinib n'est pas le seul inhibiteur de tyrosine kinase toxique pour la vasculature, mais que le dasatinib et le nilotinib le sont également, et que ces traitements affectent plus particulièrement les artères. Dans un second temps, par une revue de la littérature et par réalisation d'expérimentations *in vitro*, nous avons déterminé que le mécanisme sous-jacent de la toxicité vasculaire avec ces trois traitements est différent. Le ponatinib induit une toxicité importante sur les cellules endothéliales qui pourrait être responsable des occlusions artérielles associées à ce traitement. Le dasatinib est également toxique pour ce type cellulaire, mais à un moindre degré, alors que le nilotinib a un profil vasculaire différent. Il dérégule le métabolisme glucidique et lipidique et a un impact sur les fonctions plaquettaires.

L'identification du mécanisme précis sous-jacent aux occlusions artérielles induites par les inhibiteurs de tyrosine kinase est un élément central afin d'améliorer la gestion de la toxicité vasculaire et d'orienter la sélection du traitement, la surveillance vasculaire et d'anticiper des occlusions artérielles. Les recherches futures devraient s'orienter sur la validation de biomarqueurs prédictifs de cette toxicité vasculaire.

Remerciements

Un des plaisirs dans l'accomplissement de cette thèse est de pouvoir remercier ceux qui y ont contribué.

Mes premiers mots seront pour le Professeur Jonathan Douxfils, promoteur de ce travail de longue haleine et présent depuis les premières heures de ce projet. Merci pour les nombreuses heures que tu y as dédié, les nombreuses relectures et ton apport scientifique toujours très pertinent. Merci également pour les opportunités que tu as pu me proposer et qui ont contribué à mon évolution. Je souhaite également remercier le Professeur François Mullier, copromoteur de cette thèse, pour le support apporté ainsi que la confiance accordée. Finalement, ce projet n'aurait pu voir le jour sans le Professeur Jean-Michel Dogné, directeur du département de pharmacie de l'Université de Namur qui a été à l'initiation du projet et qui m'a permis de persister au sein du département de pharmacie. Merci pour l'apport de votre précieuse expertise en pharmacovigilance et pour votre soutien dans la réalisation de cette thèse.

Il m'est important de remercier chaleureusement les membres de mon comité d'accompagnement qui m'ont épaulé durant ces six dernières années. Merci aux Professeurs Pierre Sonveaux, Philippe Hainaut et Carlos Graux pour votre bienveillance et pour l'ensemble des conseils prodigués qui ont permis de fournir un travail d'une meilleure qualité. Je souhaite également remercier les membres de mon jury, Professeur Saglio et Professeur Havelange, pour leur temps consacré à la relecture de mon manuscrit ainsi que pour m'avoir fait profiter de leurs connaissances.

Le monde de l'expérimentation *in vitro* m'était totalement inconnu à mon arrivée au département de pharmacie. Il m'a donc fallu une formation solide et beaucoup de support pour répondre à mes nombreuses questions. Pour cela, un grand merci à Adeline et Anne-Sophie, la petite équipe de culture cellulaire des années 2017-2018. Votre soutien m'a été indispensable. Merci également à Céline, Julie et Lionel pour la participation aux nombreuses

réunions concernant mon projet et pour le partage de votre expérience et expertise. Enfin, mes sincères remerciements vont à Élise pour les nombreuses heures passées au laboratoire pour ce projet.

Je souhaite également remercier d'autres départements de l'UNamur qui ont contribué à ce travail. À ce titre, je remercie l'URBM pour l'accès au cytomètre ainsi que l'URPhyM pour la formation à la technique de la zymographie.

Ces remerciements seraient incomplets si je n'en adressais pas à l'ensemble des personnes qui m'ont aidé dans la conception de l'étude clinique « BAT² ». À ce titre, je remercie plus particulièrement le Professeur Vekemans et le Professeur Havelange qui ont été capables d'envisager une modification de leur routine clinique dans l'optique de participer à cette étude. J'adresse également ma gratitude au Docteur Michael Dupont pour avoir pris le temps d'évaluer la faisabilité de cette étude au sein de son service.

Ce travail de thèse a été réalisé en parallèle de mes activités pédagogiques. À ce titre, toute ma reconnaissance va à Christelle, Lütfiye et Marie pour avoir assuré avec beaucoup de professionnalisme et de dévotion la bonne tenue des séances de travaux pratiques. L'enseignement est un des fondements de la société et grâce à vous et à votre envie de transmettre vos connaissances, il a été d'une excellente qualité. Merci également à Philippe d'avoir pris le relai de la préparation des séances de travaux pratiques dans des conditions peu favorables.

Il m'est très important de remercier l'ensemble de l'équipe du département de pharmacie pour les moments de détente partagés. Ce sont par ces moments que les affinités se nouent, facilitant les collaborations et échanges autour des divers projets. Mes plus chaleureux remerciements s'adressent à Adeline, Anne-Sophie, Céline, Christelle, Julie et Lütfiye qui ont été une grande source de réconfort dans des périodes plus compliquées. Un merci plus particulier à Christelle pour tous les moments de partage. Notre entente n'aura connu aucune faille et c'est un réel plaisir de te compter comme amie. Nos discussions me manqueront.

Merci à Jonathan D. de m'avoir ouvert les portes de QUALIblood. C'est une expérience qui m'a permis de développer de nouvelles compétences et connaissances. Merci à l'ensemble de l'équipe QUALIblood pour les bons moments partagés. Un remerciement plus particulier à Laure pour ton dynamisme communicatif. Cela a toujours été un plaisir de travailler ensemble.

Mes plus sincères remerciements vont évidemment à ma famille et mes amis qui m'ont permis de mener à bien ce doctorat dans les meilleures conditions. Un merci particulier à mon papa qui est probablement le premier à croire en moi. Merci à toutes les personnes qui m'ont permis de vivre une installation sereine en Belgique. À ce titre, je remercie ma belle-famille et particulièrement Martine et Dominique ; merci pour le soutien que vous nous apportez au quotidien. Ces remerciements ne peuvent s'achever sans une pensée pour ma grand-mère, Colette, qui enseignait la science avec passion.

Pour clore cette section de la meilleure manière, je tiens à remercier Guillaume, mon conjoint. Merci de rendre notre quotidien si joyeux. De cet amour est né Gabriel, un rayon de soleil additionnel qui me donne d'autant plus l'envie d'être une meilleure version de moi-même et de lui offrir le meilleur des avenirs.

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List of abbreviations

5HT	5-hydroxytryptamine
7-AAD	7-aminoactinomycin D
8-oxo-dG	8-hydroxy-2'-deoxyguanosine
ABC	ATP-binding cassette
ABI	Ankle-brachial index
ABL	Abelson
ACC	American College of Cardiology
ADP	Adenosine diphosphate
AE	Adverse event
AHA	American Heart Association
ALI	Acute lung injury
AMI	Acute myocardial infarction
AOE	Arterial occlusive event
AP	Acute phase
Apobec1	Apolipoprotein B mRNA editing enzyme catalytic subunit 1
aPTT	Activated partial thromboplastin time
Arg	Abelson-related gene
ASCVD	Atherosclerotic cardiovascular disease
ASMC	Arterial smooth muscle cell
ATH	Arterial hypertension
ATP	Adenosine triphosphate
BAEC	Bovine aortic endothelial cell
BAL	Bronchoalveolar level
BAoSMC	Bovine aortic smooth muscle cell
BCR	Breakpoint cluster region
bid	*bis in die*
BP	Blast phase
BrdU	Bromodesoxyuridine
BTK	Bruton's tyrosine kinase
Ca	Calcium
CCyR	Complete cytogenetic response
CEC	Circulating endothelial cell
CETP	Cholesteryl ester transfer protein
CFSE	Carboxyfluorescein succinimidyl ester
CI	Confidence interval
CM	Chylomicron

CML	Chronic myeloid leukemia
COL	Collagen
CP	Chronic phase
CRP	C-reactive protein
CSFR	Colony stimulating factor receptor
CXCL1	(C-X-C motif) ligand 1
DDR-1	Discoidin domain receptor tyrosine kinase 1
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DVT	Deep venous thrombosis
EC	Endothelial cell
ECG	Electrocardiogram
ECM	Extracellular matrix
EdU	5-ethynyl-2'-deoxyuridine
ELISA	Enzyme-linked immunoabsorbent assay
ELISPOT	Enzyme-linked immunospot
ELTS	European treatment and outcomes study long-term survival
EMA	European medicine agenda
EndMT	Endothelial to mesenchymal transition
EPC	Endothelial progenitor cell
Eph	Ephrin
ESC	European Society of Cardiology
ET-1	Endothelin 1
EU-SmPC	European Summary of Product Characteristics
FA	Fatty acid
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FcR	Fc receptor
FDA	Food and drug administration
FEM	Fixed-effect model
FITC	Fluorescein isothiocyanate
FXII	Factor XII
GLUT	Glucose transporter
GP	Glycoprotein
GTT	Global thrombosis test
HaOSMC	Human aortic smooth muscle cell
HCAEC	Human coronary artery endothelial cells
HCASMC	Human coronary artery smooth muscle cell

HCtAEC	human carotid artery endothelial cell
HDL	High-density lipoprotein
hERG	Human Ether-à-go-go-Related Gene
HLA	Human leukocyte antigen
HMEC-1	Human microvascular endothelial cell
HMGB1	High–mobility group box 1
HMGCoA reductase	3-hydroxy-3-methyl-glutaryl-coenzyme A reductase
HPAEC	Human pulmonary artery endothelial cell
HRMEC	Human retinal microvascular endothelial cells
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cells
HVSMC	Human vascular smooth muscle cell
IC_{50}	Half maximal inhibitory concentration
ICAM-1	Intercellular Adhesion Molecule 1
IDL	Intermediate-density lipoprotein
IFN-γ	Interferon gamma
IL	Interleukin
iPSC	Induced pluripotent stem cells
IRIS	International randomized study of interferon and STI571
IRS	Insulin receptor substrate
ITT	Intention-to-treat
JNK	c-Jun N-terminal kinase
LAT	Linker for activation of T-cells
Lck	Lymphocyte-specific protein tyrosine kinase
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
LRP	Lipoprotein receptor-related protein
MCP	Monocyte chemoattractant protein-1
MEKK1	MAPK/ERK kinase kinase 1
MIP-1	Macrophage inflammatory protein 1
MMP	Matrix metalloproteinase
MMR	Major molecular response
MPO	Myeloperoxidase
MR	Molecular response

NCCN	National Comprehensive Cancer Network
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor-kappa B
NG-TKI	New generation tyrosine kinase inhibitor
NO	Nitric oxide
OR	Odds ratio
OS	Overall survival
oxLDL	Oxidized low-density lipoprotein
PAI-1	Plasminogen activator inhibitor-1
PAOD	Peripheral arterial occlusive disease
PAR	Protease-activated receptor
PARP	Poly(ADP-ribose) polymerase
PASMC	Pulmonary artery smooth muscle cell
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDK1	Pyruvate Dehydrogenase Kinase 1
PE	Pulmonary embolism
PFA	Platelet function assay
PFS	Progression-free survival
Ph+	Philadelphia positive
PI3K	Phosphoinositide 3-kinase
PLC	Phospholipase C
PRP	Platelet-rich plasma
PS	Phosphatidyl serine
PSGL-1	P-selectin glycoprotein ligand-1
PT	Prothrombin time
p-y	Patient-year
qd	*quaque die*
QoL	Quality-of-life
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RAGE	Receptor for advanced glycation endproducts
RBC	Red blood cell
RCT	Randomized clinical trial
RhoA	Ras homolog gene family, member A
RoB 2	Risk of bias 2

ROCK	Rho-associated protein kinase
ROS	Reactive oxygen species
SAE	Serious adverse event
SD	Standard deviation
SDF-1	Stromal cell-derived factor 1
SEER	Surveillance, epidemiology, and end results
SEM	Standard error of the mean
SIK	Salt inducible kinase
SMC	Smooth muscle cell
SR	Scavenger receptor
STAMP	Specifically Targeting the ABL Myristoyl Pocket
TCR	T-cell receptor
TFPI	Tissue factor pathway inhibitor
Tie-2	Tunica interna endothelial cell kinase
TIMP	Tissue inhibitor of metalloproteinase
TKI	Tyrosine kinase inhibitor
TLR	Toll-like receptor
TM	Thrombomodulin
TNF	Tumor necrosis factor
t-PA	Tissue plasminogen activator
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TXA2	Thromboxane A2
VCAM-1	Vascular Cell Adhesion Molecule 1
VE-cadherin	Vascular endothelial cadherin
VEGFR	Vascular endothelial growth factor receptor
VLDL	Very low-density lipoprotein
VSMC	Vascular smooth muscle cell
VTE	Venous thromboemblism
vWF	Von Willebrand factor

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Introduction

Chapter 1. Introduction

During ponatinib phase 1 (NCT00660920) and phase 2 (NCT01207440) clinical trials, at least 27% of patients developed arterial or venous thrombosis, causing the temporal suspension of ponatinib marketing in the United States, the early termination of the phase 3 clinical trial (NCT01650805) and the implementation of a risk management plan.(Food and Drug Administration 2013; European Medicines Agency 2013b) Several and serious cases of peripheral arterial occlusive diseases were also reported with nilotinib, another BCR-ABL tyrosine kinase inihibitor (TKI) used for the treatment of chronic myeloid leukemia (CML).(Food and Drug Administration 2014c; Levato et al. 2013; Le Coutre et al. 2011) At the beginning of this thesis, little was known regarding the cardiovascular risk with imatinib, dasatinib and bosutinib, three other BCR-ABL TKIs also used in the treatment of CML, but no signals were identified during clinical trials. This thesis aimed at assessing the risk of thromboembolic events associated with the use of the different BCR-ABL TKIs on the market and to evaluate the biological mechanisms possibly explaining the risk in the CML population.

Chronic Myeloid Leukemia

Chronic myeloid leukemia is a myeloproliferative neoplasm, characterized by unregulated growth of myeloid cells in the bone marrow without the loss of their capacity to differentiate.(Jabbour and Kantarjian 2014) Consequently, the bone marrow and the peripheral blood cell profiles show an increased number of granulocytes and their immature precursors, including occasional blast cells. Chronic myeloid leukemia represents 10 to 20% of adult leukemias and has a yearly incidence of 1 to 2 in 100,000 in the general population. Chronic myeloid leukemia incidence increases by age, with a mean age at diagnosis around 60 years (**Figure 1.1**).(Hoglund, Sandin, and Simonsson 2015; Bethesda).

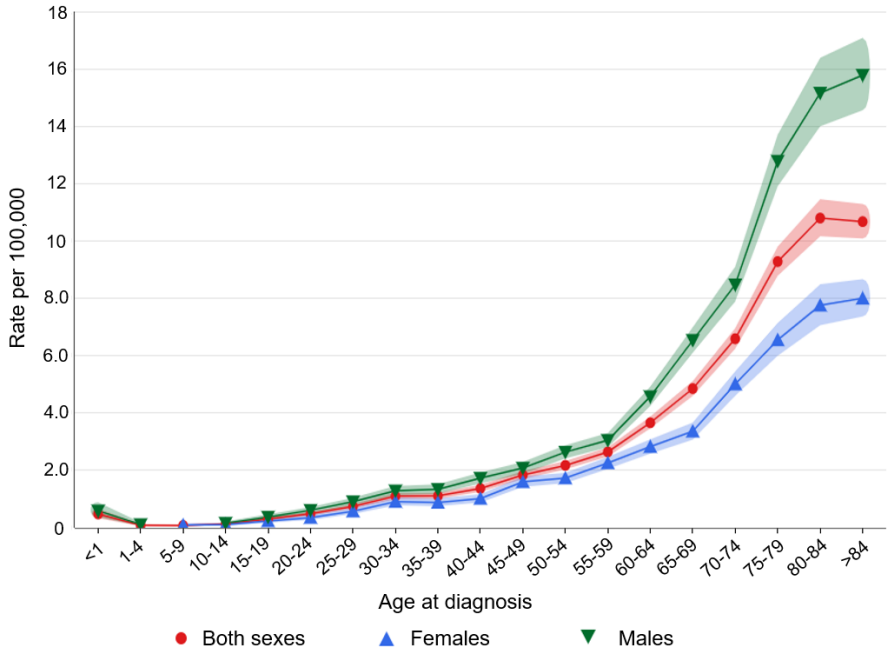

Figure 1.1 | **SEER incidence rates of CML by age at diagnosis.** The incidence rate of CML increases by age in both females and males. Males are more subject to CML than females. Rates are per 100,000. Incidence rates were based on data from 2013 to 2017 from the SEER 21 areas. SEER: Surveillance, Epidemiology, and End Results; CML: chronic myeloid leukemia.
Adapted from **https://seer.cancer.gov/explorer consulted on 2021/03/16**

More than 90% of cases result from a cytogenetic aberration known as the Philadelphia chromosome.(An et al. 2010; Nowell and Hungerford 1960) The Philadelphia chromosome is the result of a translocation between chromosome 9 and chromosome 22, inducing the translocation of the Abelson (*abl*) proto-oncogene on chromosome 9 near to the breakpoint cluster region (*bcr*) gene on chromosome 22.(Price et al. 2013; Rowley 1973) The translocation of the *bcr* gene near to the *abl* gene results in the formation of a chimeric fusion protein, BCR-ABL.(Price et al. 2013) The ABL kinase is regulated by 2 mechanisms: (1) phosphorylation of critical tyrosine residues, (2) autoinhibition by binding of N-term myristate into the kinase domain.(Hoffbrand et al. 2016) The junction of BCR to ABL in the BCR-ABL kinase has several actions leading to its constitutive activation (**Figure 1.2**): (1)

the kinase dimerizes through a coiled-coil domain in BCR, facilitating the tetramerization of the kinase. This tetramerization induces the proximity of the critical tyrosine residues that auto-phosphorylate and subsequently, auto-activate the kinase activity by disrupting the SH3-linker interaction.(Hoffbrand et al. 2016; Smith, Yacobi, and Van Etten 2003) (2) The N-term myristate is lost and replaced by BCR, inducing the loss of the autoinhibition of the kinase activity (**Figure 1.2**). Through these 2 actions, BCR-ABL is constitutively activated and its expression in the cell leads to dysregulation of numerous signaling pathways involved in cell proliferation, apoptosis and adhesion to the marrow stroma.

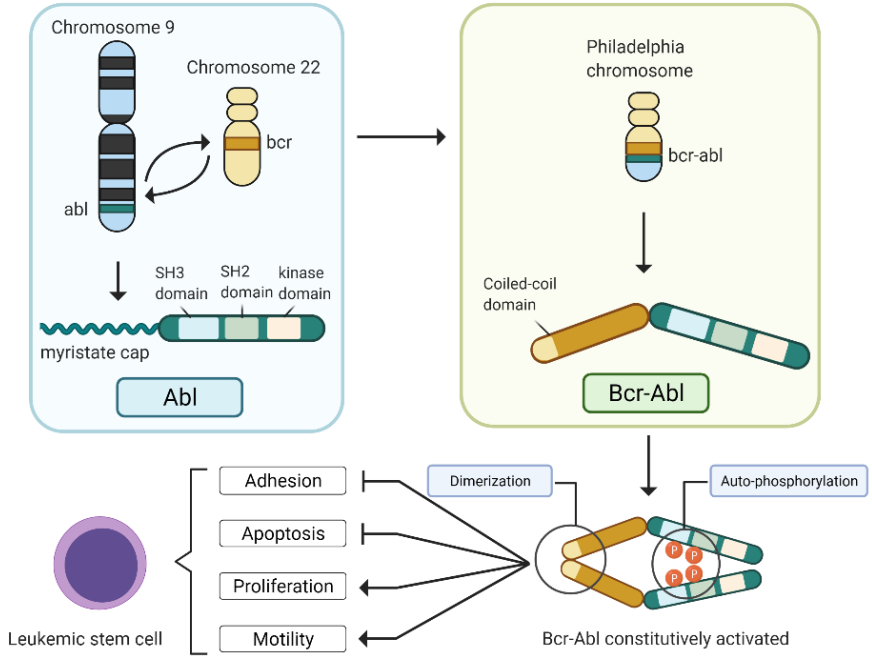

Figure 1.2 | **Schematic representation of CML pathogenesis.** The pathogenesis of CML is the consequence of the translocation between chromosome 9 and chromosome 22 that induces the creation of a BCR-ABL dysregulated tyrosine kinase. The expression of BCR-ABL by itself deregulates numerous signaling pathways and promotes the development of CML. Created with BioRender.

In non-cancer cells, the *abl* gene encodes an ABL non-receptor tyrosine kinase, a crucial and conserved kinase involved in a wide variety of cellular processes. ABL is localized both in the cytoplasm and in the nucleus, in which it has numerous functions. In the cytoplasm, ABL transduces signals to regulate actin remodeling which impacts cell adhesion and migration.(Wang 2014) In the nucleus, ABL also has a prominent role as it transduces DNA damage signal to regulate transcription, DNA repair and to promote apoptosis.(Wang 2014) Interestingly, ABL is expressed in all mammalian cell types and possesses additional functions in the vasculature (**Figure 1.3**). The depletion of ABL in Abl-related gene (Arg)-null mice revealed that ABL is implicated in the vasculature development and that the loss of these two kinases (i.e. ABL and Arg) increased endothelial cell apoptosis, contributing to the vascular dysfunction.(Chislock, Ring, and Pendergast 2013; Wang 2014) Recent studies also pointed toward a central role of ABL and Arg in the regulation of vascular permeability.(Rizzo, Aman, et al. 2015) Most studies indicate that ABL has a barrier disruptive effect and that inhibition of ABL induces barrier protective effects. However, some studies assessing different agonists found that ABL also possesses opposite effects on the preservation of the endothelial barrier (i.e. barrier protective effects) (**Figure 1.3**).(Rizzo, Aman, et al. 2015) Interestingly, ABL is also implicated in shear stress-induced endothelial activation (Li et al. 2019) and in the production of pro-inflammatory cytokines and chemokines following exposure to inflammatory agents.(Bohio et al. 2020) Oppositely to ABL, the BCR protein that is encoded by the *bcr* gene has less clear functions. BCR possesses an intrinsic kinase activity and has been identified as a major regulator of RhoA activity in keratinocytes.(Dubash et al. 2013) Early studies also suggest its involvement in neural development and memory formation.(Oh et al. 2010)

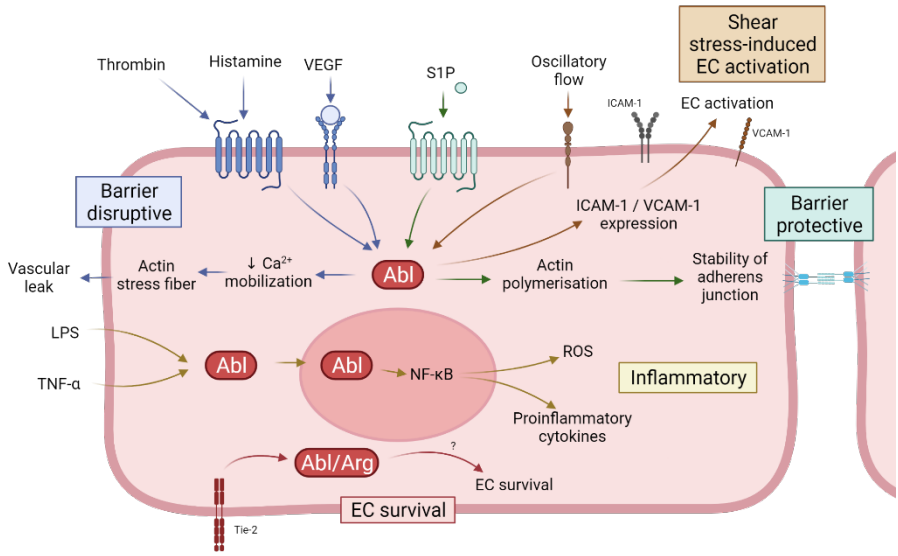

Figure 1.3|Role of ABL in endothelial cells. ABL is implicated in the regulation of numerous functions in endothelial cells including the shear stress-induced EC activation (in brown), the regulation of EC survival (in red), inflammatory function (in yellow) and opposite roles in the barrier function of EC (in green and blue). Created with BioRender

Chronic myeloid leukemia progresses through 3 distinct phases: the chronic, accelerated, and blast phases, mainly defined on the percentage of blast cells in the blood and bone marrow (**Figure 1.4**).(Jabbour and Kantarjian 2014) In the chronic phase (CP), the number of granulocytes increases but granulocytes present normal morphology and functions.(Hoffbrand et al. 2016) The myeloid maturation is normal or slightly elevated. The acute phase is characterized by an increased number of blasts due to impaired differentiation or drug resistance, blast cells representing 10 to 19% of blood cells.(National Comprehensive Cancer Network 2020) Finally, the disease evolves to the blast phase, a state close to acute leukemias in which bone marrow failed due to absence of maturation or differentiation, and the number of blasts exceeds 20% of blood cells.(National Comprehensive Cancer Network 2020) Once the blast phase arises, the death of the patient without treatment usually occurs within one year due to infection or bleeding. The

natural evolution of CML without treatment is 5 to 10 years until patient death.

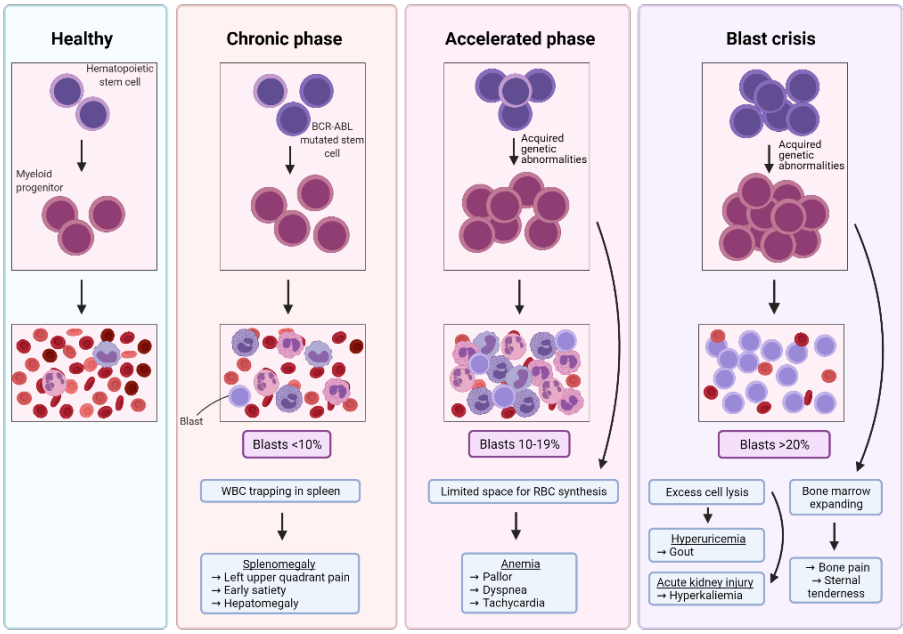

Figure 1.4 | **Phases of CML development.** Chronic myeloid leukemia is divided in three phases, the chronic phase, the accelerated phase and the blast crisis. The classification of patients with CML in these three phases is mainly based on the percentage of blasts in the blood and bone marrow. Created with BioRender.

Chronic Myeloid Leukemia Therapy

History of Chronic Myeloid Leukemia Management

First treatment of CML to control the symptoms and improve patient quality-of-life (QoL) started in 1865 with the first public use of arsenic to treat leukemia, followed by numerous attempts of diverse therapeutic strategies that were mainly unsuccessful (**Figure 1.5**).(Mughal et al. 2016) It was only in 1953, with the introduction of the alkylating agent busulfan that the overall survival of CML patients improved. Busulfan was then progressively replaced by hydroxyurea in the mid-1960s due to its mutagenic potential.(Mughal et al. 2016) However, busulfan remained highly toxic and

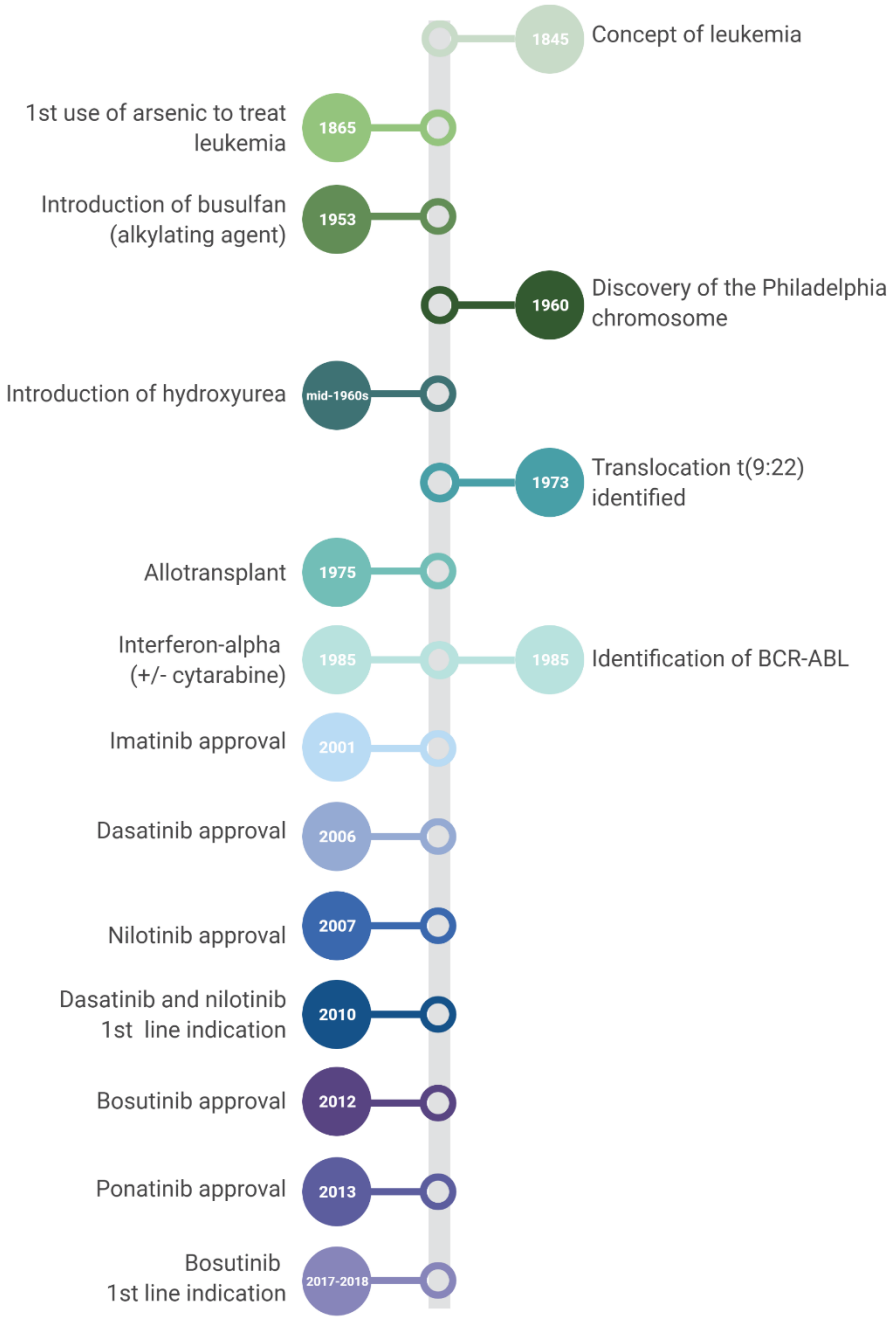

Figure 1.5 | **Milestones in the discovery and treatment of CML.** Historical treatment of CML relied on conventional chemotherapy. The revolution in the treatment of CML was the remarkable efficacy of BCR-ABL TKIs at the start of the 21st century. Created with Biorender.

these two conventional chemotherapy allowed only a control of the disease during a short period of time (**Figure 1.6**). In the mid-1970s, allogeneic stem-cell transplantation was explored and became the standard treatment choice for younger patients with a HLA-identical sibling donor.(Bjorkholm et al. 2011; Bennour, Saad, and Sennana 2015) In 1985, the introduction of interferon-α in therapeutics allowed the achievement of complete cytogenetic response (i.e. absence of Philadelphia chromosome positive cells) in a fraction of CML patients. Interferon-α also improved long-term survival and increased the 8-year survival of CML patients from less than 15% to 42 to 65%, depending on the studies.(Bjorkholm et al. 2011; Bennour, Saad, and Sennana 2015; Granatowicz et al. 2015) However, interferon-α was also associated with numerous serious adverse effects, including neurotoxicity and liver dysfunction.

First-Generation BCR-ABL TKI

The recognition that the BCR-ABL chimeric protein is a pivotal contributor to CML in the 1980s shifted the focus of novel drug development towards the targeting of BCR-ABL.(Mughal et al. 2016) A drug discovery program was launched in 1987 to assay thousands of molecules for their biological activity towards BCR-ABL. By this program, imatinib was identified as a molecule able to bind the inactive forms of BCR-ABL by occupying its ATP-binding pocket, thereby preventing the ATP binding and the conformational switch to BCR-ABL active form.(Mughal et al. 2013)

Imatinib was tested in patients with CML in 1998, in the International Randomized Study of Interferon and STI571 (IRIS) trial, a phase III clinical trial comparing imatinib to the combination of interferon-α and low-dose cytarabine. In this study, imatinib demonstrated superior efficacy than the combination of interferon-α plus cytarabine by improving cytogenetic and hematological response and progression-free survival (PFS) at 18 months (92.1% vs. 73.5% respectively).(Levi et al. 2003) Remarkably, patients treated with imatinib had minimal adverse effects, and imatinib was much better tolerated than the association interferon-α plus cytarabine.(Levi et al. 2003)

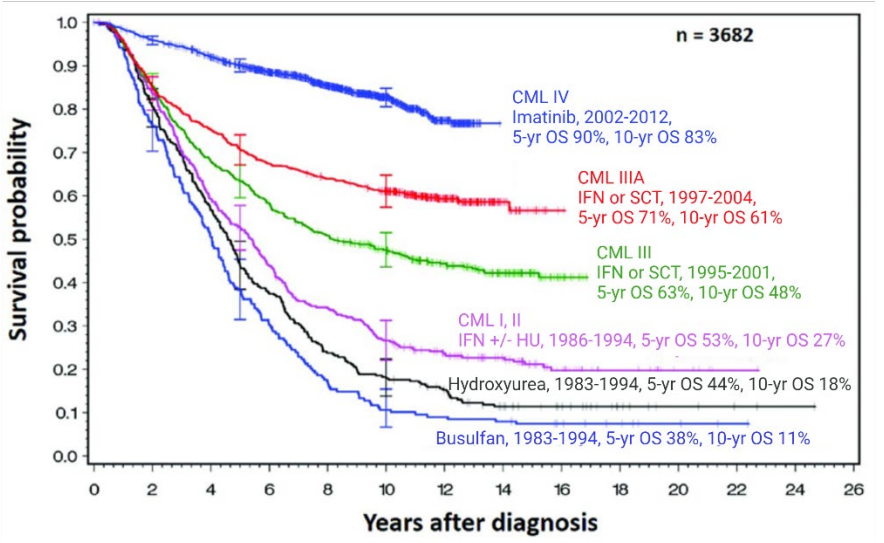

Figure 1.6 | Evolution of survival probability of patients with CML over time. This figure depicts the limited efficacy of conventional chemotherapy (i.e. busulfan and hydroxyurea) the impressive efficacy of imatinib on overall survival of CML patients. Adapted from (Hehlmann 2016).

Based on these impressive results, imatinib approval was granted in 2001 by the Food and drug administration (FDA) and the European medicine agency (EMA). As of today, imatinib is still used to treat CML, strengthening the impressive efficacy and safety profile of this compound.(Gambacorti-Passerini and Piazza 2015) Imatinib has been reported to significantly improve the 10-year overall survival of patients with CML in chronic phase (**Figure 1.6**).(Jabbour and Kantarjian 2014) This approach fundamentally revolutionized the treatment of CML by replacing relatively non-specific antineoplastic drugs targeting DNA or its synthesis which were known to be relatively toxic drugs by a safer agent with a favorable pharmacodynamic. It demonstrated that a complete understanding of the disease's molecular drivers may led to the development of successful targeted therapies with reduced toxicity.

However, imatinib is far from perfect and therapeutic resistance emerged soon after the introduction of the drug into clinical practice (Iqbal and Iqbal

2014) whereas another fraction of patients were intolerant to imatinib (i.e. development of adverse events that cannot be managed through dose reduction or treatment of symptoms). The rates of patients resistant and intolerant to imatinib vary between sources from 5 to 30% for imatinib intolerance and up to 45% for imatinib resistance.(Gisoo Barnes 2015)

Imatinib resistance is defined as primary or secondary resistance based on the achievement or not of a preset hematological and/or cytogenetic milestone following TKI introduction. Different mechanisms of resistance have been identified and can be classified as BCR-ABL dependent and BCR-ABL independent. The most predominant BCR-ABL dependent resistance mechanism is the occurrence of a mutation in the *bcr-abl* gene, interfering with critical hydrogen bonds that forms between the BCR-ABL kinase domain and the TKI.(Massimino et al. 2020) To date, more than 100 mutations in the BCR-ABL kinase domain have been identified to decrease the binding affinity of TKIs without disturbing the binding of the usual substrate. Other types of BCR-ABL dependent resistance mechanisms have been reported including differential drug trafficking (i.e. dysfunction of drug import and export pumps on the target cell membrane) and *bcr-abl* gene amplification.(Alves et al. 2021) BCR-ABL independent mechanisms of resistance are found in patients treated with a TKI and despite BCR-ABL inhibition, the CML cells retain the ability to survive and proliferate. This phenomenon can occur due to epigenetic changes, clonal evolution (i.e. acquisition of additional cytogenetic abnormalities) or by the activation of alternative signaling pathways (e.g. deregulation of apoptotic proteins or overexpression of kinases that promote survival and proliferation).(Iqbal and Iqbal 2014)

Second- and Third-Generation BCR-ABL TKIs

Second-generation TKIs have rapidly been developed to overcome this resistance and are more potent than imatinib in *in vitro* assays.(Mughal et al. 2016) Nilotinib is a second-generation BCR-ABL TKIs designed to increase imatinib selectivity and activity. Nilotinib binds BCR-ABL with 20 times the affinity of imatinib and has activity against many imatinib-resistant mutants.

Dasatinib is a dual Src/ABL inhibitor. It inhibits a higher number of tyrosine kinases than imatinib and has a greater activity *in vitro* against BCR-ABL than nilotinib (**Table 1.1**) (i.e. 300-fold more potent than imatinib).(Mughal et al. 2016) Compared to imatinib, dasatinib binds to BCR-ABL regardless of its conformation (i.e. active and inactive conformation). Bosutinib is the third and last second generation BCR-ABL TKIs. Similarly to dasatinib, bosutinib is a dual Src/ABL inhibitor. Bosutinib appears to be able to overcome many kinase domain mutations thanks to its different chemical structure.(Mughal et al. 2016) Dasatinib and nilotinib were approved in 2006 and 2007 respectively for patients with CP-CML resistant or intolerant to imatinib, whereas bosutinib has been licensed in 2012. The approval of these 3 novel therapies was not conditioned by increased overall survival, but based on an enhancement of surrogate outcomes such as quicker and deeper achievement of cytogenetic and molecular responses (i.e. reduction in the BCR-ABL transcript levels). They obtained the first-line indication in 2010 for dasatinib and nilotinib and in 2017 (FDA) and 2018 (EMA) for bosutinib (**Figure 1.5**).(Cross et al. 2015)

However, the T315I mutation provides resistance to both imatinib and all second generation TKIs. This mutation is a threonine to isoleucine substitution in the ATP-binding domain inducing a steric hindered and impairing the binding of BCR-ABL TKIs (**Figure 1.7**). The high prevalence of this mutation (i.e. 10 to 20% of all the resistance) and the lack of therapeutic strategies to overcome this mutation remains important issues in CML management. To answer this need, ponatinib, a "next generation" BCR-ABL TKI was developed using a structure-based approach with the purpose of potently inhibiting all mutants of BCR-ABL, including the T315I mutant.(Mughal et al. 2016) Ponatinib contains a triple carbon-carbon liaison conferring it a long and flexible arm able to bind the ATP-binding pocket of BCR-ABL even in presence of the T315I mutation. Ponatinib inhibits ABL, Src and a variety of other kinases.(Mughal et al. 2016) This TKI was classified as a third-generation BCR-ABL TKI and was licensed in 2013 for patients with

CML resistant or intolerant to dasatinib or nilotinib or for patients with the T315I mutation.[1]

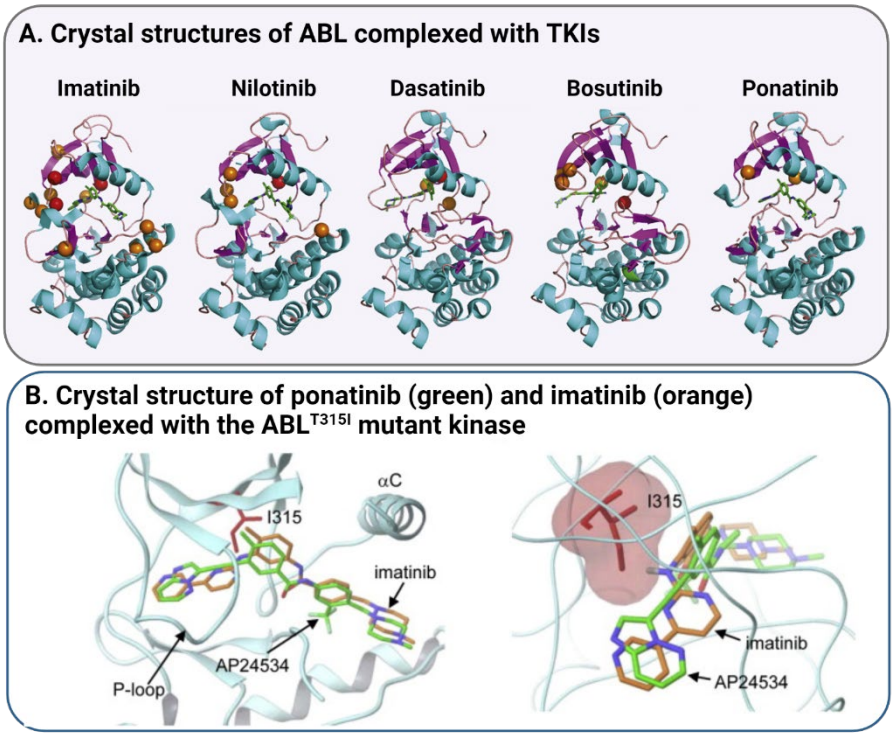

Figure 1.7 | Binding of BCR-ABL TKIs with ABL and impact on the T315I mutation. A. Crystal structures of ABL in complex with the five BCR-ABL TKIs. **B.** Superposition of crystal structures of ponatinib (green) and imatinib (orange) complexed with the ABL^{T315I} mutant kinase. This superposition highlights the effect of the T315I mutation on the imatinib binding. Adapted from (Braun, Eide, and Druker 2020) and (O'Hare et al. 2009)

[1] A sixth BCR-ABL TKIs so-called radotinib is commercially available but this TKI is only encountered in Asia and will not be discussed in details in this thesis.

Table 1.1 | **Main characteristics of approved BCR-ABL TKIs.** Type I inhibitors bind to the active and inactive conformation of BCR-ABL. Type II inhibitors bind to the inactive conformation of BCR-ABL only.

	Imatinib	**Dasatinib**	**Nilotinib**	**Bosutinib**	**Ponatinib**
Generation	First	Second	Second	Second	Third
Authorization year	2001	2006	2007	2012	2013
Type of inhibitor	Type II	Type I	Type II	Type I	Type I
Approval for patients with CML in chronic phase	First line (2003)	First line (2010)	First line (2010)	First line (2017/2018)	Intolerant/resistance to 2nd generation TKI OR T315I mutation
Daily dosage in adults (recommended)	400 mg qd	100 mg qd	300 mg bid	500 mg qd	45 mg qd
Efficacy against T315I mutation	No	No	No	No	Yes
BCR-ABL cellular IC_{50} (nM)*	100-500	0.8-1.8	10-25	42	0.5
Number of off-targets† $n_{inhibited}/n_{total}$ (%)	63/457 (14%)	137/438 (31%)	69/337 (20%)	126/293 (43%)	37/135 (27%)

* Results from cellular assays.(Rossari, Minutolo, and Orciuolo 2018)

† Results from personal data.

AP: acute phase; bid: bis in die; BP: blast phase; CP: chronic phase; IC_{50}: half-maximal inhibitory concentration; qd: quaque die; TKI: tyrosine kinase inhibitor

Treatment Choice

In 2015, at the start of this thesis, international guidelines did not recommend a particular TKIs between imatinib, dasatinib and nilotinib for initial treatment of chronic phase CML.(Price et al. 2013) The two other new generation BCR-ABL TKIs, ponatinib and bosutinib, were reserved for the treatment of patients with CML resistant or intolerant to first-line treatment, and in patients with the T315I mutation for ponatinib.(National Comprehensive Cancer Network 2016a) Since then, bosutinib has been approved for first-line treatment of chronic phase CML patients. Consequently, imatinib and second-generation TKIs are appropriate options to treat CML in chronic phase (**Table 1.1**). Current guidelines recommend selecting the TKI used for treatment initiation based on a risk score, patient comorbidities, toxicity profile of TKI, possible drug interactions and patient preference.(National Comprehensive Cancer Network 2020) The risk score should be determined using one of the three following scoring systems: the Sokal score, the Hasford score (Euro) or the European Treatment and Outcome Study long-term survival (ELTS) score.(Hasford et al. 2011; Hasford et al. 1998; Sokal et al. 1984) All of these grading systems allow a classification of the disease into 3 groups: low-, intermediate- and high-risk groups. Because disease progression is more frequent in patients with intermediate- and high-risk score and that second-generation TKIs demonstrate a lower risk of disease progression than imatinib, second-generation TKIs should be preferred for these patients.(National Comprehensive Cancer Network 2020) Recently, it has been proposed by the European LeukemiaNet group that the emergence of additional chromosome abnormalities in Philadelphia positive cells and the fiber content in the bone marrow biopsies are two predictive risk factors. Therefore, the European LeukemiaNet group recommends managing patients with high-risk additional chromosome abnormalities as high-risk patients.(Hochhaus, Baccarani, et al. 2020)

TKI Discontinuation

These therapies were assumed to be continued indefinitely to prevent disease relapses. However, several studies demonstrated that a portion of patients may discontinue TKI therapy without relapse after achievement of deep molecular responses.(Etienne et al. 2017; Atallah et al. 2021; Saussele et al. 2018; Hochhaus, Masszi, et al. 2017) Reassuringly, almost all the patients that relapsed responded to TKI re-initiation and very few cases of progression to accelerated phase or blast phase were reported.(Atallah and Schiffer 2020) The different studies reported that approximately 50% of patients that discontinued TKI (after the achievement of predefined milestones) remain in remission 2 years after the discontinuation.(Atallah and Schiffer 2020)

To date, guidelines to treat CML include TKI discontinuation and recommend attempting TKI discontinuation in patients without history of accelerated phase or blast crisis, treated during at least 5 years with imatinib or 4 years with a second-generation TKI and with a continued deep molecular response (BCR-ABL level < 0.01% international scale) for at least 2 years.(National Comprehensive Cancer Network 2020; Hochhaus, Baccarani, et al. 2020) In order to increase the number of patients eligible for TKI discontinuation, second-generation TKIs are of interest as they permit to increase the rate of treatment-free remission of 10 to 20% compared to imatinib.(Atallah and Schiffer 2020)

Cardiovascular Toxicity of BCR-ABL TKIs

During the clinical development of ponatinib, a high incidence of arterial occlusive events including peripheral artery occlusive diseases has been observed leading to the temporal suspension of its marketing authorization in the United States (US)[2] and the early termination of the phase III clinical

[2] Ponatinib has been approved by the FDA in December 2012 through an accelerated approval program in order to address the important unmet need for patients with CML that is resistant to or intolerant of prior TKI therapy.

trial (NCT01650805).(Food and Drug Administration 2013; Gainor and Chabner 2015) This brought the FDA to include a boxed warning in the prescribing information for arterial thrombosis indicating that 8% of patients treated by ponatinib experienced serious arterial thrombosis.(Food and Drug Administration, 2013)

Serious cases of peripheral arterial occlusive diseases were also reported in CML patients treated with nilotinib in clinical trials and in post-marketing experience.(Food and Drug Administration 2014c; Quintas-Cardama, Kantarjian, and Cortes 2012) At the beginning of this thesis, the relationship between nilotinib and vascular occlusive events remained speculative.(Aichberger et al. 2011a) Several retrospective studies demonstrated higher incidence of cardiovascular events with nilotinib compared to imatinib.(Levato et al. 2013; Aichberger et al. 2011a; Quintas-Cardama, Kantarjian, and Cortes 2012; Gambacorti-Passerini and Piazza 2011) However, these reports were not considered conclusive due to the small number of patients and their retrospective design.(Gambacorti-Passerini and Piazza 2011) No signal of increased risk of vascular thrombosis and embolism was found in clinical trials with imatinib and dasatinib, however, some cases were also reported post-marketing.(Food and Drug Administration 2014a, 2014b) Nevertheless, in light of the expected life span of CML patients under TKIs and the low thrombotic risk of the disease itself in comparison with other myeloproliferative disorders, the long-term safety of these products is particularly important.

Objectives

This thesis aimed at investigating the vascular toxicity associated with BCR-ABL TKIs in the treatment of CML. We first assessed the relative risk of vascular occlusive events with 2nd and 3rd generation BCR-ABL TKIs compared with imatinib in CML through a meta-analysis of randomized clinical trials (RCTs), in order to ascertain the vascular risk of nilotinib and other 2nd generation BCR-ABL TKIs. We also aimed to differentiate arterial from venous occlusive events to guide mechanistic investigations. Finally, we

aimed to help in the implementation of recommendations for frontline treatment of chronic phase CML patients to determine if 2nd generation TKIs should be favored over imatinib in these patients.

Secondly, we investigated some key aspects of the pathophysiology underlying the vascular occlusive events associated with BCR-ABL TKIs in CML. To guide the mechanistic studies, we perform a comprehensive review of the literature to generate mechanistic hypotheses (**Chapter 3**) and then investigate the impact of BCR-ABL TKIs on the viability and major functions of endothelial cells *in vitro* (**Chapter 4**). The understanding of the mechanism(s) by which new generation BCR-ABL TKIs induce or promote arterial occlusive events will improve the clinical use of these therapies. This could help in the selection of patients to whom the prescription of these drugs should be avoided and to guide the clinicians with the best strategies to minimize the risk of thrombotic events.

Part A: Clinical Evaluation of the Efficacy and the Vascular safety of BCR-ABL TKIs

Chapter 2. Long-Term Survival, Vascular Occlusive Events and Efficacy Biomarkers of First-Line Treatment of CML: A Meta-Analysis

The vascular toxicity encountered with ponatinib during its clinical development and the case-reports of peripheral arterial occlusive disease with nilotinib questioned about the assessment of the vascular safety profile of the 2nd and 3rd generation BCR-ABL TKIs.(Le Coutre et al. 2011; Food and Drug Administration 2014c; Levato et al. 2013) Following these observations, we decided to extend this assessment to all BCR-ABL TKIs and to provide recommendations on how to minimize the risk.(Moslehi and Deininger 2015; Douxfils, Haguet, et al. 2016a) The vascular safety of the TKIs was assessed through a meta-analysis of randomized clinical trials.(Douxfils, Haguet, et al. 2016b) This meta-analysis demonstrates that new generation BCR-ABL TKIs are associated with a 3-fold increased risk of vascular occlusive events compared with imatinib in patients with CML (OR, 3.45; 95%CI, 2.30-5.18).(Douxfils, Haguet, et al. 2016a) Stratification by treatment specifies that nilotinib (OR, 3.42; 95%CI, 2.07-5.63), dasatinib (OR, 3.86; 95%CI, 1.33-11.18) and ponatinib (OR, 3.47; 95%CI, 1.23-9.78) are all associated with a significant increase of the risk, whereas a trend was demonstrated for bosutinib (OR, 2.77; 95%CI, 0.39-19.77).(Douxfils, Haguet, et al. 2016a) New-generation TKIs improve the rate of major molecular response (MMR) (OR, 2.22; 95%CI, 1.87 to 2.63) but no statistical difference in overall survival at 1 year was found (overall OR, 1.20; 95%CI, 0.63-2.29). This study was the first that demonstrates a significant increase in the rate of vascular occlusive events associated with the use of dasatinib, nilotinib and ponatinib compared to imatinib.

Following this publication, Yun et al. responded to the article through a letter to the editor in which they proposed an analysis of the risk of vascular occlusive events with high-dose imatinib, and a comparison between patients previously treated with imatinib and naive-treatment patients.(Yun,

Vincelette, and Abraham 2016) They concluded that the cautions about vascular safety should be extended to high-dose imatinib and prior exposure to imatinib. In response to this letter, we performed the same analysis using similar criteria and statistical methodology as in our meta-analysis but we disagreed with their estimates.(Douxfils, Haguet, and Dogne 2016) In this analysis, we found that the risk of vascular occlusive events is slightly higher in patients treated with new generation TKIs when they are naive of treatment (OR_{PETO}: 3.51; 95%CI: 2.29 to 5.39) compared to patients previously treated with imatinib (OR_{PETO}: 2.98; 95%CI: 0.84 to 10.62). No difference was seen in the incidence of patients receiving high dose imatinib (i.e. 800 mg once daily) (1.61%) and patients treated with new generation TKIs (1.67%) (OR_{PETO}: 1.09; 95%CI: 0.10 to 11.80). However, the limited statistical power of this analysis precluded firm conclusions.(Douxfils, Haguet, and Dogne 2016)

Subsequent to this first meta-analysis, we aimed to discriminate the burden of arterial versus venous risk. We performed an update of our previous meta-analysis and distinguished arterial and venous occlusive events in order to conduct mechanistic studies and to determine the adapted strategies to minimize the vascular risk.(Haguet et al. 2017) In this meta-analysis, the screening of 243 additional abstracts and 219 clinical trials led to the identification of 12 clinical trials comparing patients with Philadelphia chromosome positive CML treated with new generation BCR-ABL TKIs and patients treated with imatinib. Statistical analysis indicated that overall, new generation TKIs induce higher rates of arterial (OR_{PETO}: 3.46; 95%CI: 2.35 to 5.10) than imatinib. Stratification by treatment demonstrated that ponatinib (OR_{PETO}: 3.26; 95%CI: 1.12 to 9.50), nilotinib (OR_{PETO}: 3.69; 95%CI: 2.29 to 5.95) and dasatinib (OR_{PETO}: 3.32; 95%CI: 1.37 to 8.01) are all associated with increased risk of arterial occlusive events. This sub-analysis highlighted the predominant part of arterial occlusive events (4.78%) compared with venous thromboembolism (0.96%) with new generation BCR-ABL TKIs and guide our mechanistic investigations toward an arterial impact of BCR-ABL TKIs rather than venous. Perspectives were therefore to achieve additional mechanistic studies to assess the underlying pathophysiological mechanisms and to assess

the benefits of prophylactic treatments (i.e. anti-platelet therapies and/or statins) through clinical trials where cardiovascular outcomes are properly investigated.(Haguet et al. 2017)

Recently, numerous studies challenged the position of imatinib by concluding that 2nd generation TKIs are superior for frontline treatment of CML based on the higher rate of surrogate outcomes. (Cortes 2018; Gurion et al. 2013; Chen et al. 2018; Fachi et al. 2018; Larson et al. 2014) However, survival benefit has never been demonstrated with 2nd generation TKIs compared with imatinib, possibly due to the shorter follow-up (with the exception of the 5-year follow-up results of a nilotinib trial). Since the first meta-analysis that assessed overall survival at 1 year, data with longer follow-up have been published. Therefore, we updated again our meta-analysis to assess the long-term efficacy of these treatments using a patient-relevant outcome approach (i.e. overall survival).(Haguet, Graux, et al. 2020) The cardiovascular safety of TKIs was still considered, with a special attention for bosutinib for which prior meta-analysis precluded firm conclusions due to insufficient power.

Original article in Cancers (Basel) 12(5)

Long-Term Survival, Vascular Occlusive Events and Efficacy Biomarkers of First-Line Treatment of CML: A Meta-Analysis

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Summary

Large randomized clinical trials and prior meta-analyses indicate that second-generation BCR-ABL tyrosine kinase inhibitors (TKIs) improve surrogate biomarkers in patients with chronic myeloid leukemia (CML) without providing survival benefits. The objective is to evaluate the long-term efficacy and the occurrence of vascular occlusion with second-generation BCR-ABL TKIs compared with imatinib in patients with CML. Three scientific databases, a clinical registry and abstracts from congress were searched to identify all randomized controlled trials that compared a second-generation BCR-ABL TKI to imatinib in patients with CML. Outcomes extracted were overall survival, major molecular response and complete cytogenetic response, arterial occlusive events and venous thromboembolism. These data were synthesized by odds ratios using a fixed-effect model. This meta-analysis included 4659 participants from 14 trials. Second-generation BCR-ABL TKIs did not improve overall survival compared with imatinib, even at longer follow-up (OR, 1.17 (95% CI, 0.91–1.52)). They improved surrogate biomarkers at 12 and 24 months but increased the risk of arterial occlusion (OR_{PETO}, 2.81 (95% CI, 2.11–3.73)). The long-term benefits of second-generation TKIs are restricted to surrogate outcomes and do not translate into prolonged survival compared to imatinib. Given the long-term use, frontline therapy should be chosen carefully, with special attention to the patients' quality of life and cardiovascular risks.

Keywords: *protein kinase inhibitors; overall survival; meta-analysis; leukemia; myelogenous chronic; BCR-ABL positive; arterial occlusive disease*

Introduction

Rationale

Treatment of chronic myeloid leukemia (CML) has significantly changed over the last two decades with the development of tyrosine kinase inhibitors (TKIs) targeting BCR-ABL. Today, five BCR-ABL TKIs are approved to treat CML (a sixth BCR-ABL TKI, radotinib, is approved in Korea only). Four of them are indicated for use in newly diagnosed chronic phase CML patients (Radich et al. 2018). The optimal choice is challenging for physicians. The first-generation TKI, imatinib is a well-known safe and effective drug, whereas second-generation TKIs (i.e., dasatinib, nilotinib or bosutinib) provide faster molecular responses but are considered less safe than imatinib (Cortes 2018; Hantel and Larson 2018). Evidence-based guidelines recommend basing the decision of the frontline therapy on the treatment aim, the treatment cost and the TKI safety profiles (Radich et al. 2018; Baccarani et al. 2015; Hochhaus, Saussele, et al. 2017). The use of a second-generation TKI over imatinib is particularly recommended for patients with intermediate- or high-risk Sokal scores. Second-generation TKIs are also recommended for young patients because of the higher probability of treatment-free remission with these TKIs (Radich et al. 2018).

Despite their benefit in molecular and cytogenetic responses, second-generation TKIs have not demonstrated survival benefits over imatinib in clinical trials (Cortes, Saglio, et al. 2016; O'Brien et al. 2018), possibly because of the short follow-up. Meta-analyses have been performed to compare the efficacy of second-generation BCR-ABL TKIs with imatinib in patients with CML (Douxfils, Haguet, et al. 2016b; Fachi et al. 2018; Chen et al. 2018; Hoffmann et al. 2017; Gurion et al. 2013). All concluded that second-generation TKIs provide better surrogate outcomes (i.e., molecular and cytogenetic responses) but no survival benefit (Chen et al. 2018; Douxfils, Haguet, et al. 2016b). However, overall survival analyses were restricted to data at one year and showed a high rate of survival. This limits the probability

of demonstrating a significant benefit of second-generation TKIs in terms of survival (Douxfils, Haguet, et al. 2016b). Since then, additional data from randomized clinical trials (RCTs) with longer follow-up have been published (Cortes, Saglio, et al. 2016; O'Brien et al. 2018; Hochhaus et al. 2016). The 5-year report of the ENESTnd trial, a large phase 3 trial, revealed that nilotinib induces survival benefits compared with imatinib (Larson et al. 2014). In regard to safety, a prior meta-analysis assessed the risk of vascular occlusion of second-generation BCR-ABL TKIs plus the third-generation TKI ponatinib because of a signal during ponatinib development that led to the subsequent discontinuation of the phase 3 trial (European Medicines Agency 2013a; Cortes, Kim, et al. 2018). That study concluded that a greater risk of arterial occlusion was observed compared to the risk with imatinib (Haguet et al. 2017). Subgroup analyses indicate that an increased risk exists with two of the three second-generation TKIs (dasatinib and nilotinib). The lack of data did not allow firm conclusions about bosutinib, recently approved as a first-line drug for chronic phase CML (Haguet et al. 2017; Committee for Medicinal Products for Human Use of the European Medicines Agency ; U.S. Food and Drug Administration). Since then, the results from an additional phase 3 study have been published on bosutinib. The inclusion of data on long-term follow-up permits a more global approach of the benefit-risk profile of second-generation BCR-ABL TKIs.

Objectives

The study proposed is the first meta-analysis aimed to assess the long-term overall survival, major molecular response (MMR) and cytogenetic response (CCyR) of second-generation BCR-ABL TKIs compared with imatinib in patients with CML in RCTs. This meta-analysis also compares the occurrence of arterial and venous occlusions with first-line BCR-ABL TKIs in CML patients.

Materials and Methods

Search Methods

This meta-analysis was performed in accordance with the protocol for meta-analyses published in 2015 (Douxfils, Haguet, et al. 2016b) and complied with the PROSPERO protocol 2014:CRD42014014147. Three scientific databases (PubMed (from 1966), Scopus (from 1995) and CENTRAL (Cochrane Central Register of Controlled Trials; from 1996)), a clinical trial registry (clinicaltrials.gov) and abstracts from the last 3 years of 3 meetings (i.e., ASCO, ESMO and ASH congresses) were searched up to January 14, 2019, using the search strategy reported in **Method S1**. Only English publications were considered. This meta-analysis follows the PRISMA statement (**Table 2.4**).

Data Collection

Study Selection

Two researchers (HH and JD) independently screened all titles and abstracts identified from the literature search to determine potentially eligible studies. We included RCTs that compared a second-generation TKI approved for first-line CML treatment with imatinib in patients with CML. The full texts of these potentially relevant articles were then assessed independently by the same researchers (HH and JD) based on predefined inclusion and exclusion criteria. Disagreements were resolved through discussion with a third author (JMD).

Data Extraction

The data extraction was performed for each study independently by 2 researchers (HH and JD) using a standard data extraction form. The outcomes extracted were overall survival, MMR and CCyR at predefined time points (12 months, 24 months, 36 months, 48 months and 60 months, when available), as well as arterial occlusive events and venous thromboembolism. **Method S2** presents the list of terms considered as arterial occlusive events and venous thromboembolisms. Reviewers took special care to extract

responses at a specific time point rather than the cumulative response (i.e. the rate of response achieved by the time point, regardless of whether they lost the response/discontinued or not). The number of patients alive was also preferred over the estimated overall survival (Kaplan–Meier analysis), and the overall survival with the longest study follow-up was included.

When discrepancies occurred between sources, a conservative approach was adopted. For arterial occlusive events and venous thromboembolism analyses, data were extracted from the adverse event report rather than from secondary analyses performed by the study sponsor (i.e. vascular safety analyses performed as secondary outcomes) when possible, to limit the risk of bias.

Data Analysis

Data Synthesis

We conducted meta-analyses of dichotomous variables with the fixed-effect model (FEM) by computing odds ratios (ORs) and 95% confidence intervals (CIs). For the arterial occlusive event and venous thromboembolism analyses, ORs were computed using the Peto method because the rate of events was low (Bradburn et al. 2007). Statistical analyses were performed using Review Manager (RevMan) version 5.3 (Copenhagen, 2014, https://training.cochrane.org/online-learning/core-software-cochrane-reviews/revman/revman-5-download). In addition to ORs, anticipated absolute effects were computed using a GRADE profiler (GRADEpro GDT, McMaster University, 2015). This measure was expressed as a risk difference with its 95% CI and is based on the baseline risk (i.e. the risk in the comparison group) and the relative effect of the intervention.

Subgroup Analysis and Investigation of Heterogeneity

Stratification per treatment was performed, as well as per TKI dose when data were available. Heterogeneity among trials was quantified by the I^2 statistic,

with an I^2 value < 25% reflecting mild heterogeneity, 25%–50% reflecting moderate heterogeneity and > 50% reflecting severe heterogeneity.

Sensitivity Analysis

Sensitivity analyses were performed as a repeat of the meta-analysis by substituting previously treated patients in order to explore their impact on MMR and CCyR. Sensitivity analyses were also performed by subtracting trials with high-dose imatinib (i.e. greater than 400 mg per day).

Assessment of Risk of Bias in Included Studies

The risk of bias was assessed using the Cochrane Collaboration's risk of bias 2 (RoB 2) tool for RCT (Higgins et al. 2011). To assess the risk of reporting bias, we carried out an assessment of publication bias using funnel plots. Funnel plot asymmetry was evaluated using Egger's regression tests (Sterne et al. 2011).

Results

Eligible Studies and Study Characteristics

The literature search yielded 918 records. After titles and abstract screening, 113 full texts were considered for further investigation. The full-text screening excluded 93 articles. Major exclusion reasons were the lack of pertinent data, outdated data or an inappropriate study design. Finally, we identified 14 clinical trials reported in 20 abstracts and articles that met the inclusion criteria, involving a total of 4659 participants (Choi et al. 2012; Cavalli et al. 2018; Deininger et al. 2018; Gambacorti-Passerini et al. 2017; Cortes, Mauro, et al. 2018; Saglio, Kim, et al. 2010; Cortes, Jiang, et al. 2018; Cortes, Saglio, et al. 2016; Radich et al. 2012; Hochhaus et al. 2016; Kwak et al. 2017; Lipton et al. 2016; O'Brien et al. 2018; Hughes et al. 2017; Cortes, De Souza, et al. 2016; Kantarjian et al. 2012; Brummendorf et al. 2015; Wang et al. 2015; O'Brien et al. 2014; Kantarjian et al. 2009; Hughes et al. 2014; Hjorth-Hansen et al. 2015). **Figure 2.1** shows the flow of studies in the systematic review process and

Table 2.3 lists the key characteristics of the 14 studies. All included studies are RCTs.

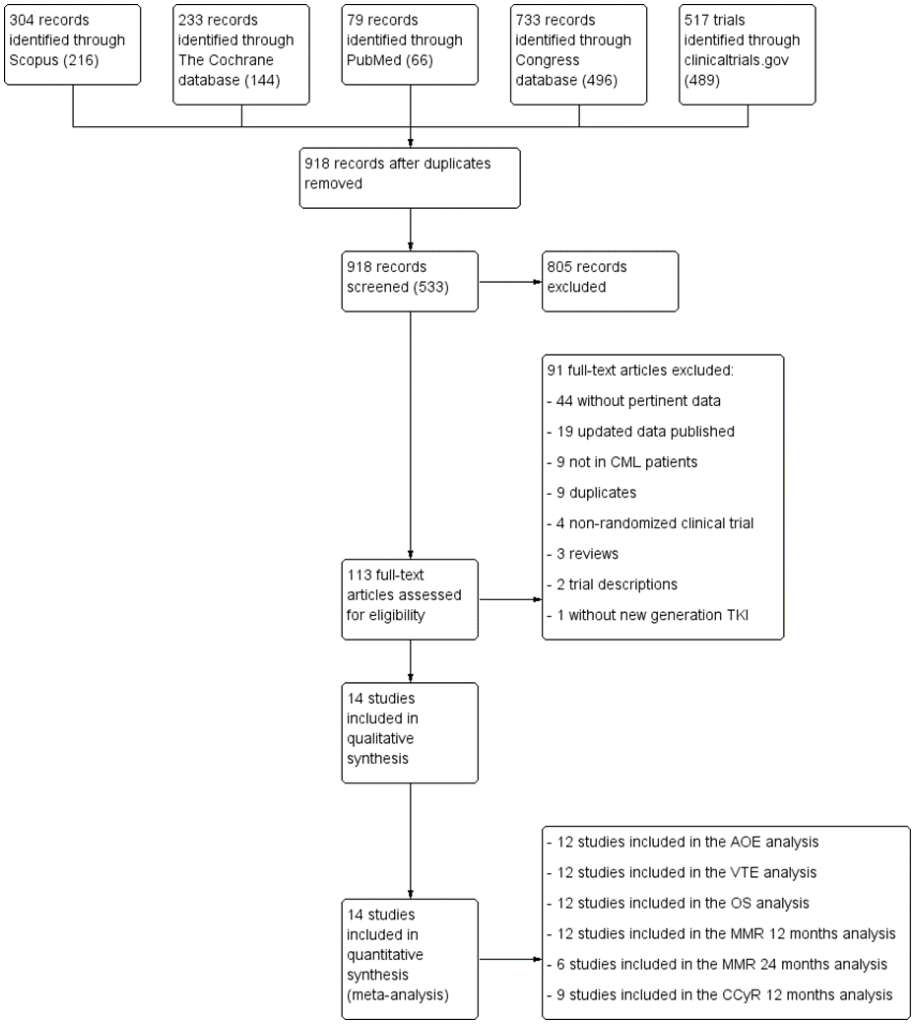
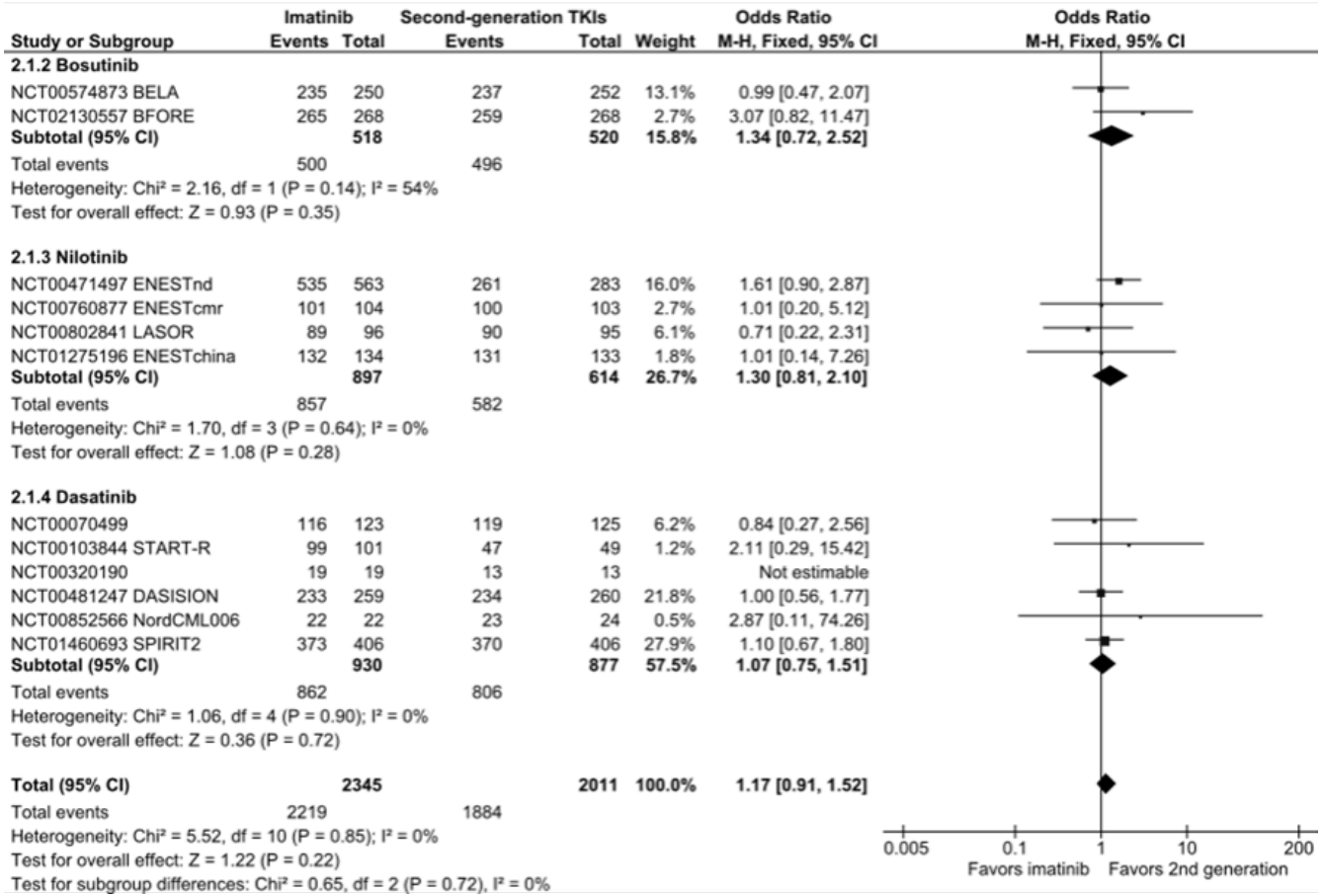


Figure 2.1 | **PRISMA flow diagram of the study selection process.** The risk of bias for each of the 14 included studies is shown in **Figure 2.4**.

AOE: arterial occlusive events; CCyR: complete cytogenetic response; MMR: major molecular response; OS: overall survival; TKI: tyrosine kinase inhibitor; VTE: venous thromboembolism.


Imatinib
Second-generation TKIs
Odds Ratio
Odds Ratio
Study or Subgroup
Events Total
Events Total Weight
M-H, Fixed, 95% CI
M-H, Fixed, 95% CI
2.1.2 Bosutinib
NCT00574873 BELA 235 250 237 252 13.1% 0.99 [0.47, 2.07]
NCT02130557 BFORE 265 268 259 268 2.7% 3.07 [0.82, 11.47]
Subtotal (95% CI) 518 520 15.8% 1.34 [0.72, 2.52]
Total events 500 496
Heterogeneity: Chi² = 2.16, df = 1 (P = 0.14); I² = 54%
Test for overall effect: Z = 0.93 (P = 0.35)
2.1.3 Nilotinib
NCT00471497 ENESTnd 535 563 261 283 16.0% 1.61 [0.90, 2.87]
NCT00760877 ENESTcmr 101 104 100 103 2.7% 1.01 [0.20, 5.12]
NCT00802841 LASOR 89 96 90 95 6.1% 0.71 [0.22, 2.31]
NCT01275196 ENESTchina 132 134 131 133 1.8% 1.01 [0.14, 7.26]
Subtotal (95% CI) 897 614 26.7% 1.30 [0.81, 2.10]
Total events 857 582
Heterogeneity: Chi² = 1.70, df = 3 (P = 0.64); I² = 0%
Test for overall effect: Z = 1.08 (P = 0.28)
2.1.4 Dasatinib
NCT00070499 116 123 119 125 6.2% 0.84 [0.27, 2.56]
NCT00103844 START-R 99 101 47 49 1.2% 2.11 [0.29, 15.42]
NCT00320190 19 19 13 13 Not estimable
NCT00481247 DASISION 233 259 234 260 21.8% 1.00 [0.56, 1.77]
NCT00852566 NordCML006 22 22 23 24 0.5% 2.87 [0.11, 74.26]
NCT01460693 SPIRIT2 373 406 370 406 27.9% 1.10 [0.67, 1.80]
Subtotal (95% CI) 930 877 57.5% 1.07 [0.75, 1.51]
Total events 862 806
Heterogeneity: Chi² = 1.06, df = 4 (P = 0.90); I² = 0%
Test for overall effect: Z = 0.36 (P = 0.72)
Total (95% CI) 2345 2011 100.0% 1.17 [0.91, 1.52]
Total events 2219 1884
Heterogeneity: Chi² = 5.52, df = 10 (P = 0.85); I² = 0%
Test for overall effect: Z = 1.22 (P = 0.22)
Test for subgroup differences: Chi² = 0.65, df = 2 (P = 0.72), I² = 0%
0.005
0.1
1
10
200
Favors imatinib
Favors 2nd generation

▲ **Figure 2.2 | Forest plot of overall survival in patients with chronic myeloid leukemia treated with second-generation TKIs versus imatinib.**

Overall Survival

Of the 14 included studies, 12 reported overall survival data. There was no significant difference between second-generation TKIs and imatinib in the outcome of overall survival (OR, 1.17 (95% CI: 0.91–1.52); **Figure 2.2**). This result was consistent between TKIs (subgroup difference, $I^2 = 0\%$; **Figure 2.2)** and between nilotinib dose regimens (300 mg BID: OR, 1.21 (95% CI: 0.66 to 2.24); 400 mg BID: OR, 1.53 (95% CI: 0.86–2.74); **Figure 2.5**). The anticipated absolute effect of overall survival was 0.9% superior (95% CI: −0.6–2.1) with second-generation TKIs over imatinib (survival rate of 93.7%, **Table 2.1**).

The omission of clinical trials performed in previously treated CML patients did not significantly change the results (OR, 1.20 (95% CI: 0.92 to 1.57); **Figure 2.6**). Only two studies reported overall survival in clinical trials comparing high-dose imatinib to second-generation TKIs. Their analysis demonstrated no survival benefit of high-dose imatinib compared to second-generation TKIs (**Figure 2.6** and **Table 2.5**).

Efficacy

The MMR rate at 12 and 24 months and the CCyR rate at 12 months were reported in 12, 6 and 9 studies respectively. Of these three efficacy outcomes, all favored second-generation TKIs (MMR 12 months: OR, 2.01 (95% CI: 1.77–2.30); MMR 24 months: OR, 1.40 (95% CI: 1.17–1.67)); CCyR 12 months: OR, 1.50 (95% CI: 1.31–1.72); **Figure 2.7**), even compared with high-dose imatinib (**Figure 2.6**). These results were consistent between studies for MMR at 12 and 24 months (respectively, $I^2 = 20\%$ and 0%), but the I^2 statistic indicated moderate heterogeneity for CCyR at 12 months ($I^2 = 48\%$). This inconsistency was mainly due to the heterogeneity between nilotinib ($I^2 = 81\%$) and bosutinib trials ($I^2 = 64\%$). Differences between these study designs were related to TKI dose and population (i.e. patients previously treated vs. treatment-naïve patients; **Table 2.3**). The stratification by nilotinib dose regimen demonstrated similar results between 300 and 400 mg BID (**Figure 2.5**), and the omission of clinical trials performed in previously treated CML patients did not significantly change the results (**Figure 2.6**).

Table 2.1 | Overall survival in patients with CML receiving second-generation BCR-ABL TKIs.

BCR-ABL TKI No. of Patients (No. of Studies)	Odds Ratio (95% CI)	Anticipated Absolute Effects (95% CI)		
		Risk with Imatinib	Risk with 2nd-Generation TKI	Risk Difference with 2nd-Generation TKI
All 2nd Generation TKI **No. of Patients: 4356** (12 Studies)	**1.17** **(0.91 to 1.52)**	**93.7%**	**94.6%** **(93.1 to 95.8)**	**+0.9%** **(−0.6 to +2.1)**
Bosutinib **No. of Patients: 1038** (2 Studies)	1.34 (0.72 to 2.52)	95.4%	96.5% (93.7 to 98.1)	+1.1% (−1.7 to +2.7)
Nilotinib **No. of Patients: 1511** (4 Studies)	1.30 (0.81 to 2.10)	94.8%	95.9% (93.6 to 97.4)	+1.2% (−1.1 to +2.7)
Dasatinib **No. of Patients: 1807** (6 Studies)	1.07 (0.75 to 1.51)	91.9%	92.4% (89.5 to 94.5)	+0.5% (−2.4 to +2.6)

Vascular Occlusion

The risk of arterial occlusive events was increased with second-generation TKIs compared with imatinib (OR_{PETO}, 2.81 (95% CI: 2.11–3.73); **Figure 2.3**) and high-dose imatinib (**Figure 2.6**). With imatinib, 23 out of 1000 patients developed an arterial occlusion, whereas this meta-analysis estimated that this rate increased to 61 out of 1000 patients with second-generation TKIs (95% CI: 46–79, **Table 2.2**). There was a trend toward an increased risk of venous thromboembolism (OR_{PETO}, 1.74 (95% CI: 0.82–3.66); **Figure 2.7**).

The risk of arterial occlusive events was consistently higher for second-generation TKIs and the difference was significant for nilotinib (OR, 3.89 (95% CI: 2.47–6.11)) and dasatinib (OR, 2.68 (95% CI: 1.72–4.17)). Only bosutinib was not associated with a significantly increased risk of arterial occlusive events compared with imatinib (OR, 1.61 (95% CI: 0.85–3.07)). However, this result became significant when simulating recruitment similar to that with dasatinib and nilotinib (i.e. repeating the analysis until the same number of patients was obtained). Arterial occlusive events occurred more frequently with nilotinib 400 mg BID than with nilotinib 300 mg BID (300 mg BID: 3.32 (95% CI: 1.56–7.09); 400 mg BID: 5.19 (95% CI: 3.14–8.57); **Figure 2.5**).

Risk of Bias

The analysis of the risk of bias is reported in the **Figure 2.4**. Four studies of the 12 included in the arterial occlusive event analysis were considered to have high concern. Indeed, because of the discovery of the risk of vascular occlusion with another BCR-ABL TKI (i.e., ponatinib) before these four studies were conducted, the reporting and the subjectivity of similar events (e.g. chest pain, phlebitis) with the investigated treatments might have been affected. All of the 14 included trials were at moderate risk of bias because of the high rate of treatment discontinuation or switching and the possibility of modifying the treatment dose. Additional risks of potential bias were the lack of details of the randomization process, the selection of the reported outcome and the open-label design. Funnel plots and Egger's tests indicated no evidence of publication bias (**Figure 2.8**).

Table 2.2 | Arterial occlusive events in patients with CML receiving second-generation BCR-ABL TKIs.

BCR-ABL TKI **No. of patients** (Studies)	**Odds Ratio** **(95% CI)**	**Anticipated Absolute Effects (95% CI)**		
		Risk with Imatinib	Risk with 2nd Generation TKI	Risk Difference with 2nd Generation TKI
All 2nd Generation TKI **No. of patients: 4328** (12 RCTs)	**OR 2.81** **(2.11 to 3.73)**	**2.3%**	**6.1%** **(4.6 to 7.9)**	**+3.8%** **(+2.4 to +5.7)**
Bosutinib **No. of patients: 1032** (2 RCTs)	OR 1.61 (0.85 to 3.07)	2.9%	4.6% (2.5 to 8.4)	+1.7% (−0.4 to +5.5)
Nilotinib **No. of patients: 1494** (4 RCTs)	OR 3.89 (2.47 to 6.11)	1.3%	4.9% (3.2 to 7.5)	+3.6% (+1.9 to +6.2)
Dasatinib **No. of patients: 1802** (6 RCTs)	OR 2.68 (1.72 to 4.17)	2.5%	6.5% (4.3 to 9.7)	+4.0% (+1.7 to +7.2)

Study or Subgroup	Imatinib Events	Imatinib Total	Second-generation TKIs Events	Second-generation TKIs Total	Weight	Peto Odds Ratio Peto, Fixed, 95% CI
1.1.2 Bosutinib						
NCT00574873 BELA	5	248	3	251	4.1%	1.68 [0.42, 6.79]
NCT02130557 BFORE	19	268	12	265	15.3%	1.59 [0.77, 3.29]
Subtotal (95% CI)		**516**		**516**	**19.5%**	**1.61 [0.85, 3.07]**
Total events	24		15			
Heterogeneity: Chi² = 0.00, df = 1 (P = 0.95); I² = 0%						
Test for overall effect: Z = 1.46 (P = 0.15)						
1.1.3 Nilotinib						
NCT00471497 ENESTnd	58	556	6	280	27.6%	3.23 [1.88, 5.53]
NCT00760877 ENESTcmr	16	101	2	103	8.6%	5.58 [2.13, 14.65]
NCT00802841 LASOR	5	96	0	93	2.6%	7.47 [1.27, 43.98]
NCT01275196 ENESTchina	1	133	0	132	0.5%	7.33 [0.15, 369.61]
Subtotal (95% CI)		**886**		**608**	**39.4%**	**3.89 [2.47, 6.11]**
Total events	80		8			
Heterogeneity: Chi² = 1.62, df = 3 (P = 0.65); I² = 0%						
Test for overall effect: Z = 5.88 (P < 0.00001)						
1.1.4 Dasatinib						
NCT00070499	12	123	0	123	6.0%	8.12 [2.55, 25.84]
NCT00103844 START-R	8	101	0	49	3.5%	4.75 [1.05, 21.58]
NCT00320190	1	19	2	13	1.4%	0.32 [0.03, 3.41]
NCT00481247 DASISION	34	258	18	258	24.5%	1.98 [1.12, 3.51]
NCT00852566 NordCML006	1	22	0	24	0.5%	8.09 [0.16, 409.34]
NCT01460693 SPIRIT2	8	406	2	406	5.2%	3.37 [0.97, 11.70]
Subtotal (95% CI)		**929**		**873**	**41.2%**	**2.68 [1.72, 4.17]**
Total events	64		22			
Heterogeneity: Chi² = 8.68, df = 5 (P = 0.12); I² = 42%						
Test for overall effect: Z = 4.37 (P < 0.0001)						
Total (95% CI)		**2331**		**1997**	**100.0%**	**2.81 [2.11, 3.73]**
Total events	168		45			
Heterogeneity: Chi² = 15.18, df = 11 (P = 0.17); I² = 28%						
Test for overall effect: Z = 7.13 (P < 0.00001)						
Test for subgroup differences: Chi² = 4.88, df = 2 (P = 0.09), I² = 59.0%						

▲ **Figure 2.3 | Forest plot of arterial occlusive events in patients with CML treated with second-generation TKIs versus imatinib.**

Discussion

Should Second-Generation TKIs Be Favored over Imatinib for Treatment-Naïve chronic phase CML Patients?

Are Second-Generation TKIs More Efficient Than Imatinib to Treat chronic phase CML?

This is the first meta-analysis of second-generation BCR-ABL TKIs that provides long-term estimates on efficacy and safety compared to imatinib. The major finding of this meta-analysis of 14 RCTs including 4659 CML patients was that second-generation BCR-ABL TKIs did not significantly improve short- or long-term overall survival compared with imatinib. Except for nilotinib, for which survival superiority was suggested in the ENESTnd trial (Larson et al. 2014), this absence of the benefit of second-generation TKI in terms of survival is in accordance with the RCT results (Cortes, Saglio, et al. 2016; O'Brien et al. 2018). Unsurprisingly, and in line with large clinical trials, surrogate outcomes such as the rates of MMR and CCyR at 12 and 24 months were improved with second-generation BCR-ABL TKIs compared to imatinib.

Based on the higher rate of surrogate outcomes with second-generation TKIs, numerous recent studies have concluded that second-generation TKIs are superior for frontline treatment of CML, challenging the position of imatinib (Cortes 2018; Gurion et al. 2013; Chen et al. 2018; Fachi et al. 2018). Surrogate outcomes have been adopted as predictive of overall survival at the time CML patients were treated with interferon-α because of the strong correlation between cytogenetic response and survival. They have then been used for TKI marketing authorization because patient-relevant outcomes (e.g., overall survival and progression-free survival) are time dependent. However, BCR-ABL TKIs and interferon-α differ in their mechanisms of action, but the validity of the surrogate outcomes to predict patient survival with TKIs has not been questioned. Different molecular responses (early molecular response and MR 4.5) have been proposed as surrogate markers for overall survival but

have failed to predict patient survival (Guilhot et al. 2015). To our knowledge, it has not been demonstrated that a potential TKI effect on survival is explained by molecular response. These biomarkers may not fully capture the action TKIs have on the overall survival. Indeed, the lack of selectivity of BCR-ABL TKIs is responsible for numerous adverse events that may affect patient survival (e.g. ischemic stroke or myocardial infarction) (Ota et al. 2018). A long-term study of patients with CML treated by second- or third- generation TKIs demonstrates that approximately a third of death was related to cardiovascular diseases (Caocci et al. 2020). This potentially explains why, even with long follow-up, improvement in surrogate outcomes with second-generation TKIs does not result in better overall survival. Consequently, surrogate outcomes should not be interpreted alone. The long-term benefits, treatment safety and patients' quality of life (QoL) should be preferred and seem more relevant because of the long-life expectancy of chronic phase CML patients, close to that of the general population (Bower et al. 2016).

Do All Second-Generation TKIs Favor Arterial Occlusion?

As imatinib and second-generation TKIs confer similar survival probability, their vascular safety is important to consider avoiding premature non-CML-related deaths or vascular-related disabilities. Our meta-analysis confirms that the risk of arterial occlusive events is higher with second-generation TKIs than with imatinib. Of 1000 chronic phase CML patients, 38 more patients (95% CI, 24–57) will develop an arterial occlusive disease with second-generation TKIs compared with imatinib. In accordance with real-life studies, the risk of arterial occlusion has reportedly been higher with nilotinib, dasatinib and ponatinib. Bosutinib has the safer vascular profile and is currently not associated with a significant increase of the risk of vascular occlusion (Caocci et al. 2019; Chai-Adisaksopha, Lam, and Hillis 2016). However, the size of the population of the bosutinib analysis is limited compared to the number of patients in the dasatinib and nilotinib subgroups. This lack of statistical evidence should therefore not be seen as evidence of an absence of a true risk. Further investigations are needed to improve the power

of the analysis. In addition, data with bosutinib were obtained from two trials sponsored by the marketing authorization holder. Assessment of the arterial occlusive event analysis indicated that higher ORs were reported in institutional or academic trials (i.e. NCT00070499 and NordCML006) than in sponsored industrial studies, raising some concerns about the risk of bias.

Imatinib and Second-Generation TKIs Have Limited Impact on Patients' QoL

The potential long-term duration of BCR-ABL TKI treatment led patients and physicians to pay more attention to treatment long-term risks and the impact on patients' QoL. However, the latter has rarely been studied in clinical trials. The few such studies report similar QoL with imatinib as in the general population among older patients (older than 59 years) (Efficace et al. 2011). However, younger patients are more affected and report limitations in work and daily activities compared to the age-matched general population (i.e. without cancer) (Efficace et al. 2011). With second-generation TKIs, quality-of-life sub analyses have been performed in four large RCTs (i.e. BFORE, ENESTnd, ENESTchina and SPIRIT2) (Beaumont et al. 2012; Yu et al. 2018; Cortes, Gambacorti-Passerini, et al. 2017; Brummendorf, Mamolo, et al. 2018; Guerin et al. 2014; Labeit et al. 2015). All these analyses reported no differences in QoL between the second-generation TKIs and imatinib (Beaumont et al. 2012; Yu et al. 2018; Cortes, Gambacorti-Passerini, et al. 2017; Brummendorf, Mamolo, et al. 2018; Guerin et al. 2014; Labeit et al. 2015).

Recommendations for Frontline Treatment of Chronic Phase CML

Imatinib and second-generation BCR-ABL TKIs are all highly effective in treating chronic phase CML and provide survival close to that of the age-matched population. Imatinib has demonstrated reassuring long-term safety and efficacy (Hochhaus, Larson, et al. 2017). At 10 years, the overall survival rate was estimated to be 83.3%, and fewer than 10% of patients developed serious adverse events (Hochhaus, Larson, et al. 2017). Second-generation TKIs increase the rate of molecular and cytogenetic responses but do not provide benefits in patient survival or QoL compared with imatinib.

The induction of faster and deeper molecular responses with second-generation TKIs compared to imatinib in treatment-naïve patients has generated interest over the last few years for these drugs because of the increasing number of candidates for TKI discontinuation (i.e. a higher number of patients who achieved rapid molecular remission) (Mahon 2017). However, in retrospect, treatment discontinuation was less successful than announced, with a higher number of patients relapsing after TKI cessation than expected. Recent studies indicate that TKI discontinuation was successful in 20% of patients (i.e. almost 40% of CML patients are eligible for treatment discontinuation, with a success rate of 50%), and criteria for trying treatment cessation are more constraining (Cortes, Rea, and Lipton 2019). In addition, patients should be treated longer (i.e. optimally more than 8 years) with a TKI before trying treatment cessation (Cortes, Rea, and Lipton 2019).[3]

Due to the safety profile of second-generation TKIs, particularly the vascular issue damage from these treatments (with the exception of bosutinib, which requires further investigation), the frontline treatment choice should not focus solely on the objective of treatment-free remission. Arterial occlusion occurred more frequently in patients with prior cardiovascular risk factors. Therefore, an estimation of 10-year arteriosclerotic cardiovascular disease risk, as recommended by the ACC/AHA, should be applied to CML patients before making a treatment decision (Jain and Davis 2019).

The treatment choice should then be made individually by carefully weighing the benefits and the risks of each TKI. These results support the current NCCN guidelines, particularly to limit the use of second-generation TKIs as frontline drugs for young patients. Finally, the treatment cost should be considered. A recent study demonstrates that the one-year health care expenditures were

[3] The minimal duration of TKI therapy prior treatment discontinuation varies in the litterature. The European LeukemiaNet group recommends in their 2020 guidelines a minimum of five years of treatment with imatinib or at least four years with a second-generation TKI.(Hochhaus, Baccarani, et al. 2020)

significantly higher among patients treated with second-generation BCR-ABL TKIs as first-line, compared with those treated with imatinib (Cole et al. 2020).

Is the BCR-ABL TKI Dose Optimal?

Modification of the TKI dose regimen has already been proposed as a potential measure to minimize the risk of arterial occlusive events, but data were insufficient to provide formal recommendations (Douxfils, Haguet, et al. 2016b). The results of our meta-analysis suggest that the efficacy of nilotinib is similar independent of the dose. However, nilotinib 400 mg BID was associated with a higher incidence of arterial occlusive events. Therefore, the use of 400 mg BID nilotinib is questionable. Dasatinib has been tested in a RCT only at 100 mg QD in newly diagnosed chronic phase CML patients. Future studies should assess the benefits of reduced doses of nilotinib and dasatinib to minimize the risk of arterial occlusion while preserving efficacy. For bosutinib, the comparison between the BELA and the BFORE trial, in which participants were treated with 500 mg and 400 mg QD, respectively, indicates a similar OR for efficacy outcomes and arterial occlusive event occurrence. A dose reduction to 300 mg QD allowed a reduction of adverse events and better tolerability. However, its effect on the efficacy and arterial occlusion has not been studied (Brummendorf, Gambacorti Passerini, et al. 2018).

Measures to Minimize the Vascular Risk With Second-Generation TKIs

Several measures have already been recommended in the literature to minimize the risk of arterial occlusion (Haguet et al. 2017; Garcia-Gutierrez et al. 2016). These recommendations aim to minimize cardiovascular risk by acting on common cardiovascular risk factors. Prevention treatment is only recommended for patients with diagnosed cardiovascular disease or diabetes. In the European Summary of Product Characteristics (EU-SmPC), recommendations to prevent arterial occlusion are only provided for nilotinib (European Medicines Agency ; European Medicines Agency ; European Medicines Agency). The SmPC of nilotinib does not suggest additional measures that evaluate cardiovascular risk or the management or monitoring

of common cardiovascular risk factors. Recent *in vitro* and *in vivo* studies suggest that nilotinib and ponatinib may induce arterial occlusion through coronary artery vasospasm by inhibiting the RAS/RAF/MEK pathway and increasing Ca^{2+} release by activating the calcium channel (Fiets et al. 2018; Hamadi et al. 2019). The use of diltiazem, a calcium channel blocker, to prevent vascular occlusion is an interesting possibility that should be further investigated in clinical settings with nilotinib and ponatinib [63].

Strengths and Limitations

A major limitation of this meta-analysis is the use of aggregate data from the studies because of the lack of access to individual data. This precludes additional calculations such as overall survival analyses based on patients' Sokal score and age and the integration of patients' comorbidities in the analysis of arterial occlusive events. In addition, access to individual data would provide the advantage that statistical analyses could be standardized between but also within studies, and allow the computation of the number of patients eligible for treatment discontinuation. Progression-free survival was not analyzed in our meta-analysis as its definition differs between studies (Kantarjian et al. 2011). Another limitation was the selection of the intention to treat population as data source, as this population does not take into account the proportion of patients who switched to another TKI.

A major strength of this meta-analysis is the integration of both published and unpublished data that limit the risk of publication bias. As much as possible, we confront the data from the different sources and use a conservative approach to select the data that have been included in the quantitative analysis. Another strength of our analysis is that we assessed the rates of molecular and cytogenetic responses at specific time points (e.g. MMR at 12 months). Most clinical trials have investigated these surrogate outcomes over a period of time, also called the cumulative response, and the reporting of these data is often unclear (i.e. no specification of the type of analysis). Due to the rapid variation of the molecular response, the cumulative incidence is

frequently higher than the response rate at a specific time point. Loss of molecular or cytogenetic responses can be the consequence of lack of compliance, treatment intolerance and drug resistance, three factors that are revealed by the analysis of responses at specific time points rather than cumulative response.

Conclusions

Since the introduction of BCR-ABL TKIs, patients' and physicians' concerns have shifted because of their high efficacy, and more attention has been paid to treatment toxicity and patients' QoL. Even with long follow-up, there is no direct evidence of a survival benefit associated with second-generation BCR-ABL TKIs. Given the long-term use of BCR-ABL TKI treatment, the therapeutic option should be selected individually and based on multiple factors, including cardiovascular safety and QoL. Future research should focus on these last two points. To date, the cardiovascular safety of bosutinib is reassuring. In light of the present results, it is anticipated that an in-depth reanalysis of the clinical trials by the marketing authorization holders together with the regulatory bodies would be needed to update the product information with the most recent data analysis. This would provide health care professionals with updated recommendations on the benefits and risks of these medicines.

Author Contributions: All authors have read and agreed to the published version of the manuscript. Conceptualization, H.H., J.D. and J-M.D.; methodology and literature search, H.H. and J.D.; software, H.H.; validation, H.H. and J.D.; writing—original draft preparation, H.H., J.D. and J-M.D.; writing—review and editing, C.G. and F.M.; supervision, J.D.

Supplemental materials

Method S1: Search strategy

A) The Cochrane Library (From November, 2016 to January 14th, 2019)

Bosutinib

1. bosutinib OR SKI-606 OR SKI606 *(84 articles)*
2. "randomized controlled trial" or "randomized trial" or "randomized clinical trial" or "randomised controlled trial" or "randomised trial" or "randomised clinical trial" *(684885 articles)*
3. #1 AND #2

"Search limits" → Content type: "Trials" + Cochrane Library publication date: between "Nov 2016" *(22 articles)*

Ponatinib

1. ponatinib or AP24534 *(63 articles)*
2. "randomized controlled trial" or "randomized trial" or "randomized clinical trial" or "randomised controlled trial" or "randomised trial" or "randomised clinical trial" *(684885 articles)*
3. #1 AND #2

"Search limits" → Content type: "Trials" + Cochrane Library publication date: between "Nov 2016" *(11 articles)*

Nilotinib

1. nilotinib or AMN107 *(301 articles)*
2. "randomized controlled trial" or "randomized trial" or "randomized clinical trial" or "randomised controlled trial" or "randomised trial" or "randomised clinical trial" *(684885 articles)*
3. #1 AND #2

"Search limits" → Content type: "Trials" + Cochrane Library publication date: between "Nov 2016" *(51 articles)*

Dasatinib

1. dasatinib or BMS-354825 or BMS354825 (332 articles)

2. "randomized controlled trial" or "randomized trial" or "randomized clinical trial" or "randomised controlled trial" or "randomised trial" or "randomised clinical trial" *(684885 articles)*
3. #1 AND #2

"Search limits" → Content type: "Trials" + Cochrane Library publication date: between "Nov 2016" *(53 articles)*

Imatinib

1. imatinib or imatinib mesylate or STI-571 or STI571 *(1097 articles)*
2. "randomized controlled trial" or "randomized trial" or "randomized clinical trial" or "randomised controlled trial" or "randomised trial" or "randomised clinical trial" *(684885 articles)*
3. #1 AND #2

"Search limits" → Content type: "Trials" + Cochrane Library publication date: between "Nov 2016" *(131 articles)*

Radotinib

1. Radotinib or IY5511 or IY-5511 *(9 articles)*
2. "randomized controlled trial" or "randomized trial" or "randomized clinical trial" or "randomised controlled trial" or "randomised trial" or "randomised clinical trial" *(703781 articles)*
3. #1 AND #2

"Search limits" → Content type: "Trials" + Cochrane Library publication date: to Jan 2019 *(5 articles)*

Total

Combination of the searches with « OR » *(179 articles)*

B) PubMed (From November, 8th 2016, to January, 14th 2019)

Dasatinib

1. Search ((dasatinib[Title]) OR BMS-354825[Title]) OR BMS354825[Title] *(1143 Articles)*
2. Search (((((randomized controlled trial) OR randomized trial) OR randomized clinical trial) OR randomised controlled trial) OR randomised trial) OR randomised clinical trial *(684679 articles)*

3. #1 AND #2 AND ("2016/11/08"[Date - Publication]: "3000"[Date - Publication]) *(7 articles)*

Nilotinib

1. Search (nilotinib[Title]) OR AMN107[Title] *(711 Articles)*
2. Search (((((randomized controlled trial) OR randomized trial) OR randomized clinical trial) OR randomised controlled trial) OR randomised trial) OR randomised clinical trial (684679 articles)
3. #1 AND #2 AND ("2016/11/08"[Date - Publication]: "3000"[Date - Publication]) *(6 articles)*

Bosutinib

1. Search ((bosutinib[Title]) OR SKI-606[Title]) OR SKI606[Title] *(136 Articles)*
2. Search (((((randomized controlled trial) OR randomized trial) OR randomized clinical trial) OR randomised controlled trial) OR randomised trial) OR randomised clinical trial *(684679 articles)*
3. #1 AND #2 AND ("2016/11/08"[Date - Publication]: "3000"[Date - Publication]) *(5 articles)*

Ponatinib

1. Search (ponatinib[Title]) OR AP24534[Title] *(210 Articles)*
2. Search (((((randomized controlled trial) OR randomized trial) OR randomized clinical trial) OR randomised controlled trial) OR randomised trial) OR randomised clinical trial *(684679 articles)*
3. #1 AND #2 AND ("2016/11/08"[Date - Publication]: "3000"[Date - Publication]) *(2 articles)*

Imatinib

1. Search (((imatinib[Title]) OR imatinib mesylate[Title]) OR STI-571[Title]) OR STI571[Title] *(6285 Articles)*
2. Search (((((randomized controlled trial) OR randomized trial) OR randomized clinical trial) OR randomised controlled trial) OR randomised trial) OR randomised clinical trial *(684679 articles)*
3. #1 AND #2 AND ("2016/11/08"[Date - Publication]: "3000"[Date - Publication]) *(21 articles)*

Radotinib

1. Search ((radotinib[Title]) OR IY5511[Title]) OR IY-5511[Title] *(19 articles)*
2. Search (((((randomized controlled trial) OR randomized trial) OR randomized clinical trial) OR randomised controlled trial) OR randomised trial) OR randomised clinical trial *(688374 articles)*
3. #1 AND #2 *(1 article)*

Total

Combination with "OR" (exemple: ((((#20) OR #18) OR #16) OR #14) OR #8) *(33 articles)*

C) Scopus (From 2016 to January 14th, 2019)

Bosutinib

(TITLE (bosutinib) OR TITLE (ski-606) OR TITLE (SKI606)) AND (ALL ("randomized controlled trial") OR ALL ("randomized trial") OR ALL ("randomized clinical trial") OR ALL ("randomised controlled trial") OR ALL("randomised trial") OR ALL ("randomised clinical trial"))

Articles published between 2016 and 2019 *(8 articles)*

Ponatinib

(TITLE (ponatinib) OR TITLE (AP24534)) AND (ALL ("randomized controlled trial") OR ALL ("randomized trial") OR ALL ("randomized clinical trial") OR ALL ("randomised controlled trial") OR ALL("randomised trial") OR ALL ("randomised clinical trial"))

Articles published between 2016 and 2019 *(14 articles)*

Nilotinib

(TITLE (nilotinib) OR TITLE (AMN107)) AND (ALL ("randomized controlled trial") OR ALL ("randomized trial") OR ALL ("randomized clinical trial") OR ALL ("randomised controlled trial") OR ALL("randomised trial") OR ALL ("randomised clinical trial"))

Articles published between 2016 and 2019 *(20 articles)*

Dasatinib

(TITLE (dasatinib) OR TITLE (BMS-354825) OR TITLE (BMS354825)) AND (ALL ("randomized controlled trial") OR ALL ("randomized trial") OR ALL

("randomized clinical trial") OR ALL ("randomised controlled trial") OR ALL("randomised trial") OR ALL ("randomised clinical trial"))

Articles published between 2016 and 2019 *(36 articles)*

Imatinib

(TITLE (imatinib) OR TITLE (imatinib mesylate) OR TITLE (STI-571) OR TITLE (STI571)) AND (ALL ("randomized controlled trial") OR ALL ("randomized trial") OR ALL ("randomized clinical trial") OR ALL ("randomised controlled trial") OR ALL("randomised trial") OR ALL ("randomised clinical trial"))

Articles published between 2016 and 2019 *(169 articles)*

Radotinib

(TITLE (radotinib) OR TITLE (iy5511) OR TITLE (iy-5511)) AND (ALL ("randomized controlled trial") OR ALL ("randomized trial") OR ALL ("randomized clinical trial") OR ALL ("randomised controlled trial") OR ALL ("randomised trial") OR ALL ("randomised clinical trial")) *(3 articles)*

Total

(TITLE (imatinib) OR TITLE (imatinib mesylate) OR TITLE (STI-571) OR TITLE (STI571) OR TITLE (dasatinib) OR TITLE (BMS-354825) OR TITLE (BMS354825) OR TITLE (nilotinib) OR TITLE (AMN107) OR TITLE (ponatinib) OR TITLE (AP24534) OR TITLE (bosutinib) OR TITLE (ski-606) OR TITLE (SKI606)) AND (ALL ("randomized controlled trial") OR ALL ("randomized trial") OR ALL ("randomized clinical trial") OR ALL ("randomised controlled trial") OR ALL("randomised trial") OR ALL ("randomised clinical trial"))

Articles published between 2016 and 2019 *(230 articles)*

D) Meeting abstracts

ASCO

- ASCO annual meeting 2017

Title:"imatinib" OR Title:"dasatinib" OR Title:"nilotinib" OR Title:"bosutinib" OR Title:"ponatinib" OR Title:"radotinib" *(22 abstracts)*

- ASCO annual meeting 2018

Title:"imatinib" OR Title:"dasatinib" OR Title:"nilotinib" OR Title:"bosutinib" OR Title:"ponatinib" *(21 abstracts)*

ESMO

- ESMO 2017

Advanced search → « presentation title »: « imatinib dasatinib nilotinib bosutinib ponatinib radotinib » *(9 abstracts)*

- ESMO 2018

Advanced search → « presentation title »: « imatinib dasatinib nilotinib bosutinib ponatinib radotinib » *(4 abstracts)*

ASH

- ASH 2016

Imatinib OR ponatinib OR nilotinib OR bosutinib OR dasatinib OR radotinib *(96 abstracts)*

- ASH 2017

Imatinib OR ponatinib OR nilotinib OR bosutinib OR dasatinib OR radotinib *(71 abstracts)*

- ASH 2018

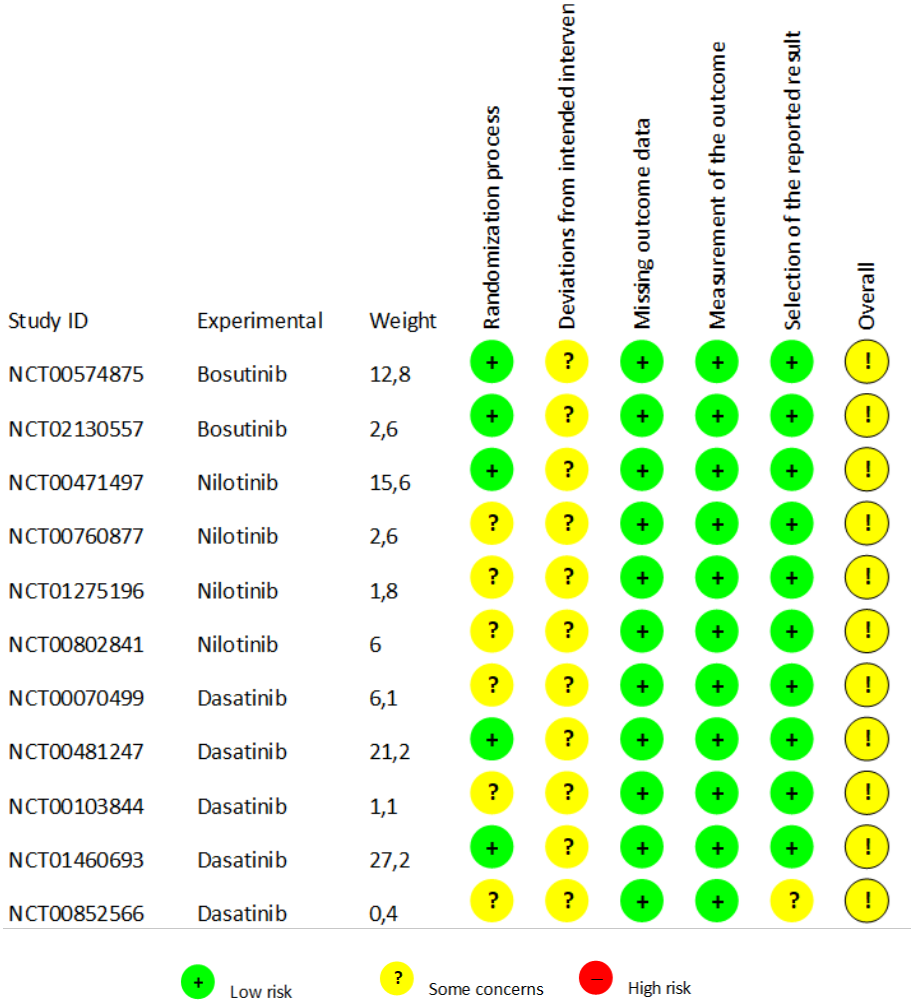
Imatinib OR ponatinib OR nilotinib OR bosutinib OR dasatinib OR radotinib *(86 abstracts)*

Method S2: List of terms considered as "arterial occlusive events" and "venous thromboembolism".

Arterial occlusive events	Venous thromboembolism
acute coronary syndrome	deep vein thrombosis
acute myocardial infarction	intermittent claudication
angina pectoris	phlebitis
angina unstable	retinal vein occlusion
aortic stenosis	retinal vein thrombosis
arterial occlusive disease	thrombophlebitis
arterial stenosis limb	varicose vein
arteriosclerosis	venous thrombosis limb
arteriosclerosis coronary artery	vasculitis
atrial thrombosis	thrombocytopenic purpura
basilar artery stenosis	pulmonary embolism
cardiogenic shock	
cardio-respiratory arrest	
cerebral infarction	
cerebrovascular accident	
cerebrovascular disorder	
chest discomfort	
chest pain	
coronary artery disease	
coronary artery occlusion	
coronary artery stenosis	
electrocardiogram ST-T segment abnormal	
femoral arterial stenosis	
iliac artery occlusion	
iliac artery stenosis	
ischaemic stroke	
myocardial ischaemia	
peripheral artery occlusive disease	
peripheral artery thrombosis	
sudden death	
transient ischemic attack	
vena cava thrombosis	
cerebrovascular ischemia	
cardiac ischemia/infarction	
cardiac arrest	
myocardial infarction	

Figure 2.4 | **Risk of bias summary.** This figure summarizes review authors' judgements about each risk of bias item for each included study. (A). Overall survival (B). Arterial occlusive event (C). MMR at 12 months (D). MMR at 24 months (E). CCyR at 12 months (F). Venous thromboembolism.

(A). Overall survival



(B). Arterial occlusive event

Study ID	Experimental	Weight	Randomization process	Deviations from intended interven	Missing outcome data	Measurement of the outcome	Selection of the reported result	Overall
NCT01650805	Ponatinib	6,6	+	?	+	+	?	!
NCT00574873	Bosutinib	3,9	+	?	+	+	?	!
NCT02130557	Bosutinib	14,3	+	?	+	?	–	–
NCT00471497	Nilotinib	25,8	+	?	+	+	?	!
NCT00760877	Nilotinib	8,1	?	?	+	+	–	–
NCT01275196	Nilotinib	0,5	?	?	+	?	–	–
NCT00802841	Nilotinib	2,4	?	?	+	+	–	–
NCT00070499	Dasatinib	5,6	?	?	+	?	?	!
NCT00481247	Dasatinib	22,9	+	?	+	+	?	!
NCT00103844	Dasatinib	3,3	?	?	+	+	?	!
NCT00320190	Dasatinib	1,3	?	?	+	+	?	!
NCT01460693	Dasatinib	4,8	+	?	+	?	?	!
NCT00852566	Dasatinib	0,5	?	?	+	+	?	!

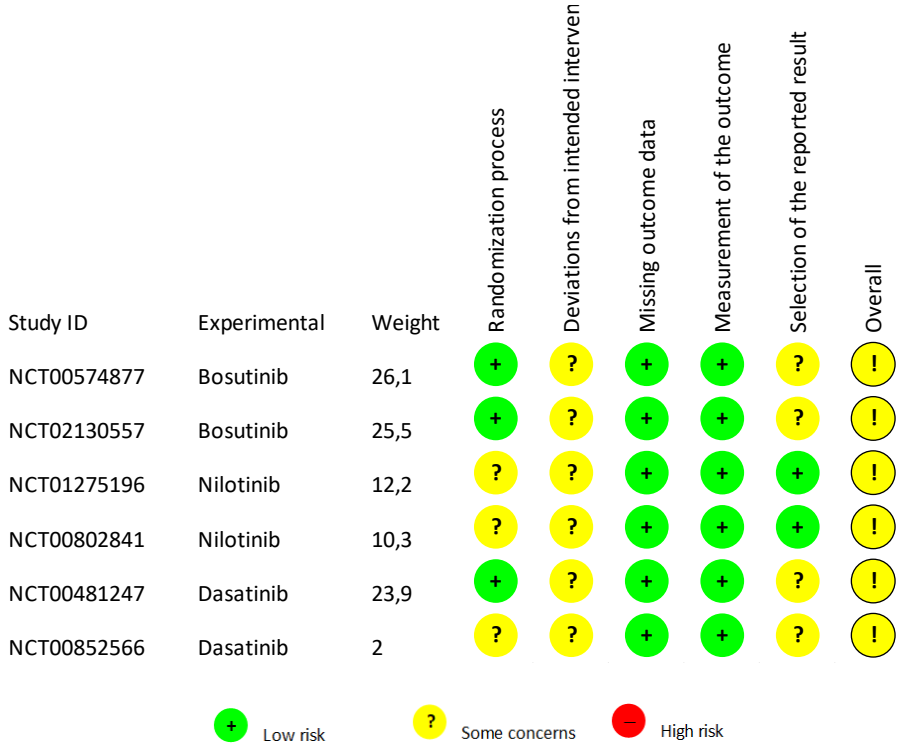
+ Low risk ? Some concerns – High risk

(C). MMR at 12 months

Study ID	Experimental	Weight	Randomization process	Deviations from intended interven	Missing outcome data	Measurement of the outcome	Selection of the reported result	Overall
NCT00574876	Bosutinib	11,8	+	?	+	+	?	!
NCT02130557	Bosutinib	15,4	+	?	+	+	+	!
NCT00471497	Nilotinib	14,2	+	?	+	+	+	!
NCT00760877	Nilotinib	0,7	?	?	+	+	?	!
NCT01275196	Nilotinib	5,3	?	?	+	+	+	!
NCT00802841	Nilotinib	4,6	?	?	+	+	+	!
NCT01400074	Nilotinib	1,5	?	?	+	+	?	!
NCT00070499	Dasatinib	4,2	?	?	+	+	+	!
NCT00481247	Dasatinib	12,1	+	?	+	+	?	!
NCT01460693	Dasatinib	21,6	+	?	+	+	?	!
NCT01593254	Dasatinib	3,1	?	?	+	+	+	!
NCT00852566	Dasatinib	0,6	?	?	+	+	+	!

+ Low risk ? Some concerns – High risk

(D). MMR at 24 months



(E). CCyR at 12 months

Study ID	Experimental	Weight	Randomization process	Deviations from intended interven	Missing outcome data	Measurement of the outcome	Selection of the reported result	Overall
NCT01650806	Ponatinib	0,1	+	?	+	+	+	!
NCT00574878	Bosutinib	14,7	+	?	+	+	+	!
NCT02130557	Bosutinib	11,6	+	?	+	+	?	!
NCT00471497	Nilotinib	14,7	+	?	+	+	+	!
NCT01275196	Nilotinib	7,9	?	?	+	+	?	!
NCT00802841	Nilotinib	7,1	?	?	+	+	+	!
NCT00070499	Dasatinib	2	?	?	+	+	+	!
NCT00481247	Dasatinib	12,6	+	?	+	+	?	!
NCT01460693	Dasatinib	22,8	+	?	+	+	?	!
NCT00852566	Dasatinib	0,1	?	?	+	+	+	!
NCT01511289	Radotinib	6,4	+	?	+	+	?	!

+ Low risk ? Some concerns – High risk

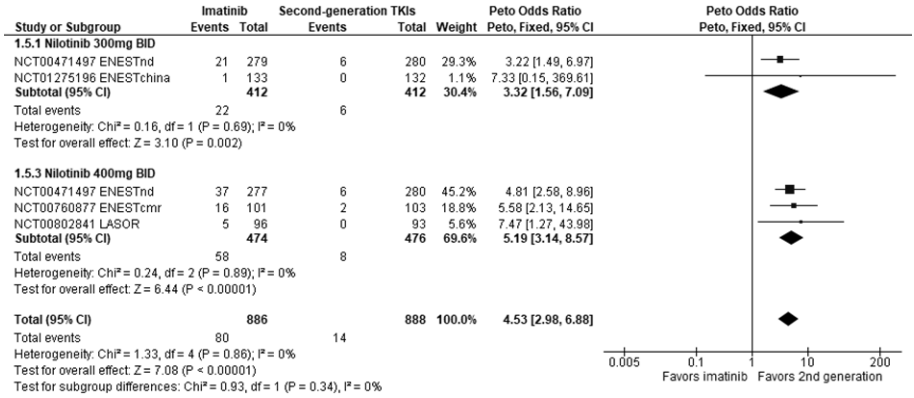
(F). Venous thromboembolism

Study ID	Experimental	Weight	Randomization process	Deviations from intended interven	Missing outcome data	Measurement of the outcome	Selection of the reported result	Overall
NCT01650806	Ponatinib	3,5	+	?	+	+	?	!
NCT02130557	Bosutinib	20,7	+	?	+	?	–	–
NCT00471497	Nilotinib	24,6	+	?	+	+	?	!
NCT00760877	Nilotinib	3,5	?	?	+	+	–	–
NCT01275196	Nilotinib	3,5	?	?	+	?	–	–
NCT00481247	Dasatinib	13,9	+	?	+	+	?	!
NCT00103844	Dasatinib	6,1	?	?	+	+	?	!
NCT01460693	Dasatinib	24,2	+	?	+	?	?	!

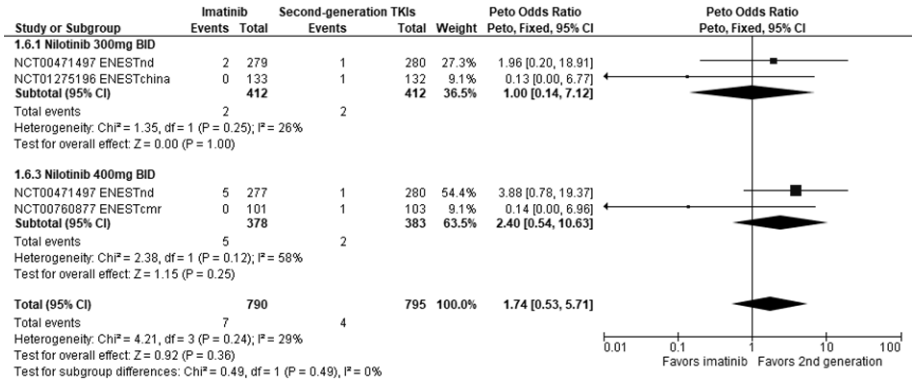
+ Low risk ? Some concerns – High risk

Figure 2.5 | **Sub-analyses by TKI dose.** (A) Forest plot of arterial occlusive events stratified by nilotinib dose. (B) Forest plot of VTE stratified by nilotinib dose. (C) Forest plot of OS stratified by nilotinib dose. (D) Forest plot of MMR at 12 months stratified by nilotinib dose. (E) Forest plot of CCyR at 12 months stratified by nilotinib dose.

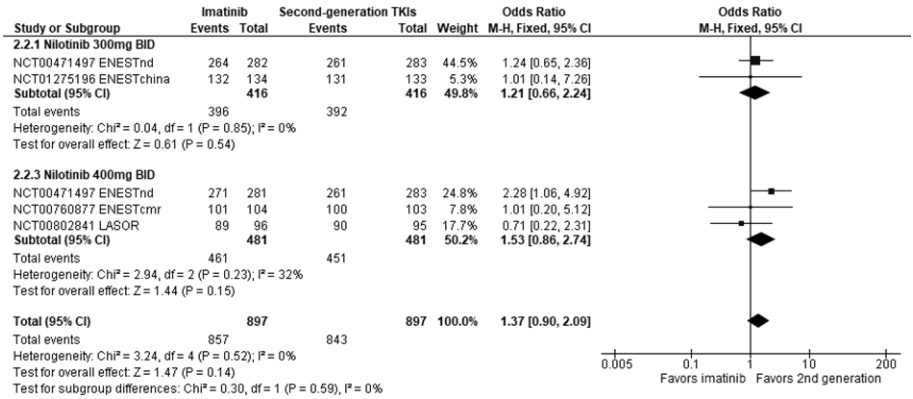
(A) Forest plot of arterial occlusive events stratified by nilotinib dose



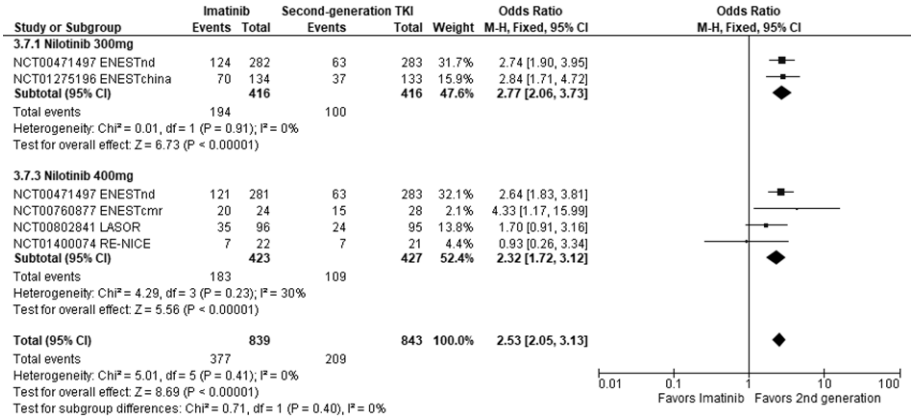
(B) Forest plot of VTE stratified by nilotinib dose



(C) Forest plot of OS stratified by nilotinib dose



(D) Forest plot of MMR at 12 months stratified by nilotinib dose



(E) Forest plot of CCyR at 12 months stratified by nilotinib dose

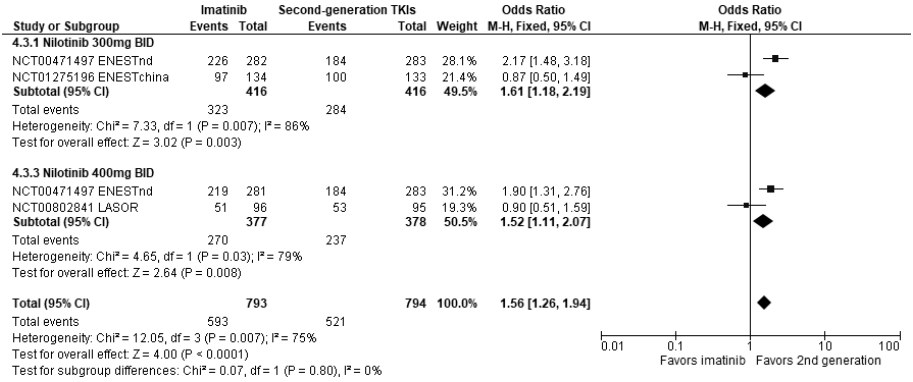
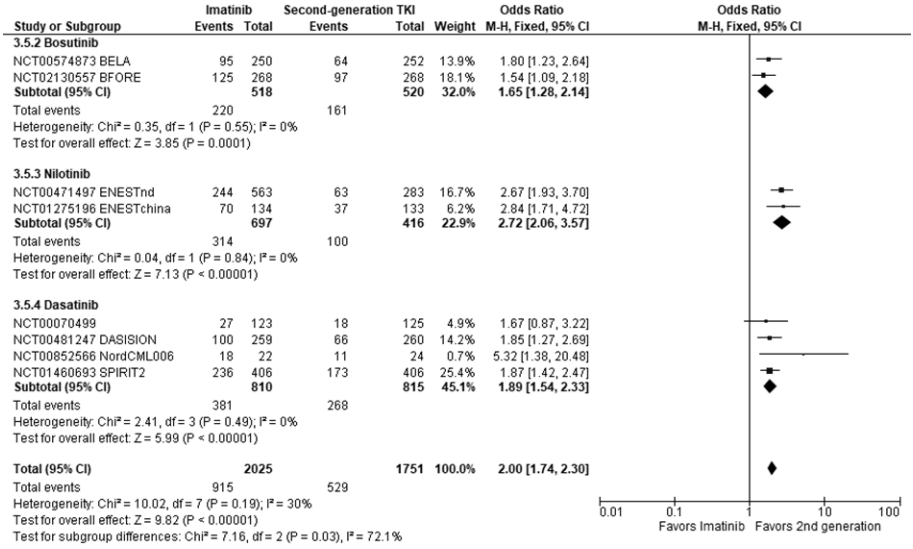
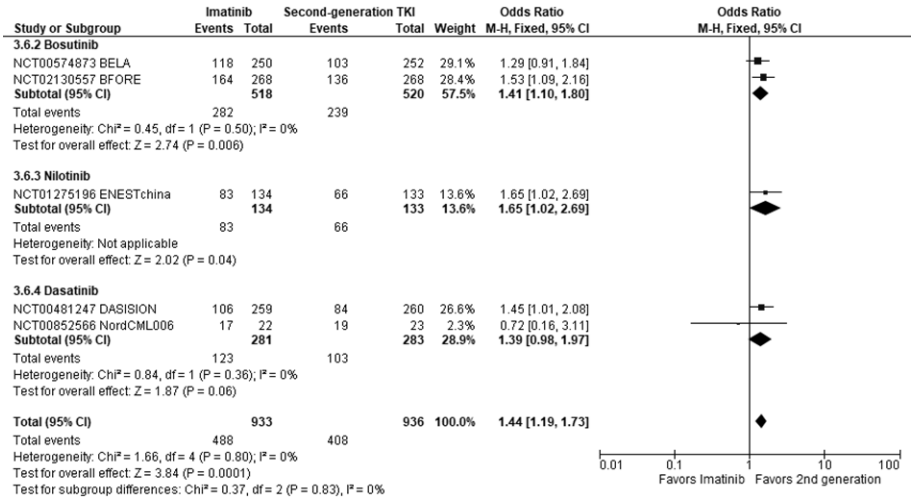


Figure 2.6 | **Sensitivity analysis.** (A) Forest plot of MMR at 12 months in treatment-naïve patients. (B) Forest plot of MMR at 24 months in treatment-naïve patients. (C) Forest plot of CCyR at 12 months in treatment-naïve patients. (D) Forest plot of OS in treatment-naïve patients (E) Forest plot of MMR at 12 months in patients with CML treated with second generation TKIs compared with high-dose imatinib. (F) Forest plot of OS in patients with CML treated with second generation TKIs compared with high-dose imatinib. (G) Forest plot of arterial occlusive events in patients with CML treated with second generation TKIs compared with high-dose imatinib.

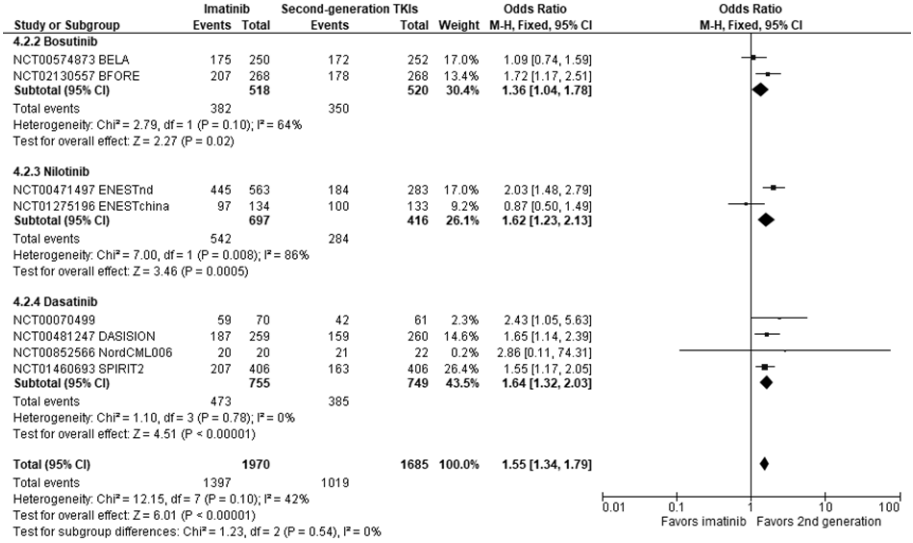
(A) Forest plot of MMR at 12 months in treatment-naïve patients.



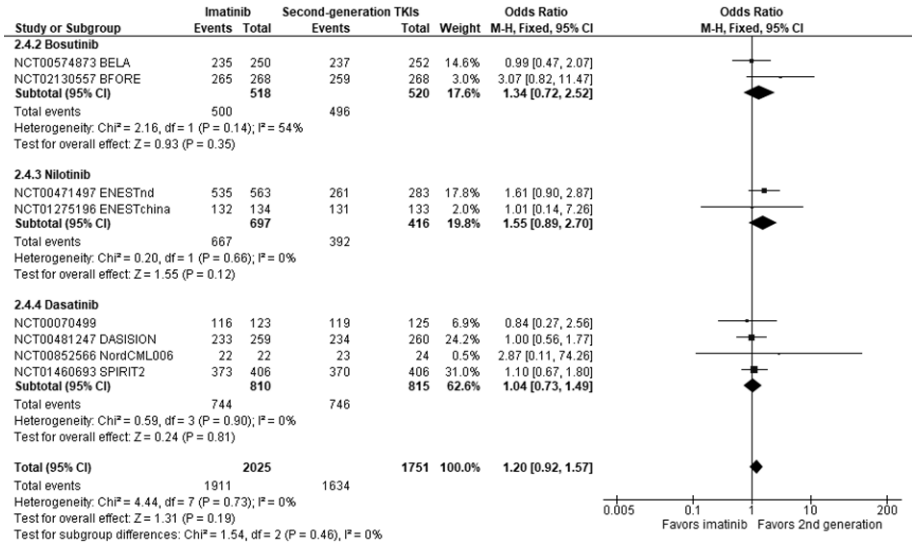
(B) Forest plot of MMR at 24 months in treatment-naïve patients.



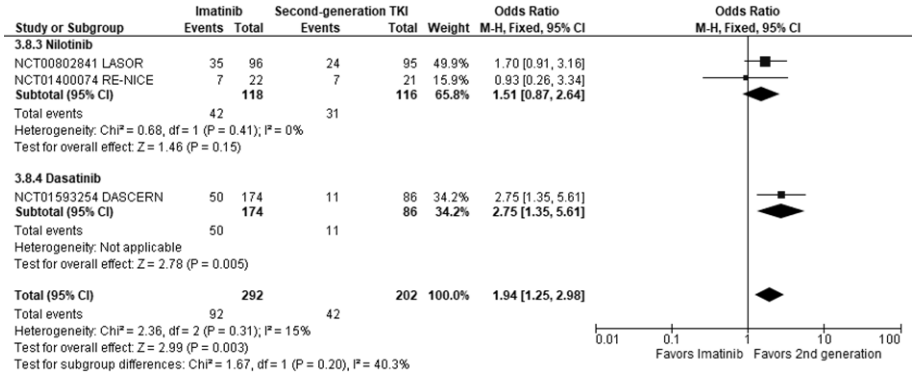
(C) Forest plot of CCyR at 12 months in treatment-naïve patients.



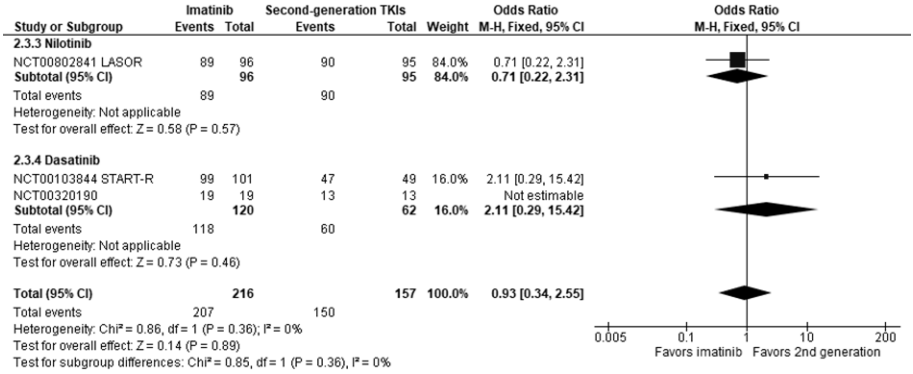
(D) Forest plot of OS in treatment-naïve patients



(E) Forest plot of MMR at 12 months in patients with CML treated with second generation TKIs compared with high-dose imatinib.



(F) Forest plot of OS in patients with CML treated with second generation TKIs compared with high-dose imatinib.



(G) Forest plot of arterial occlusive events in patients with CML treated with second generation TKIs compared with high-dose imatinib.

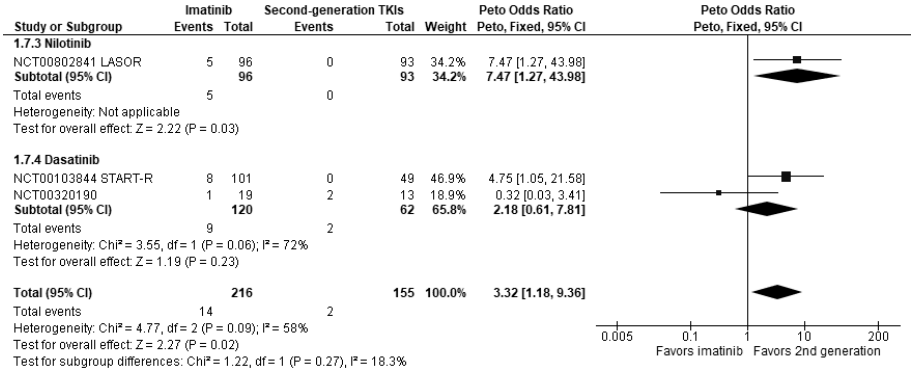
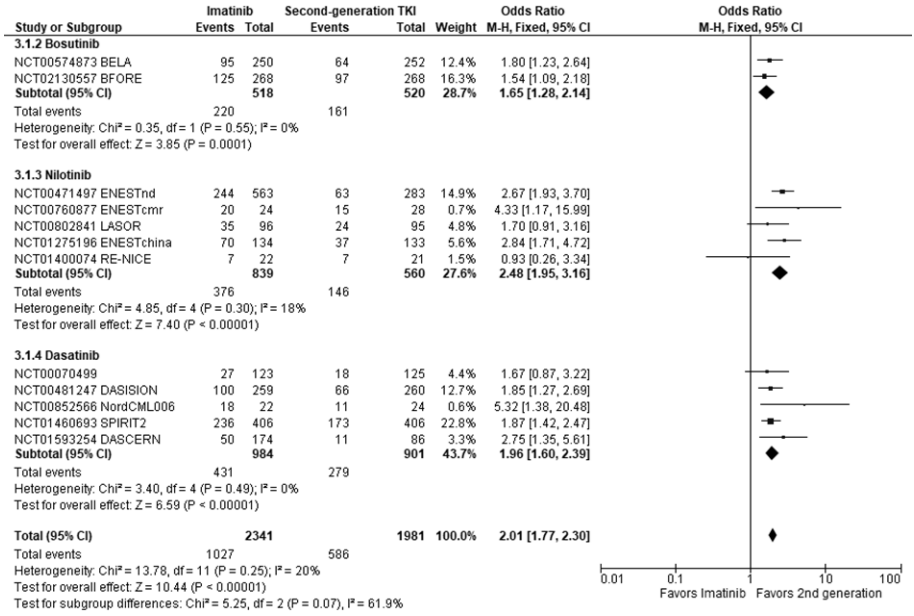
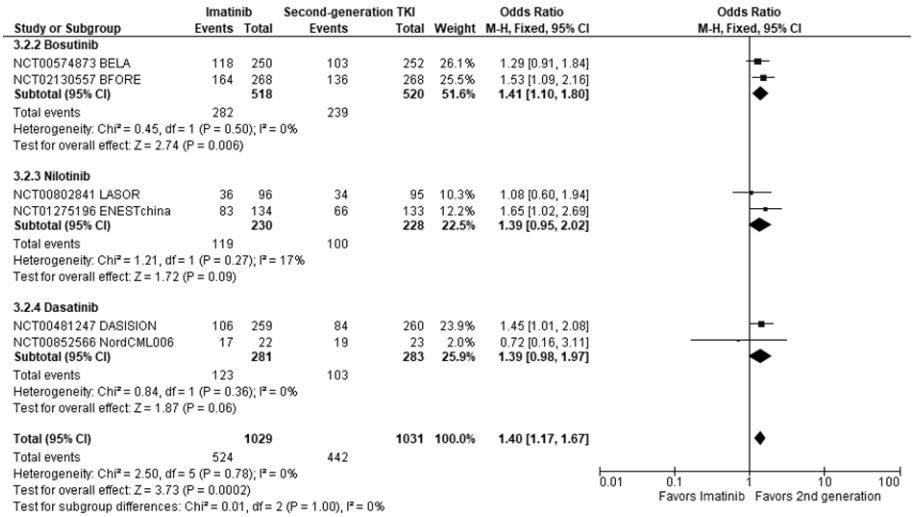


Figure 2.7 | **Forest plots of MMR, CCyR and venous thromboembolism in patients with CML treated with second generation TKIs compared with imatinib.** (A) Forest plot of MMR at 12 months. (B) Forest plot of MMR at 24 months. *(*C) Forest plot of CCyR at 12 months. (D) Forest plot of venous thromboembolism.

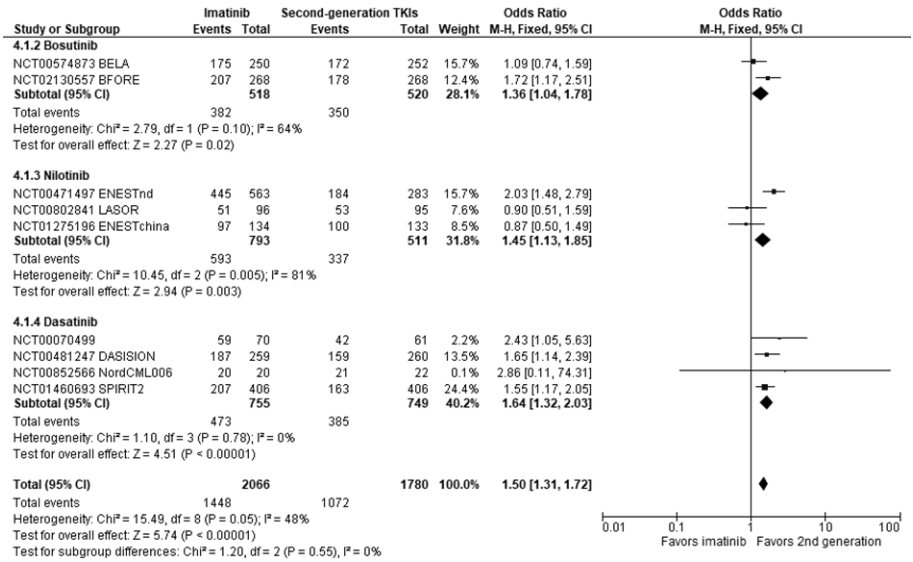
(A) Forest plot of MMR at 12 months.



(B) Forest plot of MMR at 24 months.



(C) Forest plot of CCyR at 12 months.



(D) Forest plot of venous thromboembolism.

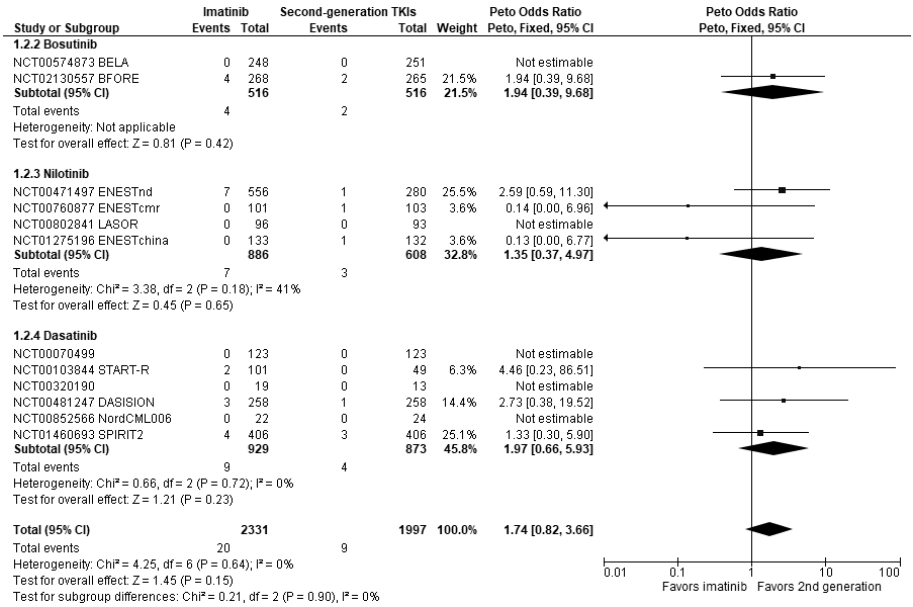
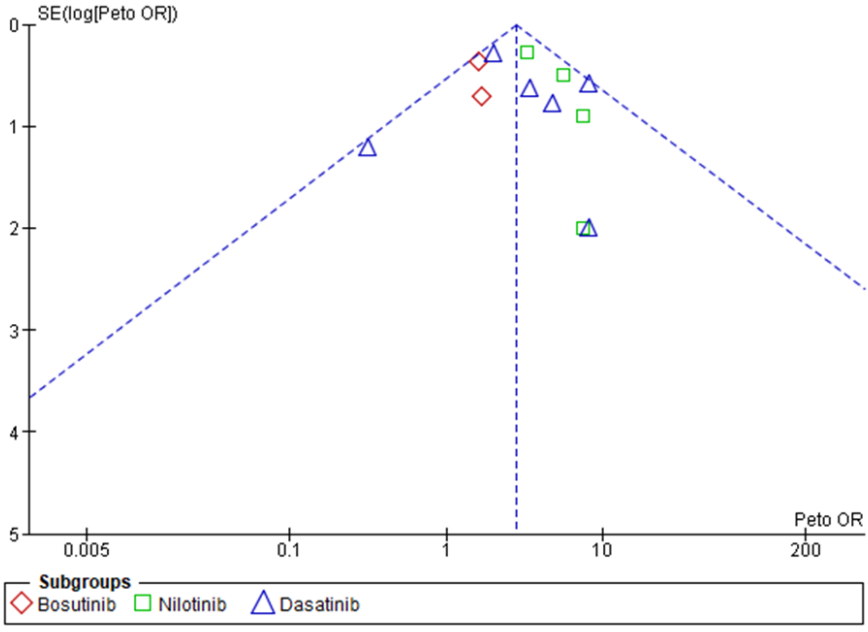
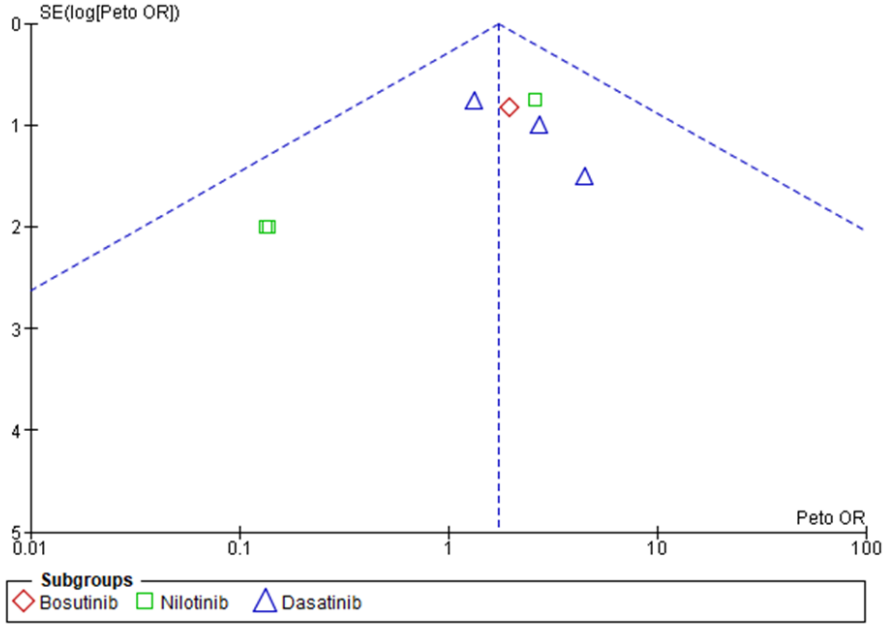


Figure 2.8 | **Publication bias assessment.** (A) Funnel plot of arterial occlusive events analysis. (B) Funnel plot of VTE analysis. (C) Funnel plot of OS analysis. (D) Funnel plot of MMR at 12 months analysis (E) Funnel plot of MMR at 24 months analysis (F) Funnel plot of CCyR at 12 months analysis.

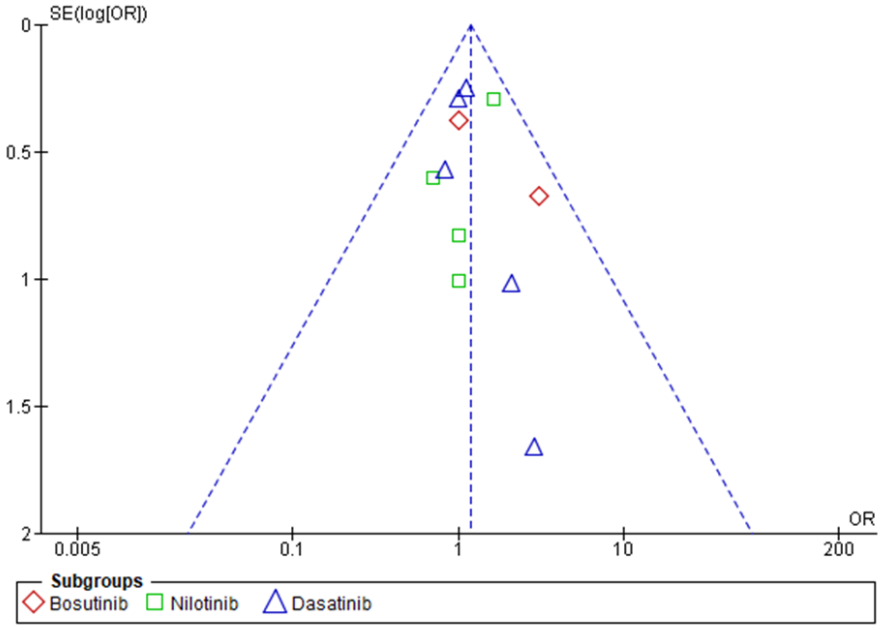
(A) Funnel plot of arterial occlusive events analysis. Egger's test for a regression intercept gave a *p*-value of 0.489 indicating no evidence of publication bias.



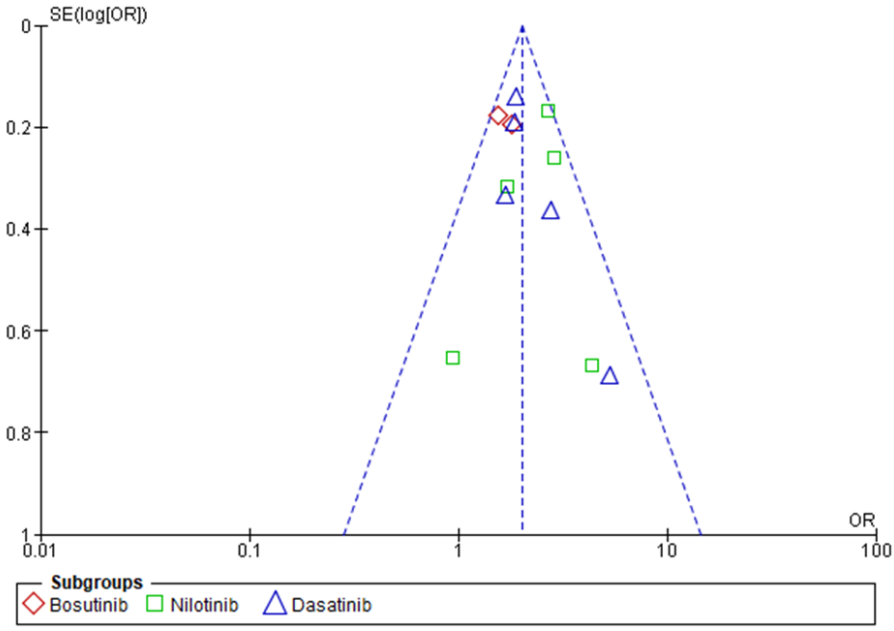
(B) Funnel plot of VTE analysis. Egger's test was not performed for this funnel plot as less than 10 studies was included in the VTE analysis.



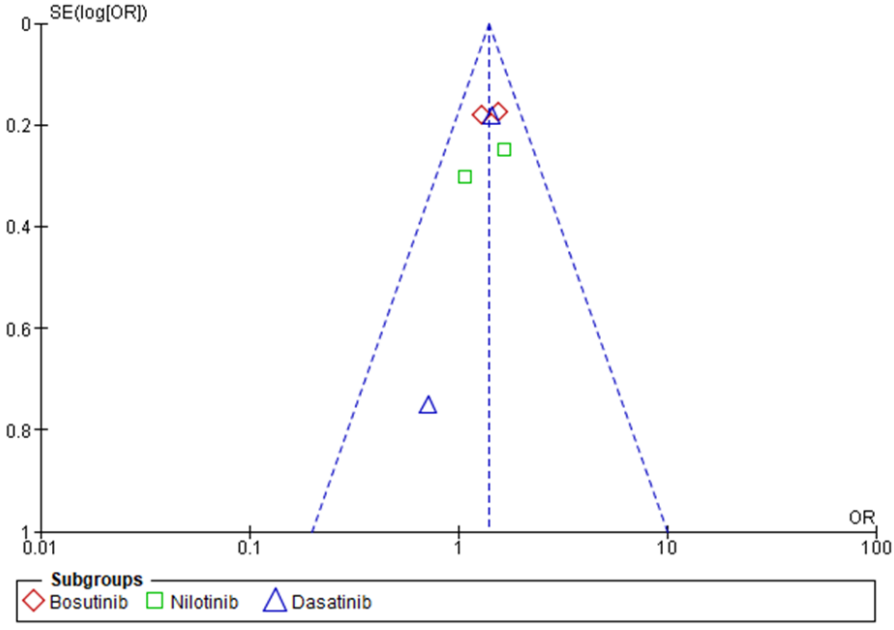
(C) Funnel plot of OS analysis. Egger's test for a regression intercept gave a *p*-value of 0.470 indicating no evidence of publication bias.



(D) Funnel plot of MMR at 12 months analysis. Egger's test for a regression intercept gave a *p*-value of 0.583 indicating no evidence of publication bias.



(E) Funnel plot of MMR at 24 months analysis. Egger's test was not performed for this funnel plot as less than 10 studies was included in the MMR at 24 months analysis.



(F) Funnel plot of CCyR at 12 months analysis. Egger's test was not performed for this funnel plot as less than 10 studies was included in the CCyR at 12 months analysis.

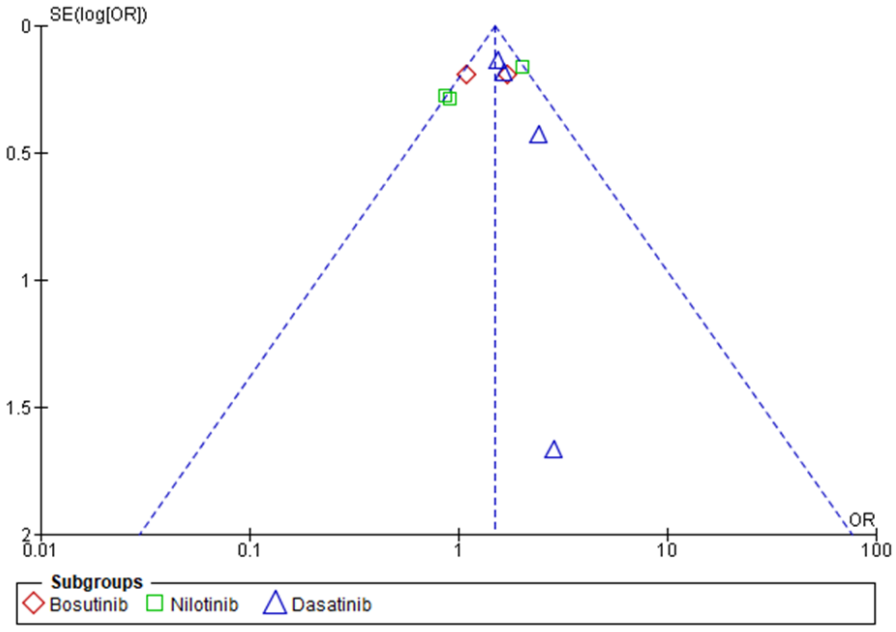


Table 2.3 | Main characteristics of the 14 included clinical trials.

NCT Study Name	Study Design	NG-TKI Dosage Frequency	Imatinib Dosage Frequency	Nb of Pts (ITT)	Population	Age (Mean ± SD)	Sex (% Male)	Primary Endpoint	Secondary Endpoint(s)
NCT00574873 **BELA**	randomized open-label	Bosutinib 500 mg QD	Imatinib 400 mg QD	502	newly diagnosed CP-CML	46.5 ± 14.61	56.6%	CCyR at 12 months	MMR at 12 months
NCT02130557 **BFORE**	randomized open-label	Bosutinib 400 mg QD	Imatinib 400 mg QD	536	newly diagnosed CP-CML	53.0	58.0%	MMR at 12 months	MMR by 18 months CCyR by 12 months OS at 12 months
NCT00760877 **ENESTcmr**	randomized open-label	Nilotinib 400 mg BID	Imatinib 400 mg QD Imatinib 600 mg QD	207	CP-CML previously treated with imatinib for at least 2 years	49.1 ± 13.16	65.7%	Rate of best CMR	OS
NCT00471497 **ENESTnd**	randomized open-label	Nilotinib 300 mg BID Nilotinib 400 mg BID	Imatinib 400 mg QD	846	newly diagnosed CP-CML	46.7	58.0%	MMR at 12 months	Durable MMR at 24 months CCyR at 12 months
NCT01275196 **ENESTchina**	randomized open-label	Nilotinib 300 mg BID	Imatinib 400 mg QD	267	newly diagnosed CP-CML Chinese patients	40.6 ± 12.82	64.4%	MMR at 12 months	MMR rate at 3, 6, 9, 12, 15, 18, 21, 24 and 36 months OS
NCT01400074 **RE-NICE**	randomized open-label	Nilotinib 400 mg BID	Imatinib 400 mg BID	43	CP-CML with suboptimal response to imatinib at a minimum dose of 400 mg daily	40.1	74.4%	Cumulative rate of MMR at 12 months	Safety analyses

NCT00802841 LASOR	randomized open-label	Nilotinib 400 mg BID	Imatinib 600 mg QD	191	CP-CML with suboptimal response to imatinib standard dose	44.4 ± 14.75	58.6%	CCyR at 6 months	MMR at 12 and 24 months CCyR at 12 and 24 months OS
NCT00070499	randomized open-label	Dasatinib 100 mg QD	Imatinib 400 mg QD	248	newly diagnosed CP-CML	48.5	59.8%	MMR rate at 12 months	2-year OS Toxicity
NCT00320190	randomized open-label	Dasatinib 100 mg QD	Imatinib 400 mg BID	32	CP-CML with suboptimal response after imatinib 400 mg daily	48.6 ± 14.85	71.9%	MMR rate at 12 months	Death, AEs, treatment-related AEs, SAEs, treatment-related SAEs and AEs leading to discontinuation On-study AEs of special interest CCyR at 6 and 12 months
NCT00103844 START-R	randomized open-label	Dasatinib 70 mg BID	Imatinib 400 mg BID	150	CP-CML resistant to imatinib at 400–600 mg daily	51 ± 13.6	50.0%	MCyR at 12 weeks	MMR CCyR after crossover AEs, SAEs, deaths and hematologic toxicities
NCT01460693 SPIRIT2	randomized open-label	Dasatinib 100 mg QD	Imatinib 400 mg QD	812	newly diagnosed CP-CML	54.4	55.6%	5-year EFS	MMR
NCT00481247 DASISION	randomized open-label	Dasatinib 100 mg QD	Imatinib 400 mg QD	519	newly diagnosed CP-CML	46.7 ± 14.2	59.2%	Best confirmed CCyR within 12 months	Participants remaining in cCCyR at 2, 3, 4 and 5 years MMR at any time OS
NCT01593254 DASCERN	randomized open-label	Dasatinib 100 mg QD	Imatinib 400 mg or more QD	260	CP-CML without optimal response to imatinib 400 mg	37.0	78.0%	MMR at 12 months of CML treatment	OS Safety profile Cyotgenetic response over time
NCT00852566 NordCML006	randomized open-label	Dasatinib 100 mg QD	Imatinib 400 mg QD	46	newly diagnosed CP-CML	55.6	47.8%	Ph-positive cells in stem cell compartments at 6 months	Molecular and cytogenetic responses at 3, 6, 12 and 18 months

NCT Study Name	Key Inclusion Criteria	Key Exclusion Criteria
NCT00574873 BELA	- Cytogenetic diagnosis of CP Ph+ CML diagnosed less than 6 months. - Diagnosis of CML chronic phase confirmed. - Adequate hepatic and renal function.	- Philadelphia negative CML. - Prior anti-leukemia treatment. - Prior stem cell transplant.
NCT02130557 BFORE	- Molecular diagnosis of CP CML of ≤ 6 months. - Adequate hepatic, renal and pancreatic function.	- Any prior medical treatment for CML, including TKIs, with the exception of hydroxyurea and/or anagrelide treatment - Any past or current Central Nervous System involvement. - Extramedullary disease only. - Major surgery or radiotherapy within 14 days of randomization. - History of clinically significant or uncontrolled cardiac disease. - History of another malignancy within 5 years (exception accepted)
NCT00760877 ENESTcmr	- Diagnosis of CML associated with BCR-ABL quantifiable by RQ-PCR - Documented CCyR by bone marrow or BCR-ABL < 1% IS in the past 12 months - Persistent disease demonstrated by 2 PCR positive tests 3 months apart both during the past 6 months. - Treatment with imatinib for at least 2 years with 400 mg or 600 mg and a stable dose - No other current or planned anti-leukemia therapies	- Evidence of rising PCR - Treatment with another investigational agent within last 6 months or TKIs other than imatinib - Prior allogeneic stem cell transplantation - Impaired cardiac function including: inability to monitor the QT interval on electrocardiogram, long QT syndrome or a known family history of long QT syndrome, clinically significant resting brachycardia (< 50 beats per minute), QTc > 450 msec on baseline ECG, other clinically significant uncontrolled heart disease (e.g., unstable angina, congestive heart failure or uncontrolled hypertension), history of or presence of clinically significant ventricular or atrial tachyarrhythmias - Administration of cytokine therapy (e.g., G-CSF, GM-CSF or SCF) within 4 weeks prior to study entry

NCT00471497 ENESTnd	- CML in CP patients within the first 6 months of diagnosis. - Diagnosis of CML in CP with confirmation of Philadelphia chromosome of (9:22) translocations	- Previously documented T315I mutation - Treatment with a TKI prior to study entry - Any medical treatment for CML with the exception of hydroxyurea and/or anagrelide - Impaired cardiac function. - Severe or uncontrolled medical conditions (i.e., uncontrolled diabetes, active or uncontrolled infection). - Use of therapeutic coumarin derivatives (i.e., warfarin, acenocoumarol, phenprocoumon) - Currently receiving treatment with any medications that have the potential to prolong the QT interval.
NCT01275196 ENESTchina	- Patients of Chinese ethnicity - Patients with CML-CP (Ph+) within 6 months of diagnosis - No evidence of extramedullary leukemic involvement, with the exception of hepatosplenomegaly - Adequate organ function	- Previously documented T315I mutations. - Treatment with TKIs prior to study entry - Treatment with IFN for more than 3 months. - Impaired cardiac function including any one of the following: complete left bundle branch block, long QT syndrome or a known family history of long QT syndrome, history of or presence of clinically significant ventricular or atrial tachyarrhythmias, clinically significant resting bradycardia (< 50 beats per minute), QTc > 450 msec, history of clinically documented myocardial infarction within past 12 months, history of unstable angina during the last 12 months, other clinically significant heart disease (e.g., congestive heart failure or uncontrolled hypertension). - Severe or uncontrolled medical conditions (i.e., uncontrolled diabetes, active or uncontrolled infection). - History of significant congenital or acquired bleeding disorder unrelated to cancer. - Major surgery within 4 weeks. - Treatment with other investigational agents within 30 days. - Another primary malignancy except if the other primary malignancy is neither currently clinically significant or requiring active intervention. - Acute or chronic liver, pancreatic or severe renal disease considered unrelated to disease. - Current intake of any medications that have the potential to prolong the QT interval.

NCT00802841 LASOR	- Ph+ CML in CP - No evidence of extramedullary leukemia involvement, with the exception of hepatosplenomegaly - Suboptimal response to 400 mg imatinib	- Prior AP or BC CML. - Prior therapy with imatinib in combination with any other CML drug other than Hydroxyurea and/or Anagrelide. - Imatinib therapy started more than 12 months after the date of the original diagnosis. - Unable to tolerate imatinib at 400 mg. - Previous treatment with any other TKI except Glivec and/or CML therapy other than IFN, hydroxyurea, and /or anagrelide; - Myelotoxicity ≥ Grade 2 present at the time of randomization, - Previously documented T315I mutations. - Impaired cardiac function including: long QT syndrome or family history of long QT syndrome, clinically significant resting brachycardia (< 50 bpm), QTcF >450 msec on screening ECG, myocardial infarction ≤ 12 months prior to the first dose of study drug, other clinically significant heart disease (e.g., CHF, uncontrolled hypertension, unstable angina, significant ventricular or atrial tachyarrhythmias). - Currently receiving treatment with any medications that have the potential to prolong the QT interval. - History of another primary malignancy that is currently clinically significant or currently requires active intervention. - Any other clinically significant medical or surgical condition which, according to investigators' discretion, should preclude participation. - Use of investigational agent within 28 days prior to enrollment.

NCT01400074 **RE-NICE**	- Diagnosis of Ph+ CML in CP - Patients with suboptimal molecular response	- Late CP who started imatinib more than 6 months after diagnosis. - Prior AP or BP CML. - Previously documented T315I mutations. - Intolerance to imatinib 400 mg daily. - Patients treated with imatinib more than 400 mg daily. - Achieved prior MMR or CCyR on imatinib and lost response to entering the study. - Previous treatment with interferon or any other TKI except imatinib (allow hydroxyurea or anagrelide). - Impaired cardiac function. - Treatment with inhibitors of CYP3A4 or medications well documented to prolong the QT interval are contraindicated. - Any other malignancy that is clinically significant or requires active intervention. - Severe or uncontrolled medical conditions. - History of significant congenital or acquired bleeding disorder unrelated to cancer. - Previous radiotherapy to ≥ 25% of the bone marrow. - Major surgery within 4 weeks or who have not recovered from prior surgery. - Treatment with other investigational agents within 30 days.
NCT00070499	- CML in CP - Registration on this study within 180 days after the date of first being diagnosed with CML. - With an electrocardiogram within 42 days, and without any of the following cardiac symptoms: uncontrolled angina, congestive heart failure or myocardial infarction within 6 months, diagnosed or suspected congenital long QT syndrome, history of clinically significant ventricular arrhythmias, prolonged corrected QT interval or uncontrolled hypertension	- Prior treatment for CML with the exception of hydroxyurea and/or anagrelide. - Any prior chemotherapy regimen for peripheral blood stem cell mobilization. - Major surgery within 28 days before registration, or without having fully recovered from any other prior major surgery. - Other prior malignancy (exceptions are allowed). - History of significant bleeding disorder unrelated to cancer, including congenital bleeding disorders and acquired bleeding disorder within one year.

NCT00320190	- CP Ph+ CML demonstrating only a suboptimal response	- Previous diagnosis of AP or BC CML - Uncontrolled or significant cardiovascular disease - History of significant bleeding disorder unrelated to CML - Concurrent malignancies - Intolerance of imatinib 400 mg - Prior treatment with imatinib at a dose higher than 400 mg - Prior stem cell transplantation and/or high-dose chemotherapy for CML
NCT00103844 START-R	- Subjects with CP Ph+ CML. - Subjects have not been treated with imatinib at a dose >600 mg/day. - Subjects developed resistance to disease while receiving an imatinib dose 400–600 mg/day. - Able to tolerate imatinib at the highest dose the subject had received in the past. - Adequate renal and hepatic function.	- Prior treatment with imatinib at a dose >600 mg/day. - Subjects who have previously identified specific BCR-ABL mutations. - Previous diagnosis of AP or BC CML. - Intolerance to imatinib at any dose. - Subjects who are eligible and willing to undergo transplantation during the screening period. - Serious uncontrolled medical disorder or active infection. - Uncontrolled or significant cardiovascular disease. - Uncontrolled hypertension. - Evidence of organ dysfunction. - Use of imatinib within 7 days. - Use of interferon or cytarabine within 14 days. - Use of a targeted small molecule anticancer agent within 14 days. - Subjects taking certain medications that are accepted to have a risk of causing Torsades de Pointes. - Subjects taking medications that irreversibly inhibit platelet function or anticoagulants. - Prior therapy with BMS-354825.

NCT01460693 SPIRIT2	- Enrolled within 3 months of initial diagnosis of CML-CP - Cytogenetic confirmation of the Philadelphia chromosome or variants of (9;22) translocations - No evidence of extramedullary leukaemic involvement, with the exception of the hepatosplenomegaly.	- Patients with Ph-negative, BCR-ABL-positive disease. - Any prior treatment for CML with: any TKI; busulphan; interferon-alpha; homoharringtonine; cytosine arabinoside; any other investigational agents (hydroxycarbamide and anagrelide are the only drugs permitted). - Patients who received prior chemotherapy. - Patient who have had any form of prior haemopoietic stem cell transplant, either autograft or allograft. - Patients with International normalized ratio (INR) or partial thromboplastin time (PTT) > 1.5 x IULN, with the exception of patients on treatment with oral anticoagulants. - Patients with uncontrolled medical disease such as diabetes mellitus, thyroid dysfunction, neuropsychiatric disorders, infection, angina, or Grade 3/4 cardiac problems as defined by the New York Heart Association Criteria. - Patients who have undergone major surgery within 4 weeks, or who have not recovered from prior major surgery. - Patients with a history of another malignancy either currently or within the past five years (exception).
NCT00481247 DASISION	- CP Philadelphia Chromosome-positive CML	- Pleural Effusion - Uncontrolled cardiovascular disease - Significant bleeding disorder unrelated to CML - Prior treatment with interferon/imatinib/dasatinib/anti-CML systemic treatments except anagrelide/hydroxyurea
NCT01593254 DASCERN	- CP-CML Ph+ patients with complete hematologic response (CHR) but without one log BCR-ABL reduction (BCR-ABL level >10% IS) 3 months of imatinib 400 mg treatment. - Currently tolerating imatinib 400 mg QD. - Adequate renal function - Adequate hepatic function	- Previous diagnosis of AP or BC - Subjects with clonal evolution in Ph+ cells observed in ≥2 metaphases at baseline bone marrow cytogenetic test, unless the same abnormalities were present at diagnosis. - Subjects with less than CHR after 3 months of imatinib treatment or lost CHR after initial achievement - Documented T315I/A, F317L, or V299L mutations - A serious uncontrolled medical disorder or active infection that would impair the ability of the subject to receive protocol therapy

NCT00852566 NordCML006	- CML in CP - No evidence of extramedullary leukemia apart from hepatosplenomegaly - Ph+ or variants must be demonstrated by BM cytogenetics, FISH or PCR. - Previously untreated CML in CP, with the exception of hydroxyurea or anagrelide - Enrolled in this study within 90 days after the date of first being diagnosed with CML - Adequate hepatic function - Adequate renal function	- A serious uncontrolled medical disorder or active infection that would impair the ability of the subject to receive protocol therapy. - Known pleural effusion at baseline. - Uncontrolled or significant cardiovascular disease - History of significant bleeding disorder unrelated to CML. - Prior chemotherapy for peripheral stem cell mobilization. - Prior or concurrent malignancy. - Any prior treatment with interferon, dasatinib or imatinib - Any other prior systemic treatments, with anti-CML activity [except for anagrelide, or hydroxyurea (HU)]. - Current uptake of drugs that are generally accepted to have a risk of causing Torsades de Pointes.

Abbreviations: AE: adverse event; AP: acute phase; BP: blast phase; CCyR: complete cytogenetic response; CML: chronic myeloid leukemia; CP: chronic phase; ITT: intention-to-treat; MMR: major molecular response; OS: overall survival; Ph+: Philadelphia positive; SAE: serious adverse event; SD: standard deviation; TKI: tyrosine kinase inhibitors.

Table 2.4 | PRISMA checklist.

Section/Topic	#	Checklist Item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	21
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	21
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	22-23
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	23
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	24
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	24
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	24

Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	Method S1
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	24
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	24-25
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	24
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	26
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	25
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I2) for each meta-analysis.	25
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	26
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	25-26
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	26 Figure 2.1

Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	Table 2.3
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	32 Figure 2.4
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	27-32 Figures 2.2; 2.3 and 2.6
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	27-32
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	27-32 Figure 2.8
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	27-32 Figure 2.7
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	36-40
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	41
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	42
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	42

Table 2.5 | Detailed statistics in Figure 2.6
(A). Table Detailed statistics for MMR at 12 months.

BCR-ABL TKI **№ of Participants** (Studies)	**Odds Ratio (95% CI)**	**Anticipated Absolute Effects (95% CI)**		
		Risk with Imatinib	Risk with 2nd Generation TKI	Risk Difference with 2nd Generation TKI
All NG-TKIs **№ of Participants: 4322** (12 Studies)	**OR 2.01 (1.77 to 2.30)**	**29.6%**	**45.8% (42.6 to 49.1)**	**+16.2% (+13.1 to +19.6)**
Bosutinib **№ of Participants: 1038** (2 Studies)	OR 1.65 (1.28 to 2.14)	31.0%	42.5% (36.5 to 49)	+11.6% (+5.5 to +18)
Nilotinib **№ of Participants: 1399** (5 Studies)	OR 2.48 (1.95 to 3.16)	26.1%	46.7% (40.7 to 52.7)	+20.6% (+14.7 to +26.6)
Dasatinib **№ of Participants: 1885** (5 Studies)	OR 1.96 (1.60 to 2.39)	31.0%	46.8% (41.8 to 51.7)	15.8% more (+10.8 to +20.8)

(B). Detailed statistics for MMR at 24 months.

BCR-ABL TKI **№ of Participants** (Studies)	**Odds Ratio (95% CI)**	**Anticipated Absolute Effects (95% CI)**		
		Risk with Imatinib	Risk with 2nd Generation TKI	Risk Difference with 2nd Generation TKI
All NG-TKIs **№ of Participants: 2060** (6 Studies)	**OR 1.40 (1.17 to 1.67)**	**42.9%**	**51.2% (46.8 to 55.6)**	**+8.4% (+3.9 to +12.7)**
Bosutinib **№ of Participants: 1038** (2 Studies)	OR 1.41 (1.10 to 1.80)	46.0%	54.5% (48.3 to 60.5)	+8.6% (+2.4 to +14.5)
Nilotinib **№ of Participants: 458** (2 Studies)	OR 1.39 (0.95 to 2.02)	43.9%	52.1% (42.6 to 61.2)	+8.2% (−1.3 to +17.4)
Dasatinib **№ of Participants: 564** (2 Studies)	OR 1.39 (0.98 to 1.97)	36.4%	44.3% (35.9 to 53)	+7.9% (−0.5 to +16.6)

(C). Detailed statistics for CCyR at 12 months.

BCR-ABL TKI **№ of Participants** (Studies)	**Odds Ratio (95% CI)**	**Anticipated Absolute Effects (95% CI)**		
		Risk with Imatinib	Risk with 2nd Generation TKI	Risk Difference with 2nd Generation TKI
All NG-TKIs **№ of Participants: 3846** (9 Studies)	**OR 1.50 (1.31 to 1.72)**	**60.2%**	**69.4% (66.5 to 72.3)**	**+9.2% (+6.3 to +12)**
Bosutinib **№ of Participants: 1038** (2 Studies)	OR 1.36 (1.04 to 1.78)	67.3%	73.7% (68.2 to 78.6)	+6.4% (+0.9 to +11.3)
Nilotinib **№ of Participants: 1304** (3 Studies)	OR 1.45 (1.13 to 1.85)	65.9%	73.7% (68.6 to 78.2)	+7.8% (+2.7 to +12.2)
Dasatinib **№ of Participants: 1504** (4 Studies)	OR 1.64 (1.32 to 2.03)	51.4%	63.4% (58.3 to 68.2)	+12.0% (+6.9 to +16.8)

(D). Detailed statistics of venous thromboembolism.

BCR-ABL TKI **№ of Participants** (Studies)	**Odds Ratio (95% CI)**	**Anticipated Absolute Effects (95% CI)**		
		Risk with Imatinib	Risk with 2nd Generation TKI	Risk Difference with 2nd Generation TKI
All NG-TKIs **№ of Participants: 4328** (12 Studies)	**OR 1.74 (0.82 to 3.66)**	**0.5%**	**0.8% (0.4 to 1.6)**	**+0.3% (−0.1 to +1.2)**
Bosutinib **№ of Participants: 1032** (2 Studies)	OR 1.94 (0.39 to 9.68)	0.4%	0.7% (0.2 to 3.6)	+0.4% (−0.2 to +3.2)
Nilotinib **№ of Participants: 1494** (4 Studies)	OR 1.35 (0.37 to 4.97)	0.5%	0.7% (0.2 to 2.4)	+0.2% (−0.3 to +1.9)
Dasatinib **№ of Participants: 1802** (6 Studies)	OR 1.97 (0.66 to 5.93)	0.5%	0.9% (0.3 to 2.7)	+0.4% (−0.2 to +2.2)

Part B: Which Mechanism(s) May Explain the Risk of Thrombosis?

Chapter 3: BCR-ABL Tyrosine Kinase Inhibitors: Which Mechanism(s) May Explain the Risk of Thrombosis?

In the previous chapter, we highlighted the risk of vascular occlusive events with 3 out of 5 commercialized BCR-ABL TKIs and then characterized that the risk is mainly arterial. However, the cellular mechanisms and signaling pathways by which these events occurred remain unknown. This chapter aims to describe potential mechanisms by which arterial occlusive events with second and third generations TKIs may occur based on a literature review of clinical, *in vivo* and *in vitro* experiment data.(Haguet et al. 2018) Data underlying the potential protective effect of imatinib on the occurrence of arterial thrombosis are also discussed. This review particularly focuses on the contribution of glucose and lipid metabolism, atherosclerosis and platelets in the occurrence of vascular adverse events with dasatinib, nilotinib and ponatinib. The lack of specificity of TKIs and the off-target receptors that might be implicated in the cardiovascular toxicity observed with these compounds are also discussed. The aim of this review was to condense information to orientate mechanistic research relying on the available clinical data and prior mechanistic investigations. The understanding of the pathophysiology of these events is elemental to help clinicians in the implementation of risk minimization strategies and to help in the development of effective drugs without vascular toxicity.

Review in TH Open 2(1): e68-e88

BCR-ABL Tyrosine Kinase Inhibitors: Which Mechanism(s) may Explain the Risk of Thrombosis?

Hélène Haguet, Jonathan Douxfils, Christian Chatelain, Carlos Graux, François Mullier and Jean-Michel Dogné

Parts of the supplemental files of this review were added to the content of the article in order to improve the readers' understanding.

Summary

Imatinib, the first-in-class BCR-ABL tyrosine kinase inhibitor (TKI), had been a revolution for the treatment of chronic myeloid leukemia (CML) and had greatly enhanced patient survival. Second- (dasatinib, nilotinib and bosutinib) and third-generation (ponatinib) TKIs have been developed to be effective against BCR-ABL mutations making imatinib less effective. However, these treatments have been associated with arterial occlusive events. This review gathers clinical data and experiments about the pathophysiology of these arterial occlusive events with BCR-ABL TKIs. Imatinib is associated with very low rates of thrombosis, suggesting a potentially protecting cardiovascular effect of this treatment in patients with BCR-ABL CML. This protective effect might be mediated by decreased platelet secretion and activation, decreased leukocyte recruitment, anti-inflammatory or anti-fibrotic effects. Clinical data have guided mechanistic studies toward alteration of platelet functions and atherosclerosis development, which might be secondary to metabolism impairment. Dasatinib, nilotinib and ponatinib affect endothelial cells and might induce atherogenesis through increased vascular permeability. Nilotinib also impairs platelet functions and induces hyperglycemia and dyslipidemia that might contribute to atherosclerosis development. Description of the pathophysiology of arterial thrombotic events is necessary to implement risk minimization strategies.

Keywords: *BCR-ABL, arterial thrombotic events, tyrosine kinase inhibitors, chronic myeloid leukemia*

Introduction

In 2001, the approval of imatinib, the first-in-class tyrosine kinase inhibitor (TKI) targeting BCR-ABL transformed the prognosis of patients with chronic-phase chronic myeloid leukemia (CML) from a life-threatening condition to a manageable and chronic disease.(Bhamidipati et al. 2013) Yet, despite satisfactory outcomes, 33% of patients did not achieve optimal response because of treatment resistance or intolerance.(Bhamidipati et al. 2013) The identification of the predominant resistance mechanism (i.e. point mutations in the kinase domain of Bcr-Abl) led to the development of second generation BCR-ABL TKIs (dasatinib, nilotinib and bosutinib respectively approved in 2006, 2007 and 2012) active against most of the BCR-ABL mutated forms.(Gorre et al. 2001; Bixby and Talpaz 2009) Second generation TKIs demonstrated no or little improvement of the overall survival compared with imatinib (Cortes, Saglio, et al. 2016; Hochhaus et al. 2016; Brummendorf et al. 2015) but 2 of these (i.e. dasatinib and nilotinib) improve surrogate outcomes and permit quicker and deeper achievement of molecular response, which is criteria to try treatment cessation (i.e. MR^4 or higher molecular response stable for at least 2 years).(Mealing et al. 2013) Based on these results, dasatinib and nilotinib were approved in 2010 for frontline management of CML, whereas bosutinib is only used after failure or intolerance of first-line BCR-ABL TKIs. Unfortunately, these treatments were ineffective against a common mutation (14% of all mutations) in the gatekeeper residue of BCR-ABL (i.e. the $T315I^4$ mutation),(Lamontanara et al. 2013; Pagnano et al. 2015; Ursan et al. 2015) requiring the development of a third generation TKI (ponatinib), efficient against this mutation. Ponatinib is currently the only treatment active against the T315I mutation and is therefore reserved to patients with this mutation or

[4] T315I: substitution at position 315 in BCR-ABL from a threonine to an isoleucine. This substitution alters the structure of the ATP-binding pocket and eliminate a crucial hydrogen bond required for binding of first- and second-generation TKIs.

to patients resistant to frontline treatments.(National Comprehensive Cancer Network 2016b)

Since its approval, the first generation TKI imatinib, has demonstrated reassuring safety profile, with a low rate of grade 3/4 adverse events and excellent tolerability.(Thanopoulou and Judson 2012; Kalmanti et al. 2015) Conversely, new generations BCR-ABL TKIs nilotinib, dasatinib, bosutinib and ponatinib are more recent and display different safety profile. Dasatinib, nilotinib and ponatinib are largely associated with fluid retention and dasatinib specifically induces a high rate of pleural effusions.(Moslehi and Deininger 2015; Breccia and Alimena 2010; European Medicines Agency, 2017b; European Medicines Agency) Nilotinib induces metabolic disorders such as dyslipidemia and hyperglycemia, whereas bosutinib safety profile is mainly characterized by gastrointestinal events (i.e. diarrhea, nausea, vomiting).(Kantarjian et al. 2014; Saglio, Kim, et al. 2010) Finally, ponatinib has been rapidly associated with a high rate of vascular occlusion.(Poch Martell et al. 2016)

Recently, meta-analyses of randomized clinical trials established that ponatinib is not the only new generation TKIs increases the cardiovascular risk.(Douxfils, Haguet, Mullier, Chatelain, Graux, and Dogné 2016; Haguet et al. 2017) The 4 new generation BCR-ABL TKIs increase the risk of vascular occlusive events compared with imatinib, especially arterial occlusive diseases, and this, in accordance to clinical trial data.(Cortes et al. 2015; Pasvolsky et al. 2015; Douxfils, Haguet, Mullier, Chatelain, Graux, and Dogné 2016; Haguet et al. 2017) However, this cardiovascular risk is controversy for dasatinib because of the low incidence (1.1 per 100 patient-year) of cardiovascular events in clinical trials.(Gora-Tybor, Medras, Calbecka, Kolkowska-Lesniak, et al. 2015; le Coutre et al. 2016) Recently, a large retrospective analysis of chronic phase CML patients treated with BCR-ABL TKIs at the *MD Anderson Cancer Center* confirmed the increased risk of vascular occlusive events with dasatinib.(Sam et al. 2016) Another controversial point is the effect of imatinib on the cardiovascular system.

Indeed, imatinib is associated with low risk of cardiovascular events and it was therefore hypothesized that imatinib prevents their occurrence.(Lassila et al. 2004; Ma et al. 2012) Clinical data indicate that most patients developing arterial occlusive events with new generation BCR-ABL TKIs are high-risk patients, but cardiovascular events also occurred in young and healthy patients. Additional information on clinical safety of BCR-ABL TKIs is described in supplementary material (**Table 3.5**). We assume that the mechanism underlying arterial thrombosis with BCR-ABL TKIs might be multiple. The predominance of arterial events raised concerns about the impact of BCR-ABL TKIs on platelet functions, atherosclerosis and metabolism, and preclude pro thrombotic states to be responsible for these events.(Fossard et al. 2016)

This review particularly focuses on the contribution of glucose and lipid metabolism, atherosclerosis and platelets in the occurrence of cardiovascular events with new generations TKIs. The last section discusses relevant off target receptors that might be implicated in the cardiovascular toxicity. The discovery of the mechanism(s) by which arterial occlusive events arose in CML patients would help in the management of patients treated with BCR-ABL TKIs and implement risk minimization measures. Discovery of the pathophysiology of these events in CML patients might also lead to the development of predictive biomarkers or to the development of new therapies with no or reduced cardiovascular toxicity profile while keeping an unaltered efficacy.

Impact on Platelet Functions

BCR-ABL TKIs are associated with both bleeding and thrombotic complications. **Table 3.1** describes experiments assessing the impact of BCR-ABL TKIs on platelet production and functions. Imatinib and dasatinib induce hemorrhagic events in patients with CML. Interestingly, dasatinib-associated hemorrhages occurred both in patients with and without thrombocytopenia.(Kostos et al. 2015) *In vitro* and *in vivo* investigations demonstrated that dasatinib affects both platelet functions (i.e. platelet

aggregation, secretion and activation) and platelet formation by impairment of megakaryocyte migration.(Mazharian et al. 2011; Alhawiti et al. 2016; Lotfi et al. 2016; Li, Grosser, and Diamond 2017) Furthermore, dasatinib decreases thrombus formation *in vitro*, *in vivo* and *ex vivo*,(Alhawiti et al. 2016) and decreases the number of procoagulant platelets (i.e. phosphatidylserine-exposing platelets).(Lotfi et al. 2016) Several dasatinib off-targets are implicated in platelet signaling and functions including members of the SFKs (e.g. Src, Lyn, Fyn, Lck and Yes) (**Figure 3.1**).(Senis, Mazharian, and Mori 2014; Gratacap et al. 2009) However, SFKs are also inhibited by bosutinib without disturbance of platelet aggregation and adhesion. Dasatinib also inhibits Syk, BTK and members of th**e** ephrin family (e.g. EphA2), all known to be involved in platelet functions.

Experimental assessments of platelet functions with imatinib demonstrate less pronounced effects on platelets. Imatinib inhibits platelet aggregation only at high doses (Alhawiti et al. 2016) and does not interfere with platelet aggregation *in vivo*.(Albrecht-Schgoer et al. 2013) However, *in vitro* studies also indicate decreased platelet secretion and activation by imatinib.(Alhawiti et al. 2016) The mechanism by which imatinib inhibits platelet functions is unknown. Oppositely to dasatinib, imatinib does not inhibit SFKs, ephrins, BTK and Syk. A hypothesis also suggests that imatinib induces bleeding disorders because of BCR-ABL rearrangements in megakaryocytic cell lines, leading to clonal expansion of dysfunctional megakaryocytes.(Shimabukuro-Vornhagen et al. 2011)

Even if ponatinib induces very few bleeding disorders, assessment of primary hemostasis in CML patients demonstrated that ponatinib induces defects in platelet aggregation. This impairment was found at all ponatinib dosage, in patients with or without low platelet counts.(Neelakantan et al. 2012) These results were in accordance with *in vitro* studies which previously demonstrated similar characteristics than dasatinib (i.e. decrease of platelet spreading, aggregation, P-selectin secretion and phosphatidylserine exposure).(Loren et al. 2015; Lotfi et al. 2016) However, *in vitro* assays tested

Table 3.1 | ***In vitro* and *ex vivo* investigations of the effects of BCR-ABL TKIs on platelet production and functions.**

Endpoints	Methods	Models	TKIs	Findings	Ref
Platelet production	Platelet count	Murine whole blood	Dasatinib	Thrombocytopenia ↘ platelet production	(Mazharian et al. 2011)
	Flow cytometry (DNA ploidy) Migration assay (Dunn chamber)	Megakaryocyte primary culture	Dasatinib	↗ megakaryocyte differentiation ↘ megakaryocyte migration ↘ proplatelet formation	(Mazharian et al. 2011)
Platelet aggregation	Born aggregometry; Light transmission aggregometry	Washed human Platelet	Imatinib	= CRP-, collagen- and thrombin-induced platelet aggregation	(Loren et al. 2015) (Gratacap et al. 2009) (Albrecht-Schgoer et al. 2013)
	Light transmission aggregometry	Human platelet (PRP)	Imatinib	↘ ADP-induced platelet aggregation ↘ collagen- and CRP-induced platelet aggregation	(Alhawiti et al. 2016)
	Light transmission aggregometry, immunostaining (PAC-1)	Human platelet (PRP); patient blood	Dasatinib	↘ ADP-, collagen-, thrombin- and CRP-induced platelet aggregation	(Alhawiti et al. 2016) (Gratacap et al. 2009) (Lotfi et al. 2016)
	Light transmission aggregometry; Born aggregometry	Human platelet (PRP); Washed human platelet	Nilotinib	= platelet aggregation	(Albrecht-Schgoer et al. 2013) (Alhawiti et al. 2016) (Loren et al. 2015)
	Born aggregometry	Washed human platelet	Ponatinib	↘ CRP-induced platelet aggregation = thrombin-induced platelet aggregation	(Loren et al. 2015)

Platelet activation	Immunostaining (PS)	Washed human platelet	Imatinib	= PS exposure	(Loren et al. 2015)
	Western blot	Human platelet lysate	Imatinib	= Src, Lyn, LAT and BTK activation	(Loren et al. 2015)
	Immunostaining (PS)	Patient blood	Dasatinib	↘ PS exposure	(Lotfi et al. 2016)
	Immunostaining (PS)	Washed human platelet	Nilotinib	= PS exposure	(Loren et al. 2015)
	Immunostaining (PS)	Patient blood	Nilotinib	↗ PS exposure	(Lotfi et al. 2016)
	Western blot	Human platelet lysate	Nilotinib	= Src, Lyn, LAT and BTK activation	(Loren et al. 2015)
	Immunostaining (PS)	Patient blood	Bosutinib	↗ PS exposure	(Lotfi et al. 2016)
	Immunostaining (PS)	Washed human platelet, patient blood	Ponatinib	↘ PS exposure	(Loren et al. 2015) (Lotfi et al. 2016)
	Western blot	Human platelet lysate	Ponatinib	↘ Src, Lyn, LAT and BTK activation	(Loren et al. 2015)
Granule release	Immunostaining (P-selectin)	Human platelet	Imatinib	↘ thrombin-, PAR-1- and CRP-mediated α-granule release = PAR-4-mediated α-granule release	(Alhawiti et al. 2016)
	Immunostaining (P-selectin)	Washed human platelet	Imatinib	= α-granule release	(Loren et al. 2015)
	Immunostaining (P-selectin)	Human platelet	Dasatinib	↘ thrombin-, PAR-1-, PAR-4- and CRP-mediated α-granule release	(Alhawiti et al. 2016)
	Immunostaining (P-selectin)	Washed human platelet	Nilotinib	= PAR-4-, CRP- and thrombin-mediated α-granule release	(Loren et al. 2015) (Alhawiti et al. 2016)

	Immunostaining (P-selectin)	Murine platelet	Nilotinib	↘ CRP-, PAR-4- and thrombin-mediated α-granule release	(Alhawiti et al. 2016)
	Immunostaining (P-selectin)	Human platelet	Nilotinib	↗ PAR-1-mediated α-granule release	(Alhawiti et al. 2016)
	Immunostaining (P-selectin)	Washed human platelet	Ponatinib	↘ α-granule release	(Loren et al. 2015)
Platelet spreading	Microscopy (platelet spreading)	Washed human platelet	Imatinib	= platelet spreading and lamellipodia formation	(Loren et al. 2015)
	Microscopy (platelet spreading)	Washed human platelet	Nilotinib	= platelet spreading and lamellipodia formation	(Loren et al. 2015)
	Microscopy (platelet spreading)	Washed human platelet	Ponatinib	↘ platelet spreading and lamellipodia formation	(Loren et al. 2015)
Thrombus formation	*In vitro* flow study, PFA-100	Human blood, murine whole blood	Imatinib	= platelet deposition and thrombus volume = closure time	(Li, Grosser, and Diamond 2017) (Alhawiti et al. 2016) (Quintas-Cardama et al. 2009)
	Ex-vivo and *in vitro* flow study	Murine whole blood, human whole blood	Imatinib	↘ thrombus volume and aggregate formation	(Alhawiti et al. 2016) (Loren et al. 2015)
	In vitro and *ex-vivo* flow study	Human blood, murine whole blood, patient whole blood	Dasatinib	↘ thrombus volume and platelet deposition	(Lotfi et al. 2016) (Li, Grosser, and Diamond 2017) (Alhawiti et al. 2016)
	PFA-100	Human whole blood	Dasatinib	↗ closure time (collagen/epinephrine activation) = closure time (collagen/ADP activation)	(Quintas-Cardama et al. 2009)

	Ex-vivo flow study	Murine whole blood, patient whole blood	Nilotinib	↗ thrombus volume (growth and stability)	(Alhawiti et al. 2016)
	In vitro flow study	Human whole blood, murine whole blood	Nilotinib	= platelet deposition and thrombus volume	(Li, Grosser, and Diamond 2017) (Loren et al. 2015) (Alhawiti et al. 2016)
	In vitro flow study	Human blood	Bosutinib	↘ platelet deposition (late)	(Li, Grosser, and Diamond 2017)
	PFA-100	Patient blood	Ponatinib	↗ closure time	(Neelakantan et al. 2012)
	In vitro flow study	Human whole blood	Ponatinib	↘ aggregate formation	(Loren et al. 2015)

ADP: adenosine diphosphate; BTK: Bruton's tyrosine kinase; CRP: C-reactive protein; DNA: deoxyribonucleic acid; LAT: linker for activation of T-cells; PAR: protease-activated receptor; PFA: platelet function assay; PRP: platelet-rich plasma; PS: phosphatidyl serine

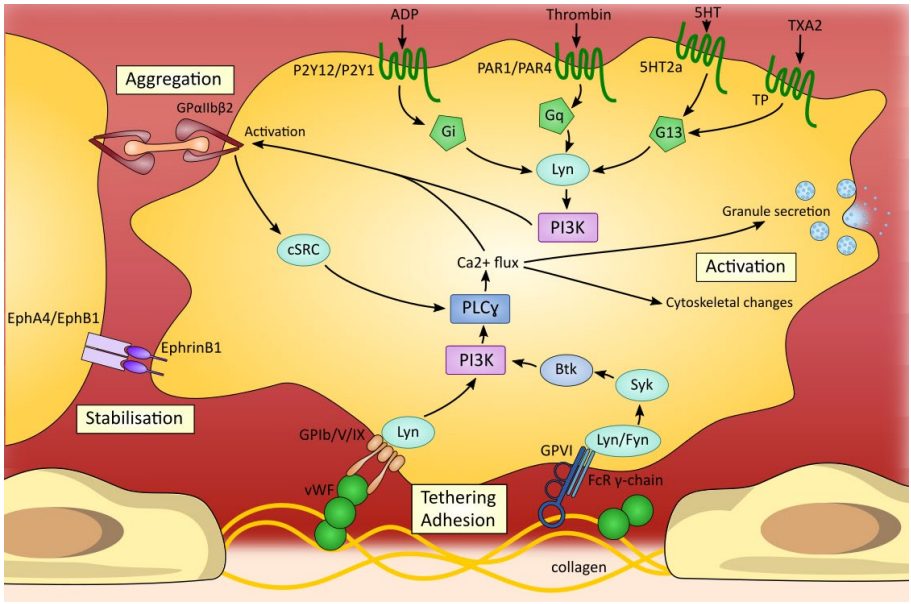


Figure 3.1 | Signaling pathways supporting platelet adhesion, activation and aggregation. Tyrosine kinases are involved in several pathways and contributes to platelet adhesion, aggregation and activation. Important players in platelet signaling are members of the Src family kinases; particularly Lyn, Fyn and cSRC. These 3 tyrosine kinases are inhibited by dasatinib which might explain platelet dysfunction encountered with this treatment. Additionally, dasatinib also inhibits BTK, Syk, EphA4 and EphB1, 4 tyrosine kinases involved in platelet activation and aggregate stabilization.

5HT: 5-hydroxytryptamine; ADP: adenosine diphosphate; Btk: Bruton's tyrosine kinase; Ca: calcium; Eph: Ephrin; FcR: Fc receptor; GP: glycoprotein; PAR: protease-activated receptor; PI3K: phosphoinositide 3-kinase; PLC: phospholipase C; TXA2: thromboxane A2; vWF: Von Willebrand factor

ponatinib at 1μM, a dose far higher than the concentration observed in patients on treatment.(Rivera et al. 2014) Nilotinib and bosutinib are not associated with bleeding disorders in CML patients. First *in vitro* studies demonstrated little or no effect on platelet aggregation and activation with these two TKIs.(Albrecht-Schgoer et al. 2013; Quintas-Cardama et al. 2009; Li, Grosser, and Diamond 2017) However, recent experiments described pro thrombotic phenotype of platelets induced by nilotinib, with increase of PAR-1 mediated-platelet secretion, adhesion and activation, without disturbing

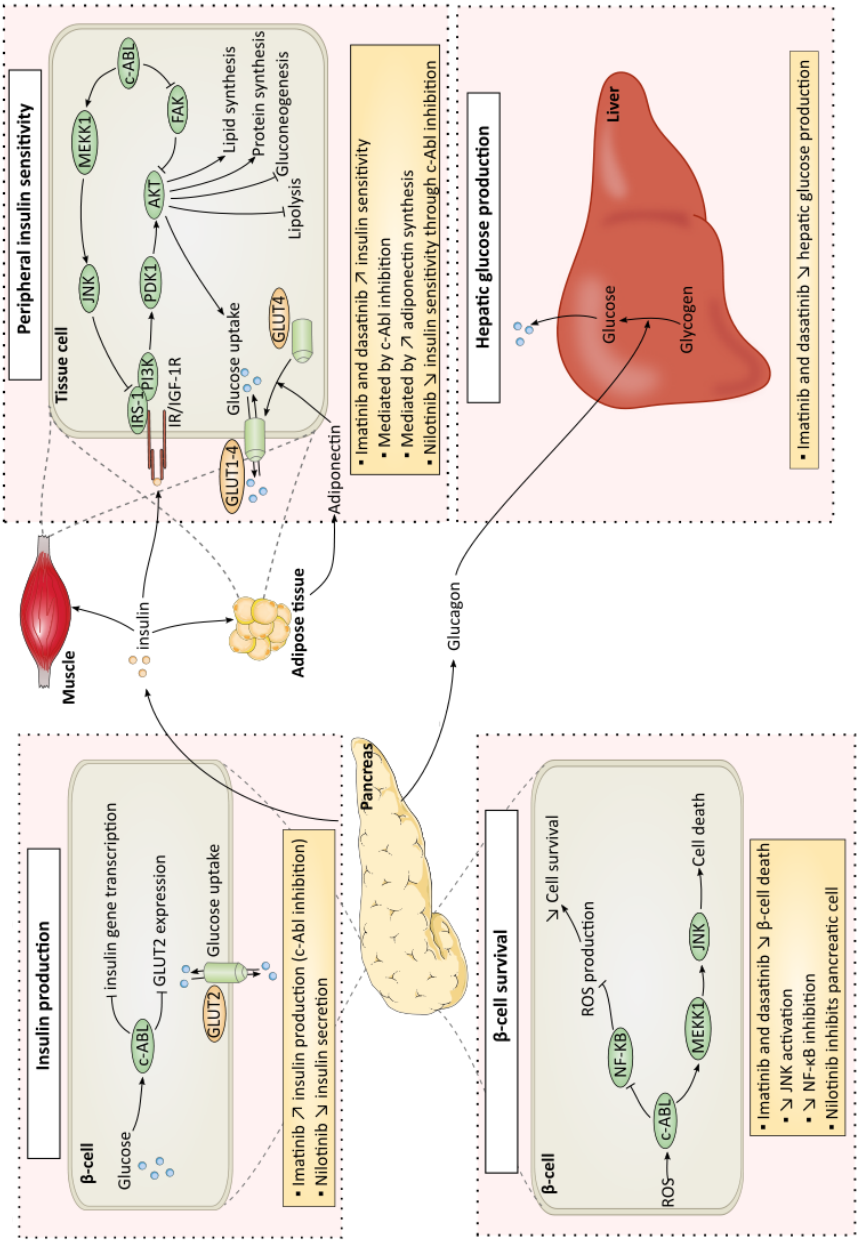
platelet aggregation.(Alhawiti et al. 2016) Additional studies demonstrated that nilotinib increases secretion of adhesive molecules as well as thrombus formation and stability *ex vivo*.(Alhawiti et al. 2016)

To summarize, dasatinib and imatinib induces hemorrhagic events through alteration of platelet functions but the molecular mechanism needs to be better determined. Ponatinib also impairs platelet functions. Therefore, no current data involves platelets in the pathogenesis of arterial thrombosis occurring with dasatinib and ponatinib. Oppositely, nilotinib might induce arterial thrombosis through alteration of platelet secretion, adhesion and activation.

Metabolic dysregulation

Glucose metabolism

BCR-ABL TKIs have contradictory effect on glucose metabolism. Imatinib and dasatinib improve glucose metabolism and type 2 diabetes management in CML patients (i.e. decrease of antidiabetic drug dosage and reversal of type 2 diabetes).(Breccia et al. 2004; Moslehi and Deininger 2015; Breccia and Alimena 2009; Agostino et al. 2011; Breccia et al. 2008; Ono et al. 2012) This clinical profile is in accordance with *in vivo* studies in which imatinib is effective to prevent the development of type 1 diabetes in prediabetic mice, without impacting the adaptive immune system.(Louvet et al. 2008) Therefore, imatinib is currently tested in clinical trials for patients suffering from type 1 diabetes mellitus (NCT01781975). The mechanism(s) by which dasatinib and imatinib improve glucose metabolism remains unknown. Global hypotheses suggest that imatinib increases peripheral insulin sensitivity, promotes β-cell survival or decreases hepatic glucose production (**Figure 3.2**).(Hagerkvist, Jansson, and Welsh 2008; Han et al. 2009; Hagerkvist et al. 2006; Hagerkvist et al. 2007) This latter hypothesis (i.e. decreased hepatic glucose production by imatinib) is not currently the preferred theory, whereas it was demonstrated that imatinib weakly affects hepatic glucose production.(Hagerkvist, Jansson, and Welsh 2008) Several targets might be


Peripheral insulin sensitivity
Tissue cell
c-ABL
MEKK1
FAK
JNK
PDK1
AKT
Lipid synthesis
Protein synthesis
Gluconeogenesis
Lipolysis
IRS-1
PI3K
IR/IGF-1R
Glucose uptake
GLUT4
GLUT1-4
Adiponectin
▪ Imatinib and dasatinib ↗ insulin sensitivity
▪ Mediated by c-Abl inhibition
▪ Mediated by ↗ adiponectin synthesis
▪ Nilotinib ↘ insulin sensitivity through c-Abl inhibition
Hepatic glucose production
Liver
Glucose
Glycogen
▪ Imatinib and dasatinib ↘ hepatic glucose production
Muscle
insulin
Adipose tissue
Glucagon
Pancreas
Insulin production
β-cell
Glucose
c-ABL
insulin gene transcription
GLUT2 expression
GLUT2
Glucose uptake
▪ Imatinib ↗ insulin production (c-Abl inhibition)
▪ Nilotinib ↘ insulin secretion
β-cell survival
β-cell
↘ Cell survival
ROS production
NF-KB
ROS
c-ABL
MEKK1
JNK
Cell death
▪ Imatinib and dasatinib ↘ β-cell death
▪ ↘ JNK activation
▪ ↘ NF-κB inhibition
▪ Nilotinib inhibits pancreatic cell

▲ **Figure 3.2** | **Effects of BCR-ABL TKIs on glucose metabolism.** Imatinib and dasatinib possess hypoglycemic effects whereas nilotinib increases blood glucose level and diabetes development. The figure describes glucose metabolism and boxes contain emitted hypotheses for effects of imatinib, dasatinib and nilotinib on glucose metabolism. Four major hypotheses have been emitted including impact on insulin production by β-cells, β-cell survival, peripheral insulin sensitivity and hepatic glucose production.
ABL: Abelson; FAK: focal adhesion kinase; GLUT: glucose transporter; IRS-1: Insulin receptor substrate 1; JNK: c-Jun N-terminal kinases; MEKK1: MAPK/ERK kinase kinase 1; NF-κB: nuclear factor-kappa B; PDK1: Pyruvate Dehydrogenase Kinase 1; PI3K: phosphoinositide 3-kinase; ROS: reactive oxygen species

involved in this metabolic effect. PDGFR has already been linked with type 1 diabetes reversal.(Louvet et al. 2008) Hägerkvist et al. hypothesized that c-Abl inhibition by imatinib promotes β-cell survival through activation of NF-κB signaling and inhibition of pro-apoptotic pathways (**Figure 3.2**).(Hagerkvist et al. 2007; Hagerkvist et al. 2006) Inhibition of c-Abl in β-cells might also increase insulin production and contribute to the glucose regulation by imatinib.(Xia et al. 2014) It was also speculated that imatinib decreases insulin resistance in peripheral tissues due to c-Abl dependent-JNK inactivation.(Hagerkvist, Jansson, and Welsh 2008) Similar hypotheses might be translated to dasatinib because of the similar off-target inhibitory profile (i.e. dasatinib also inhibits c-Abl and PDGFR). It was hypothesized that imatinib and dasatinib impact glucose metabolism through reduced adipose mass.(Hagerkvist, Jansson, and Welsh 2008; Sadiq S et al. 2017) However, clinical data do not demonstrate weight loss in CML patients and do not favor this hypothesis. In both imatinib- and dasatinib-treated patients, increased circulating adiponectin level correlate with decreased insulin resistance.(Iizuka et al. 2016; Fitter et al. 2010) This correlation might be explained by the translocation of the glucose transporter GLUT4 from the cytoplasm to the cell membrane following adiponectin signaling.(Hosch, Olefsky, and Kim 2006) Additionally, adiponectin has been related to decreased hepatic glucose production which could be an additional mechanism by which imatinib and dasatinib improve glucose metabolism.(Lihn, Pedersen, and Richelsen 2005) It was speculated that the rise of adiponectin level with imatinib and dasatinib is the consequence of increased adipogenesis subsequent to PDGFR inhibition.(Fitter et al. 2012).

Oppositely to imatinib and dasatinib, case-reports and clinical trials indicate that nilotinib increases blood glucose levels and promotes diabetes mellitus.(Aichberger et al. 2011a; Breccia et al. 2007; Castagnetti et al. 2016; Rea et al. 2012) Indeed, 20% of nilotinib treated patients developed diabetes after 3 years of treatment (Rea et al. 2012) whereas 29% of patients suffer from increase of fasting glucose after 1 year of therapy.(Castagnetti et al. 2016) However, no variations of glycated hemoglobin were reported.(Castagnetti et

al. 2016; Rea et al. 2012) Clinical data indicate no direct effect of nilotinib on β-cells, but suggest fasting insulin increase, fasting C-peptide decrease and an increase of HOMA-IR values (i.e. a model to assess insulin resistance).(Iurlo et al. 2015; Castagnetti et al. 2016; Racil et al. 2013) Therefore, the preferred hypothesis to explain the development of hyperglycemia is the manifestation of insulin resistance. Weakened insulin secretion occurred sometimes but it is likely that this impairment is the consequence of β-cell exhaustion.(Ito et al. 2013) However, *in* vitro experiments demonstrated the inhibitory effect of nilotinib on pancreatic cell growth.(Hadzijusufovic et al. 2011) Breccia et al. proposed an additional hypothesis linking development of hyperglycemia and body mass index. They suggest that the development of hyperglycemia might be the consequence of increase fat level tissue resulting in decrease peripheral insulin sensitivity.(Breccia et al. 2012) However, dietetic measures to restrict glucose exogenous uptake in patients that developed hyperglycemia were not successful (Breccia et al. 2007) and nilotinib does not induce changes in patient body weight.(Saglio, Larson, et al. 2010) Little is known regarding the mechanism by which nilotinib induces insulin resistance. Racil et al. suggested that peripheral insulin resistance is mediated by c-Abl inhibition, which is involved in insulin receptor signaling (**Figure 3.2**).(Racil et al. 2013) This hypothesis is contrary to the hypothesis described with dasatinib and imatinib in which c-Abl enhance insulin sensitivity through c-Abl inhibition. These two hypotheses describe different pathways involving c-Abl but with opposite outcomes. To date, no hypothesis is preferred, and additional studies are required to understand the opposite effect on glucose metabolism between TKIs whereas both have been attributed to c-Abl inhibition. Interestingly, Frasca et al. describe opposite role of c-Abl in insulin signaling depending of the receptor involved, the signaling pathway and the cell context.(Frasca et al. 2007) Similar investigations should be performed in the context of c-Abl inhibition by BCR-ABL TKIs. For bosutinib and ponatinib, little is known regarding their impact on glucose metabolism but no drastic changes in glucose profile have been reported during clinical trials.

Lipid Metabolism

Similarly to glucose metabolism, effects on lipid metabolism are conflicting between TKIs. Oppositely to *in vivo* study which demonstrated no impact of imatinib on total cholesterol and triglyceride levels in diabetic mice (Lassila et al. 2004) imatinib is associated in CML patients with a rapid and progressive decrease of cholesterol and triglycerides levels.(Franceschino et al. 2008; Iurlo et al. 2015; Gottardi, Manzato, and Gherlinzoni 2005; Gologan et al. 2009) First hypothesis relates the inhibition of PDGFR by imatinib (**Figure 3.3**). PDGFR is involved in the synthesis of the lipoprotein lipase (LPL) and in the regulation of the lipoprotein receptor-related protein (LRP).(Franceschino et al. 2008; Gottardi, Manzato, and Gherlinzoni 2005) However, all BCR-ABL TKIs possess inhibitory activity against PDGFR but do not share this positive impact on lipid profile. Recently, Ellis et al. described that imatinib impairs the gene expression of proteins involved in plasma lipid regulation. Indeed, in an *in vitro* model of CML cells, imatinib affects the gene expression of 4 genes implicated in lipid synthesis (HMG-CoA reductase gene and apobec1), lipid clearance (LDLR gene) and in exchange of lipids from very low-density lipoproteins (VLDL) or low-density lipoproteins (LDL) to high-density lipoproteins (HDL) (CETP gene). However, these studies were performed in a model of CML cells and need to be confirmed in more relevant models (e.g. primary cell lines, hepatocytes).(Ellis et al. 2017) Franceschino et al. suggested that imatinib decreases diarrhea-related lipid absorption due to inhibition of c-kit in interstitial Cajal cells (i.e. c-kit signaling is critical for the survival and development of these cells).(Franceschino et al. 2008) However, this hypothesis is unlikely, few patients (3.3%) developed grade 3/4 diarrhea, and patients treated with interferon-α and cytarabine developed diarrhea at a same rate and do not present lipid-level reduction in the phase 3 clinical trial (NCT00333840).

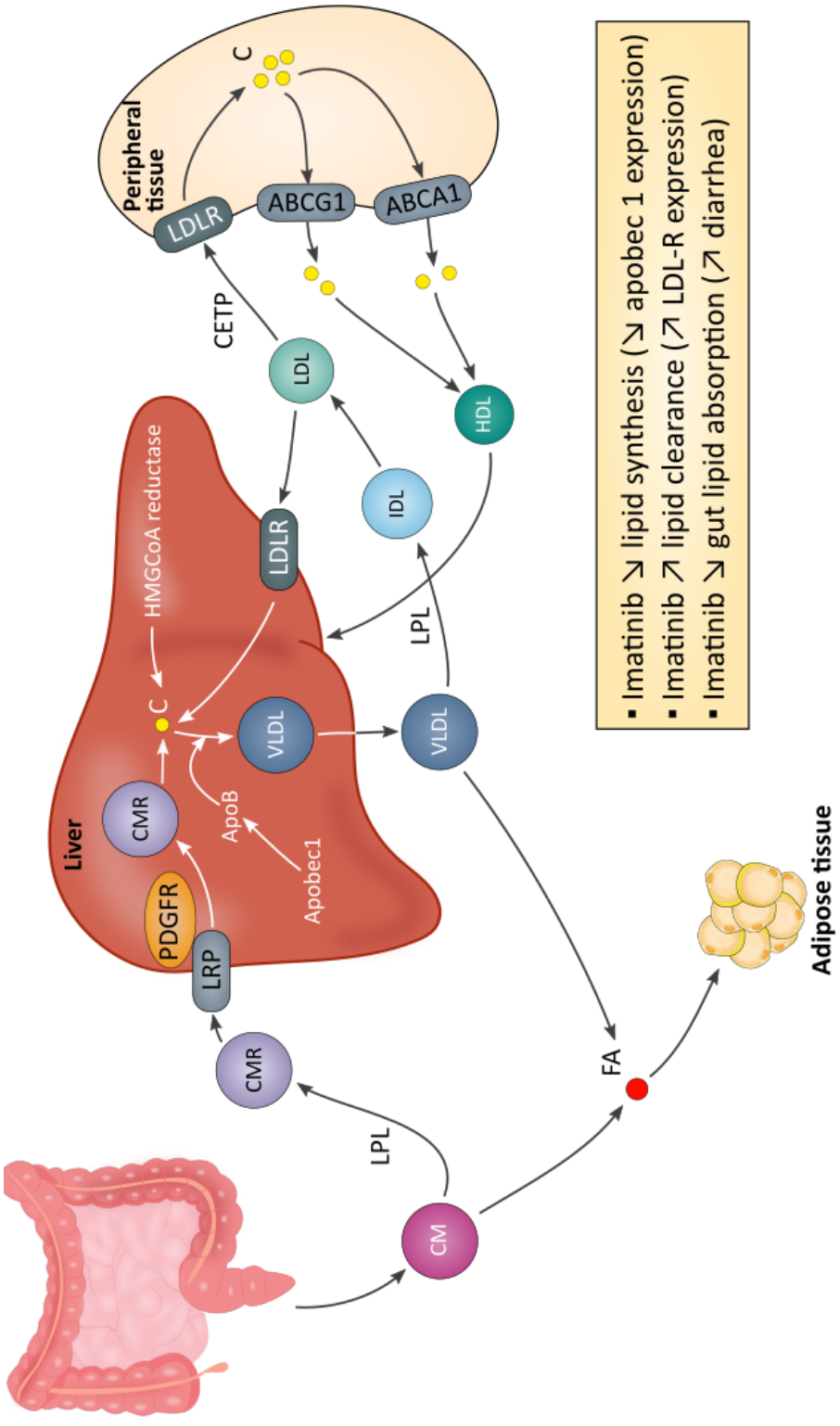
Oppositely, dasatinib and mostly nilotinib are associated with an increase of cholesterol levels.(Franklin et al. 2016; Gora-Tybor, Medras, Calbecka, Kolkowska-Lesniak, et al. 2015; Iurlo et al. 2015) Nilotinib induces quick rise

of total cholesterol, HDL and LDL (i.e. within 3 months). Nilotinib-induced dyslipidemia are responsive to statin and lipid level normalized after nilotinib discontinuation.(Rea et al. 2014) To date, the mechanism by which dasatinib and nilotinib impact lipid metabolism is unknown. Future research should determine how these treatments induce dyslipidemia. Global hypotheses could be formulated and include an increase of lipid synthesis that might be secondary to insulin resistance and hyperinsulinemia. This hypothesis is particularly relevant with nilotinib it is also associated with hyperglycemia. Dasatinib and nilotinib might also decrease blood lipid clearance (e.g. disturbance of LDLR and LPL synthesis). The development of dyslipidemia might contribute to the occurrence of arterial occlusive events that occurred with nilotinib and dasatinib. However, the relationship between impaired lipid metabolism and cardiovascular occlusive events is unknown with BCR-ABL TKIs, and there is no indication that correct management of lipid metabolism can prevent arterial thrombosis (e.g. stenosis occurred in a nilotinib treated patient despite the management of its hyperlipidemia through statin treatment).(Maurizot, Beressi, Maneglier, et al. 2014) On their side, bosutinib and ponatinib do not disturb lipid metabolism.(Rea et al. 2014; Breccia, Molica, and Alimena 2014)

Effects on atherosclerosis

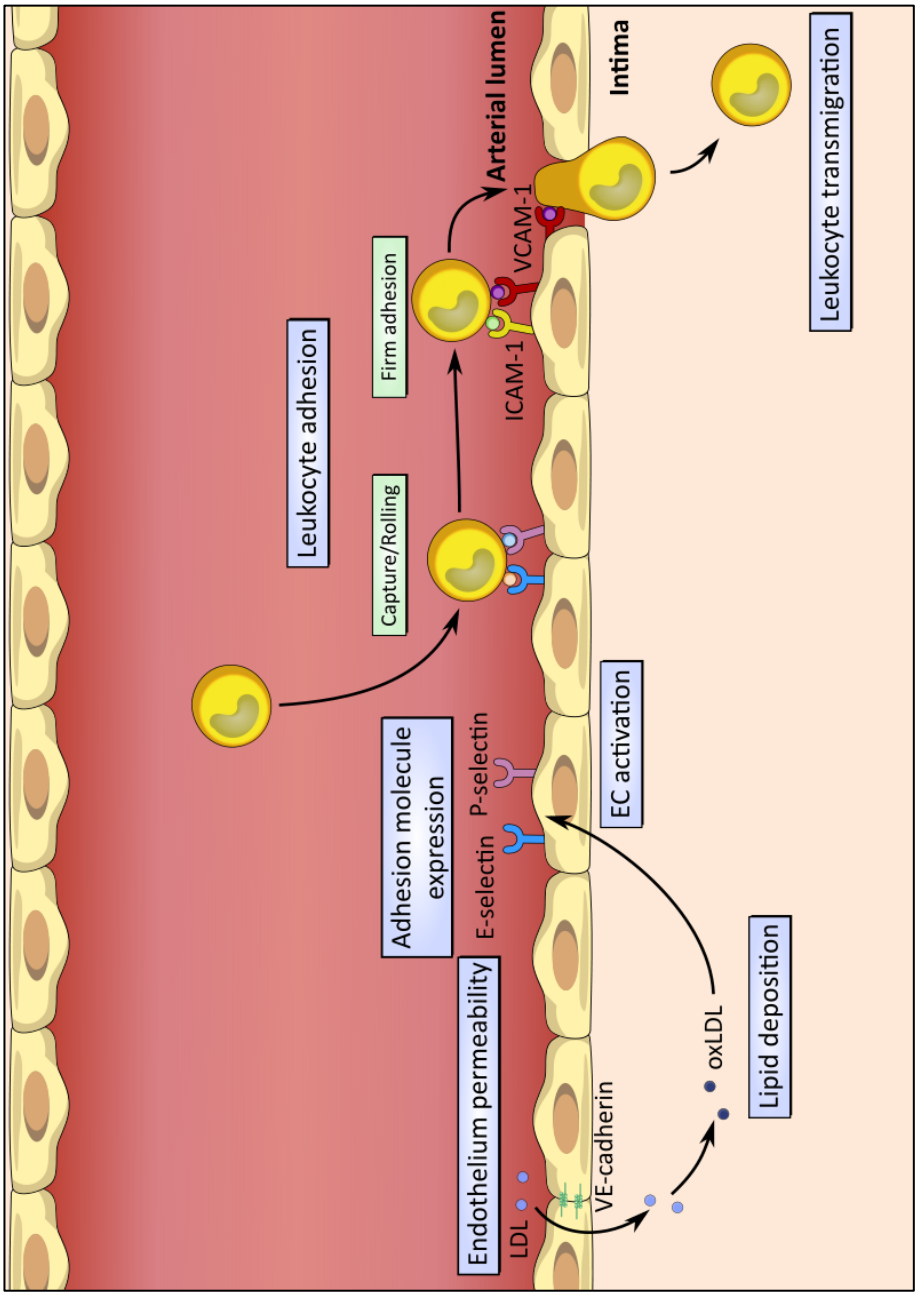
Endothelial dysfunction

Box 3.1 and **Figure 3.4** detail the role of endothelial cells (ECs) in atherosclerosis. Several *in vitro* and *in vivo* experiments assess the impact of imatinib on endothelial cell viability and functions (**Table 3.2**). These studies demonstrate that imatinib does not affect endothelial cell viability nor induce apoptosis but increases endothelial cell proliferation.(Venalis et al. 2009; Albrecht-Schgoer et al. 2013; Gover-Proaktor et al. 2017; Vallieres, Petitclerc, and Laroche 2009; Hacker et al. 2007) Only one study reports a pro-apoptotic effect of imatinib on endothelial cells but their experiments were performed


Peripheral tissue
C
LDLR
ABCG1
ABCA1
CETP
LDL
HDL
IDL
LPL
HMGCoA reductase
LDLR
C
VLDL
VLDL
Liver
CMR
ApoB
Apobec1
PDGFR
LRP
CMR
LPL
CM
FA
Adipose tissue
▪ Imatinib ↘ lipid synthesis (↘ apobec 1 expression)
▪ Imatinib ↗ lipid clearance (↗ LDL-R expression)
▪ Imatinib ↘ gut lipid absorption (↗ diarrhea)

▲ **Figure 3.3** | **Effects of BCR-ABL TKIs on lipid metabolism.** Several hypotheses have been issued to explain the imatinib-induced hypolipidemic effect. Imatinib regulates the expression of genes involved in lipid metabolism: Apobec1 that regulates ApoB expression through the introduction of a stop codon into ApoB mRNA (ApoB is essential for VLDL production), and LDLR that is implicated in lipid clearance. Imatinib-induced PDGFR inhibition influences LPL synthesis and deregulates LRP. Dasatinib and nilotinib increase cholesterol plasma levels through an unknown mechanism. Global hypotheses can be emitted and include increased hepatic lipid synthesis (possibly related to hyperinsulinemia) and decreased lipid clearance through LDLR functional defect or decreased LPL synthesis.

ABC: ATP-binding cassette; C: cholesterol; CETP: cholesterylester transfer protein; CM: chylomicron; FA: fatty acid; HMGCoA reductase: hydroxyméthylglutaryl-CoA réductase; IDL: intermediate-density lipoprotein; LDL: low-density lipoprotein; LDLR: low-density lipoprotein receptor; LPL: lipoprotein lipase; LRP: lipoprotein receptor-related protein; PDGFR: platelet-derived growth factor receptor; VLDL: very low-density lipoprotein


Arterial lumen
Intima
Leukocyte adhesion
Capture/Rolling
Firm adhesion
ICAM-1
VCAM-1
Adhesion molecule expression
E-selectin
P-selectin
Endothelium permeability
LDL
VE-cadherin
oxLDL
EC activation
Lipid deposition
Leukocyte transmigration

Box 3.1 | **Endothelial dysfunction initiates atherosclerosis**

A key event in the pathophysiology of atherosclerosis is the dysfunction of the endothelium, which results in endothelium permeability, enabling migration and trapping of lipoproteins into the intima. Modified lipoproteins in the intima activate endothelial cells and promotes the expression of cell adhesion molecules on the vessel wall, endorsing leukocyte migration into the intima.(Mestas and Ley 2008) Among adhesion molecules involved in atherosclerosis development, E-selectin, P-selectin, VCAM-1 and ICAM-1 possess major functions in leukocyte recruitment.(Moore, Sheedy, and Fisher 2013) E-selectin and P-selectin are involved in the capture and rolling of monocytes whereas ICAM-1 and VCAM-1 are implicated in leukocyte firm adhesion.(Mestas and Ley 2008) Monocyte transmigration is finally mediated by VCAM-1 and various chemokines produced by endothelial cells, macrophages and vascular smooth muscle cells.(Moore, Sheedy, and Fisher 2013)

Additionally to the role of the endothelium in early atherosclerosis development, a recent *in vivo* study reports that vascular leakage is a marker to distinguish stable from rupture-prone atherosclerotic plaques (i.e. plaques are more prone to rupture subsequently to increase leukocytes migrating to the intima).(Phinikaridou et al. 2016)

◀ **Figure 3.4** | **Endothelial dysfunction initiates atherosclerosis**
EC: endothelial cell; ICAM: intercellular adhesion molecule; LDL: low-density lipoprotein; oxLDL: oxidized low-density lipoprotein; TNF: tumor necrosis factor; VCAM: vascular cell adhesion molecule; VE-cadherin: vascular endothelial cadherin

on a cell line (i.e. EA.hy926 cells),(Vrekoussis et al. 2006) a model less reliable than primary cultures (e.g. HUVEC, HCAEC). *In vitro* studies also assessed the effect of imatinib on endothelial cell functions. In these studies, imatinib does not influence adhesion molecules expression (i.e. ICAM-1 and VCAM-1), endothelial cell migration, reactive oxygen species (ROS) production nor angiogenesis.(Venalis et al. 2009; Gover-Proaktor et al. 2017; Vrekoussis et al. 2006; Letsiou et al. 2015; Guignabert et al. 2016) Letsiou et al. suggest that imatinib decreases endothelial cell inflammation by decreasing the secretion of pro-inflammatory mediators.(Letsiou et al. 2015) The impact of imatinib on endothelial permeability is not clear. Indeed, *in vitro* studies demonstrate that imatinib increases endothelial permeability by decreasing the level of plasma membrane VE-cadherin,(Vrekoussis et al. 2006; Letsiou et al. 2015) whereas *in vivo* experiments indicate decreased vascular leak following imatinib treatment in a murine model of acute lung injury.(Rizzo, Sammani, et al. 2015) Additionally, imatinib has been tested in patients suffering from acute lung injury, a disease characterized by vascular leakage, and demonstrate promising clinical efficacy. Therefore, imatinib might positively affect atherogenesis by decreasing endothelial inflammation and reducing vascular leakage.

Nilotinib and ponatinib reduce endothelial cell proliferation and might impair endothelial regeneration.(Gover-Proaktor et al. 2017; Albrecht-Schgoer et al. 2013; Katgi et al. 2015; Hadzijusufovic et al. 2016) Additionally, ponatinib induces endothelial cell apoptosis whereas it is well recognized that high glucose concentration induces endothelial cell death (Lorenzi, Cagliero, and Toledo 1985) suggesting that nilotinib might, by this intermediary, affect endothelial cell viability. Moreover, clinical data indicate that dasatinib induces pulmonary arterial hypertension whereas imatinib is possibly beneficial in this disease.(Shah et al. 2015; Frost et al. 2015) This pathology is initiated by dysfunction or injury of pulmonary endothelial cells.(Guignabert et al. 2016)

Therefore, *in* vivo and *in vitro* studies investigated the effect of imatinib and dasatinib on pulmonary endothelial cells and demonstrate that dasatinib induces apoptosis on pulmonary endothelial cells mediated by increased mitochondrial ROS production.(Guignabert et al. 2016) Future research should assess if this effect is also found in arterial endothelial cells and ROS production should also be tested with other new generation BCR-ABL TKIs.

In addition to their effect on endothelial cell viability, nilotinib and ponatinib also influence endothelial cell functions, inhibit their migration, and decrease angiogenesis.(Albrecht-Schgoer et al. 2013; Gover-Proaktor et al. 2017) It was suggested that the anti-angiogenic effect of ponatinib is the consequence of VEGFR inhibition but this hypothesis cannot explain the anti-angiogenic effect of nilotinib (i.e. nilotinib does not inhibit VEGFR).(Gover-Proaktor et al. 2017) Nilotinib also increases adhesion molecules expression (i.e. ICAM-1, VCAM-1 and E-selectin) *in vitro* (Albrecht-Schgoer et al. 2013) suggesting that nilotinib might increase leukocyte recruitment. However, further experiments are needed to validate this hypothesis (e.g. assessment of endothelium permeability and transendothelial migration). Dasatinib also induces endothelium leakage *in vitro,* and the RhoA-ROCK pathway is involved in this phenomenon.(Dasgupta et al. 2017) It was demonstrated that RhoA activation induces the phosphorylation of myosin light chain that increases the actomyosin contractibility and disrupt endothelial barrier.(Dasgupta et al. 2017) Therefore, increased endothelium permeability is a potential mechanism by which dasatinib and nilotinib promote atherosclerosis development and arterial thrombosis. Likewise, it is plausible that ponatinib affects endothelium integrity because of its inhibitory activity against VEGFR, which is recognized as a permeability-inducing agent. Additional hypotheses suggest that inhibition of Abl kinase (i.e. Arg and c-Abl) and PDGFR might also be implicated in vascular leakage.(Vrekoussis et al. 2006) Finally, Guignabert et al. demonstrated that both in rats and in CML patients taking dasatinib, there is an increase of soluble adhesion molecules, which are well-known markers of endothelial dysfunction.(Guignabert et al. 2016)

Table 3.2 | *In vivo* and *in vitro* investigations of the effects of BCR-ABL TKIs on endothelial cell viability and major functions.

Endpoints	Methods	Models	TKIs	Findings	Ref
EC proliferation /survival	Cell counting; trypan blue staining	EA.hy 926 cell; HCAEC	Imatinib	= EC viability <10µM	(Vrekoussis et al. 2006; Hacker et al. 2007)
	Caspase assay; Annexin V staining; Hoechst staining; TUNEL assay	HMEC-1; HUVEC; Human pulmonary EC; Mouse EC	Imatinib	= EC apoptosis	(Venalis et al. 2009; Gover-Proaktor et al. 2017; Guignabert et al. 2016)
	TUNEL assay; Annexin V staining	EA.hy 926 cell	Imatinib	↗ EC apoptosis	(Vrekoussis et al. 2006)
	MTT cell proliferation assay; ^{3}H-thymidine incorporation; WST-1 assay; cell counting	HMEC-1; HUVEC; HCAEC	Imatinib	= EC proliferation	(Hacker et al. 2007; Venalis et al. 2009; Albrecht-Schgoer et al. 2013; Gover-Proaktor et al. 2017)
	Resazurin proliferation assay; PCNA expression	HUVEC; BAEC	Imatinib	↗ EC proliferation (≥1.2µM)	(Vallieres, Petitclerc, and Laroche 2009)
	Caspase assay; Hoechst staining; Annexin V staining; TUNEL assay	Human Pulmonary EC	Dasatinib	↗ EC apoptosis	(Guignabert et al. 2016)
	^{3}H-thymidine incorporation; WST-1 assay; MTT assay	HUVEC; HCAEC; HMEC-1; HCtAEC	Nilotinib	↘ EC proliferation	(Albrecht-Schgoer et al. 2013; Gover-Proaktor et al. 2017; Katgi et al. 2015)
	Annexin V staining	HUVEC	Nilotinib	= EC apoptosis	(Gover-Proaktor et al. 2017)
	Caspase assay; Annexin V staining	HCAEC; HUVEC	Ponatinib	↗ EC apoptosis	(Hadzijusufovic et al. 2016; Gover-Proaktor et al. 2017)
	^{3}H-thymidine incorporation; WST-1 assay	HUVEC; HMEC-1; EPC	Ponatinib	↘ EC proliferation	(Hadzijusufovic et al. 2016; Gover-Proaktor et al. 2017)

Oxidative stress	Fluorescent ROS detection; Immunofluorescence (8-oxo-dG)	Human Pulmonary EC; Rat lung	Imatinib	= endothelial ROS	(Guignabert et al. 2016)
	Fluorescent ROS detection; Immunofluorescence (8-oxo-dG)	Human Pulmonary EC; Rat lung	Dasatinib	↗ endothelial ROS	(Guignabert et al. 2016)
EC migration	Wound scratch assay; Microchemotaxis assay; Transwell migration assay	HMEC-1; HUVEC; EA.hy 926 cell; HCAEC	Imatinib	= EC migration	(Hacker et al. 2007; Vrekoussis et al. 2006; Venalis et al. 2009; Gover-Proaktor et al. 2017)
	Wound scratch assay	HUVEC; HCAEC; HMEC-1	Nilotinib	↘ EC migration	(Albrecht-Schgoer et al. 2013)
	Transwell migration assay	HUVEC	Nilotinib	= EC migration	(Gover-Proaktor et al. 2017)
	Transwell migration assay	HUVEC	Ponatinib	↘ EC migration	(Gover-Proaktor et al. 2017)
Angiogenesis	Tube-formation assay	HMEC-1; HUVEC	Imatinib	= angiogenesis	(Venalis et al. 2009; Gover-Proaktor et al. 2017)
	Tube-formation assay	HUVEC; HCAEC; HMEC-1	Nilotinib	↘ angiogenesis	(Albrecht-Schgoer et al. 2013)
	Tube-formation assay	HUVEC	Nilotinib	= angiogenesis	(Gover-Proaktor et al. 2017)
	Tube-formation assay	HUVEC	Ponatinib	↘ angiogenesis	(Gover-Proaktor et al. 2017)
Permeability	Permeability to albumin	EA.hy 926 cell	Imatinib	↗ endothelial permeability (10μM)	(Vrekoussis et al. 2006)
	Immunofluorescence (VE-cadherin)	EA.hy 926 cell; HPAEC	Imatinib	↘ membrane VE-cadherin (10μM)	(Vrekoussis et al. 2006; Letsiou et al. 2015)

	BAL protein levels	Mice (2-hit model of ALI)	Imatinib	↘ BAL protein levels	(Rizzo, Sammani, et al. 2015; Letsiou et al. 2015)
	Permeability to FITC-Dextran; Permeability to HRP	HMEC-1; HUVEC; Human lung microvascular EC	Imatinib	= endothelial permeability	(Dasgupta et al. 2017; Aman et al. 2012)
	Immunostaining	HUVEC	Imatinib	↘ intercellular gaps	(Aman et al. 2012)
	Evans blue/albumin extravasation	Mice	Imatinib	↘ Evans blue extravasation	(Aman et al. 2012)
	Pulmonary microvascular permeability assay; Permeability assay (FITC-Dextran)	Mice; HMEC-1; HPAEC	Dasatinib	↗ endothelial permeability	(Dasgupta et al. 2017)
	Permeability assay (FITC-Dextran)	HRMEC	Dasatinib	↘ VEGF-induced permeability	(Kim and Suh 2017)
CAM expression	Confocal microscopy; ELISA; qRT-PCR; flow cytometry	HMEC-1; Pulmonary EC (rat lung); EA.hy926	Imatinib	= ICAM-1, VCAM-1 and E-selectin expression = soluble ICAM-1, VCAM-1 and E-selectin	(Venalis et al. 2009; Guignabert et al. 2016; Sukegawa et al. 2017)
	Immunoblotting (VCAM-1)	Human lung EC	Imatinib	↘ VCAM-1 expression	(Letsiou et al. 2015)
	Confocal microscopy	Pulmonary EC (rat lung)	Dasatinib	↗ ICAM-1, VCAM-1 and E-selectin expression	(Guignabert et al. 2016)
	ELISA	Rat	Dasatinib	↗ soluble ICAM-1, VCAM-1 and E-selectin	(Guignabert et al. 2016)
	qRT-PCR; flow cytometry	EA.hy926	Dasatinib	= ICAM-1, VCAM-1 and E-selectin expression	(Sukegawa et al. 2017)
	Unknown	HUVEC	Nilotinib	↗ ICAM-1, VCAM-1 and E-selectin expression (≥1μM)	(Albrecht-Schgoer et al. 2013)

	qRT-PCR; flow cytometry	EA.hy926	Nilotinib	↗ ICAM-1, VCAM-1 and E-selectin expression	(Sukegawa et al. 2017)
Secretory	ELISA (IL-6; IL-8)	Stimulated HPAEC	Imatinib	↘ IL-8 and IL-6 (LPS induced)	(Letsiou et al. 2015)
	qRT-PCR ; ELISA (IL-1β; IL-6; TNF-α)	EA.hy926 cell ; HUVEC	Imatinib	= IL-1β, IL-6 and TNF-α expression and production	(Sukegawa et al. 2017)
	qRT-PCR ; ELISA (IL-1β; IL-6; TNF-α)	EA.hy926 cell ; HUVEC	Dasatinib	= IL-1β, IL-6 and TNF-α expression and production	(Sukegawa et al. 2017)
	qRT-PCR ; ELISA (IL-1β; IL-6; TNF-α)	EA.hy926 cell ; HUVEC	Nilotinib	= IL-6 and TNF-α expression and production ↗ IL-1β expression and production	(Sukegawa et al. 2017)
	ELISA (t-PA; PAI-1; ET-1; vWF; total NO)	HCtAEC	Nilotinib	↘ t-PA ↗ PAI-1, ET-1, vWF and total NO	(Katgi et al. 2015)
Adhesion	Unknown	HUVEC	Ponatinib	↗ adhesion to plastic surface at 1μM	(Hadzijusufovic et al. 2016)

8-oxo-dG: 8-hydroxy-2'-deoxyguanosine; ALI: acute lung injury; BAEC: bovine aortic endothelial cell; BAL: bronchoalveolar level ; EC: endothelial cell; ELISA: enzyme-linked immunosorbent assay; EPC: endothelial progenitor cell; ET-1: endothelin 1; FITC: fluorescein isothiocyanate; HCAEC: human coronary artery endothelial cell; HCtAEC: human carotid artery endothelial cell; HMEC-1: human microvascular endothelial cell; HPAEC: human pulmonary artery endothelial cell; HRMEC: human retinal microvascular endothelial cells; HUVEC: human umbilical vein endothelial cell; ICAM-1: intercellular adhesion molecule 1; IL: interleukin; LPS: lipopolysaccharide; NO: nitric oxide; PAI-1: plasminogen activator inhibitor-1; ROS: reactive oxygen species; t-PA: tissue plasminogen activator; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling; VCAM-1: vascular cell adhesion molecule 1; VE-cadherin: vascular endothelial cadherin; vWF: Von Willebrand factor

Inflammation

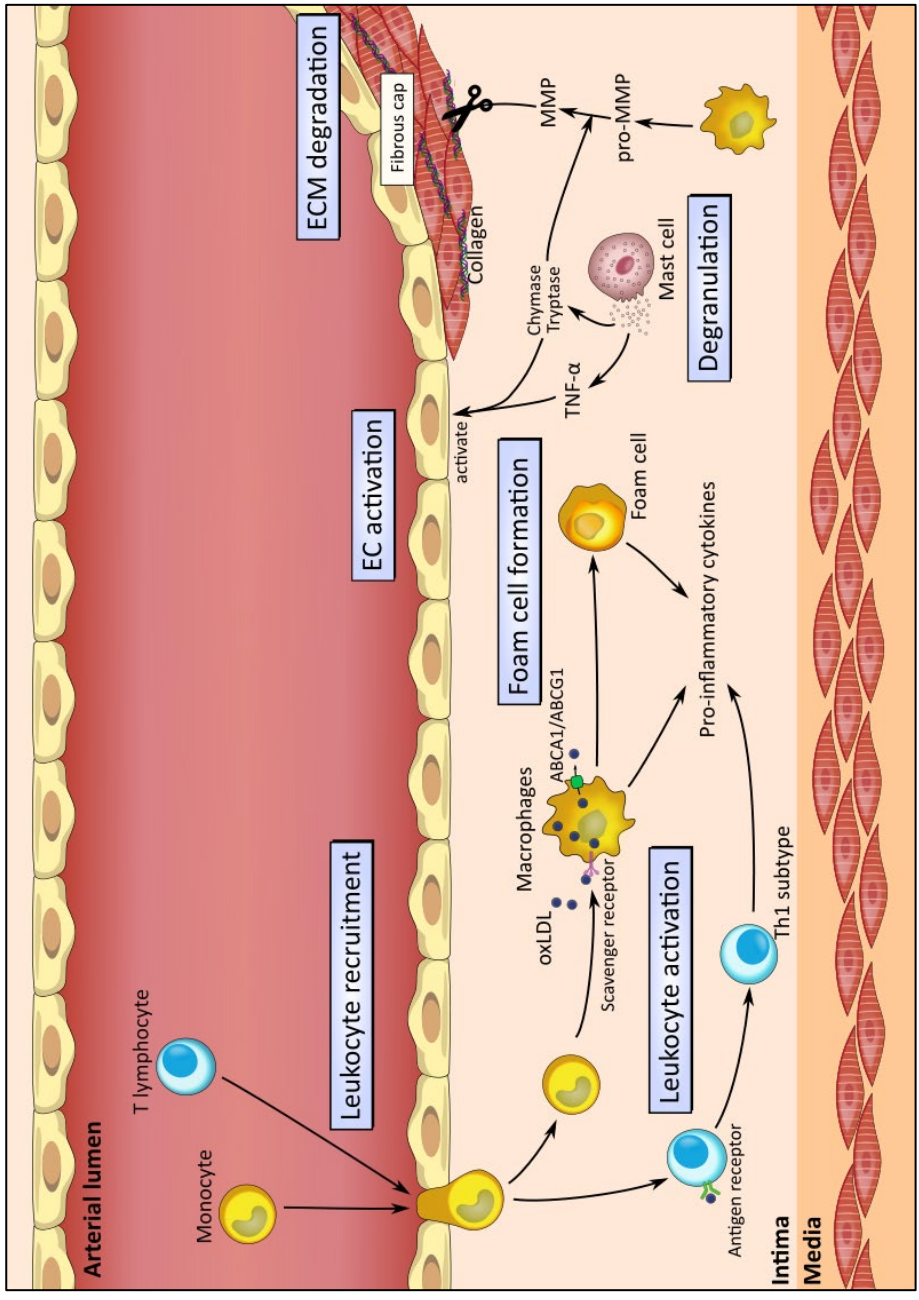
Figure 3.5 and **box 3.2** describe the role of immune cells and inflammation process during atherosclerosis. **Table 3.3** summarizes i*n vitro* studies that investigate impacts of BCR-ABL TKIs on survival, proliferation and major functions of monocytes, macrophages and T-lymphocytes. Globally, *in vitro* studies demonstrate that imatinib inhibits the development and maturation of monocytes and alters monocyte functions.(Dewar et al. 2003; Brownlow et al. 2009) Imatinib decreases production of pro-inflammatory cytokines (i.e. TNF-α and IL-6) and diminishes the potential of monocytes to phagocytose.(Dewar, Doherty, et al. 2005; Dewar, Cambareri, et al. 2005) These impacts on monocyte functions are possibly related to c-fms inhibition.(Metharom et al. 2011) Imatinib also inhibits macrophage functions *in vitro*. Imatinib decreases lipid uptake without impacting the lipid efflux and decreases activity and secretion of 2 matrix metalloproteinases (MMPs) (i.e. MMP-2 and MMP-9) on a post-transcriptional level.(Gacic et al. 2016) Additionally, imatinib inhibits T-lymphocyte activation and proliferation and decreases pro-inflammatory cytokines secretion (i.e. IFN-γ).(Cwynarski et al. 2004) The inhibition of monocytes, macrophages and T-cell functions by imatinib might prevent the development of atherosclerosis or reduce the risk of atherosclerotic plaque rupture. Effects of new generation TKIs on inflammatory cells were less studied but first experiments indicate similarities with imatinib about its impact on monocytes and macrophages. Both dasatinib and nilotinib have similar inhibitory profile on macrophage-colony formation that has been linked to CSFR inhibition.(Brownlow et al. 2008; Brownlow et al. 2009) Dasatinib also possesses anti-inflammatory functions by attenuating pro-inflammatory cytokine production (i.e. TNF-α, IL-6 and IL-12) by macrophages and increasing production of anti-inflammatory mediators (i.e. IL-10).(Ozanne, Prescott, and Clark 2015) These effects are thought to be mediated by SIK inhibition, a subfamily of three serine/threonine kinases that regulate macrophage polarization.(Ozanne, Prescott, and Clark 2015; Darling et al. 2017) Finally, dasatinib is associated

with decreased T-cell functions and particularly it decreases the production of pro-inflammatory cytokines (e.g. TNF-α, IFN-γ) and chemotactic mediators.(Schade et al. 2008) Nilotinib and bosutinib also possess anti-inflammatory activity and decrease cytokine production and T-cell activation.(Blake, Lyons, and Hughes 2009; Ozanne, Prescott, and Clark 2015) Inhibition of Lck, a tyrosine kinase implicated in T-cell receptor (TCR) signaling, is implicated in the impairment of T-cell functions by dasatinib and nilotinib.(Blake et al. 2008; Lee et al. 2010) It has been hypothesized that nilotinib decreases mast cell activity through c-kit inhibition (Aichberger et al. 2011a; Farha et al. 2014) which might result to a decrease of the vascular repair system.(Aichberger et al. 2011a; Albrecht-Schgoer et al. 2013) Clinical profile of nilotinib in patients with CML consolidates this hypothesis and demonstrates a decreased of mast cell level.(Albrecht-Schgoer et al. 2013) However, similar decreased of mast cells is also reported with imatinib without a high rate of arterial thrombosis.(Juurikivi et al. 2005)

Globally, BCR-ABL TKIs possess reassuring profile on inflammatory cells. However, impact of new generation TKIs on several functions of macrophages has not been assessed (e.g. MMP secretion and activity, lipid uptake and foam cell formation) whereas the effect of ponatinib on inflammatory cells is unknown. The assessment of lipid uptake and foam cell formation is particularly relevant with new generation TKIs because there are numerous interactions between TKIs and ABC transporters.(Sun et al. 2014; Yvan-Charvet, Wang, and Tall 2010)

Fibrous cap thickness

Figure 3.6 and **box 3.3** describe the mechanism by which atherosclerotic plaque ruptures and induces arterial thrombosis. **Table 3.4** summarizes *in vitro* and *in vivo* experiments performed on vascular smooth muscle cells (VSMCs) and fibroblasts. Imatinib decreases VSMC proliferation and growth but results are conflicting about its impact on apoptosis. Some studies demonstrate no


Arterial lumen
T lymphocyte
Monocyte
Leukocyte recruitment
EC activation
ECM degradation
Fibrous cap
Collagen
MMP
pro-MMP
Chymase
Tryptase
Mast cell
Degranulation
TNF-α
activate
Foam cell formation
Macrophages
ABCA1/ABCG1
oxLDL
Scavenger receptor
Foam cell
Pro-inflammatory cytokines
Leukocyte activation
Antigen receptor
Th1 subtype
Intima
Media

Box 3.2 | **Inflammation during atherosclerosis**

Chronic inflammation is a key feature of atherosclerotic diseases and is involved in all stages of the disease, from atheroma formation to plaque rupture.(Manduteanu and Simionescu 2012) Inflammation is initiated by retention and oxidative modification of lipids and lipoproteins in the vessel wall that results in monocyte chemotaxis and recruitment of blood immune cells (particularly monocytes and T lymphocytes) (**Figure 3.5**).(Manduteanu and Simionescu 2012) Thereafter, immune cells initiate inflammatory reactions, with transformation of monocytes to macrophages and activation of T lymphocytes, predominantly to pro-inflammatory lymphocytes (i.e. Th1 subtype).(Gorbet and Sefton 2004) Macrophages within the atheroma uptake oxidized lipoprotein by scavenger receptor (e.g. SR-A and CD36) and transform to foam cells.(Manduteanu and Simionescu 2012) Macrophages also express and secrete matrix metalloproteinases (MMPs) that degrade the extracellular matrix, thin the fibrous cap and subsequently destabilize the atherosclerotic plaque.(Silvestre-Roig et al. 2014) Macrophages, foam cells and activated T-cells release high content of pro-inflammatory mediators (e.g. IL-6, IL-12 and TNF- α), accentuating inflammation within the intima. Finally, mast cells represent only a small proportion of cells in atherosclerosis but are important players in plaque growth and destabilization.(Shi, Bot, and Kovanen 2015) During atherosclerosis, they are recruited within the intima and may degranulate, inducing release of high content of TNF-α, tryptase and chymase, two enzymes able to activate MMP proform.(Libby 2002) Episodes of acute inflammation are notably associated with rapid atherosclerosis development and play a role in plaque rupture or fissuring, increasing the inflammatory process within the plaque.(Jackson 2011)

◀ **Figure 3.5** | **Inflammation during atherosclerosis**

ABC: ATP-binding cassette; EC: endothelial cell; ECM: extracellular matrix; MMP: matrix metalloproteinase; oxLDL: oxidized low-density lipoprotein; TNF: tumor necrosis factor

Table 3.3 | *In vitro* studies on effects of BCR-ABL TKIs on proliferation, survival and major functions of monocytes, macrophages and T-lymphocytes.

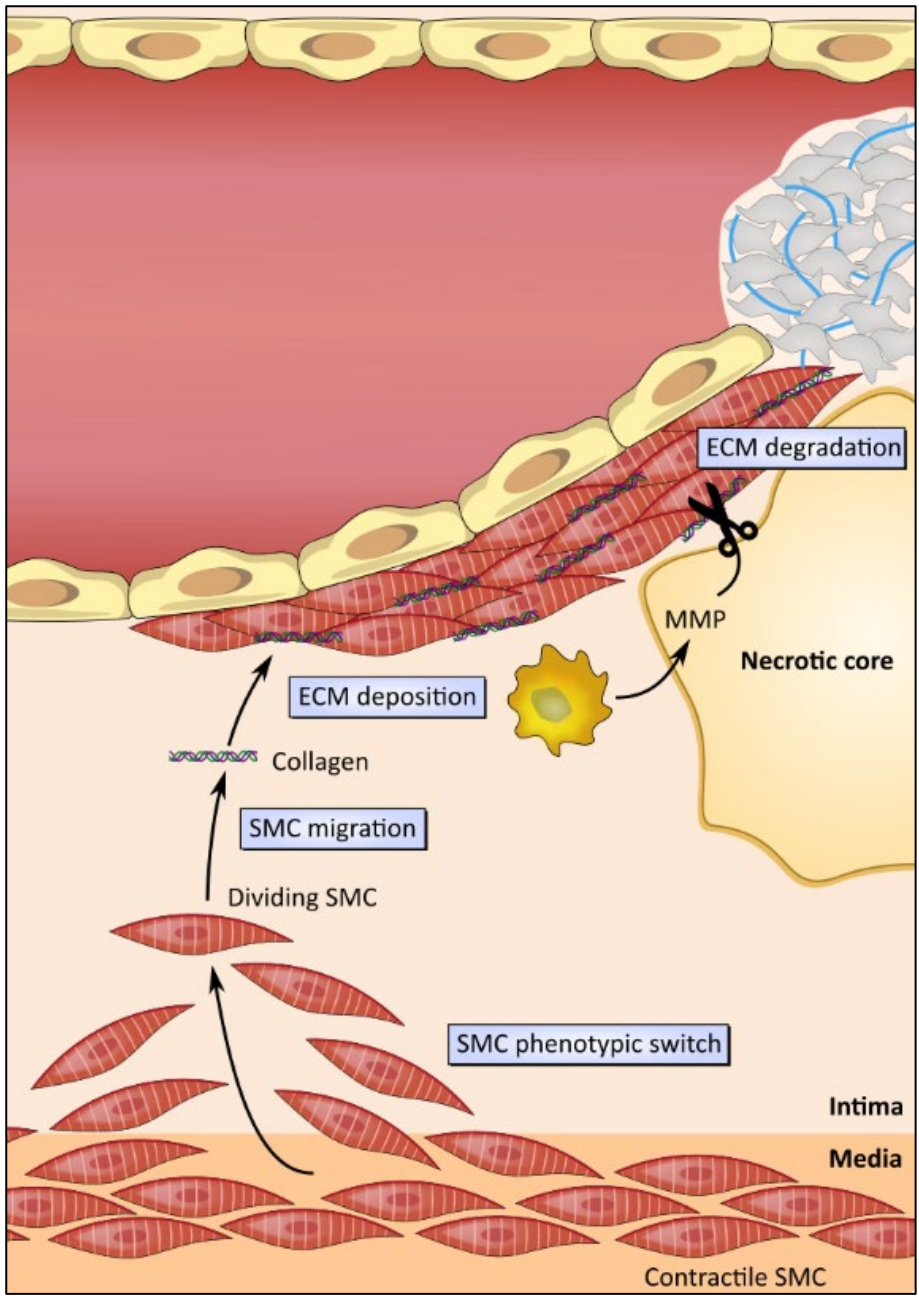
Endpoints	Methods	Models	TKIs	Findings	Ref
MONOCYTES/MACROPHAGES					
Proliferation/ survival	Propidium iodide staining	PBMC	Imatinib	= viability	(Wolf et al. 2005)
	Cell counting	Ovarian tumour ascite samples	Imatinib	↘ macrophage production	(Brownlow et al. 2009)
	Cell counting	Ovarian tumour ascite samples	Dasatinib	↘ macrophage production	(Brownlow et al. 2009)
	WST-1 assay	Human macrophages	Ponatinib	= macrophage viability	(Gover-Proaktor et al. 2017)
Monocyte differentiation	Morphology assessment	Human monocyte	Imatinib	↘ differentiation into macrophages	(Dewar et al. 2003)
Secretion	ELISA; qPCR	Human monocyte and macrophage; PBMC	Imatinib	↘ TNF-α, IL-6 and IL-8 production	(Dewar, Doherty, et al. 2005; Wolf et al. 2005)
	ELISA	PBMC; Human monocyte and macrophage	Imatinib	= IL-10 production	(Wolf et al. 2005)
	ELISA; bioplex system; nitrite assay	Raw 264.7; bone-marrow derived macrophage	Dasatinib	↘ TNF-α, IL-6, IL-12p40 and NO production	(Fraser et al. 2009; Ozanne, Prescott, and Clark 2015)
	qPCR; bioplex system	Primary macrophage (mice)	Dasatinib	↗ IL-10 production	(Ozanne, Prescott, and Clark 2015)

	Bioplex system	Bone-marrow derived macrophage	Bosutinib	↘ IL-6, IL-12p40 and TNF-α production	(Ozanne, Prescott, and Clark 2015)
	qPCR; bioplex system	Primary macrophage (mice)	Bosutinib	↗ IL-10 production	(Ozanne, Prescott, and Clark 2015)
Phagocytosis	Antigen-uptake assay	Human monocyte	Imatinib	↘ phagocytosis	(Dewar, Doherty, et al. 2005)
Cholesterol uptake	Cholesterol uptake assay	THP-1; PBMC	Imatinib	↘ LDL uptake	(Gacic et al. 2016)
	Cholesterol uptake assay	THP-1	Bosutinib	↘ LDL uptake	(Gacic et al. 2016)
MMP production/activity	Zymography	THP-1	Imatinib	↘ MMP-2 and MMP-9 secretion and activity	(Gacic et al. 2016)
T LYMPHOCYTES					
Proliferation/ survival	^{3}H-TdR incorporation; CFSE staining; triatide thymidine	Naïve CD4^{+} T-cell; Human T-cell	Imatinib	↘ T-cell proliferation	(Cwynarski et al. 2004; Seggewiss et al. 2005; Dietz et al. 2004)
	Annexin V staining; Caspase assay	Human T-cell	Imatinib	= T-cell apoptosis	(Cwynarski et al. 2004; Seggewiss et al. 2005; Dietz et al. 2004)
	Annexin V staining	Human T-cell	Imatinib	= T-cell apoptosis	
	CFSE dye	Human T-cell	Dasatinib	↘ T-cell proliferation	(Blake et al. 2008)
	Annexin V staining	PBMC; Human T-cell	Dasatinib	= T-cell viability	(Blake et al. 2008; Schade et al. 2008)
	CFSE dye	CD8^{+} T-cell; PBMC	Nilotinib	↘ T-cell proliferation	(Chen et al. 2008; Blake, Lyons, and Hughes 2009)

Secretion	ELISA	Human T-cell; $CD8^+$ and $CD4^+$ T-cell	Imatinib	↘ IFN-γ production	(Blake et al. 2008; Cwynarski et al. 2004)
	ELISA; Proteome profile array	Human T-cell; PBMC	Dasatinib	↘ TNF-α, IFN-γ, IL-2, IL-6, IL-17 production	(Schade et al. 2008; Blake et al. 2008)
	Proteome profile array	PBMC	Dasatinib	↘ chemotactic factors secretion (SDF-1, MIP-1α, MIP-1β, MCP-1, CXCL-1)	(Schade et al. 2008)
	ELISPOT assay	$CD8^+$ T-cell	Nilotinib	↘ IFN-γ production	(Chen et al. 2008)
Activation	Immunofluorescence	Human T-cell	Imatinib	↘ T-cell activation	(Cwynarski et al. 2004)
	Flow cytometry (CD25, CD69)	Human T-cell	Imatinib	= T-cell activation	(Dietz et al. 2004)
	Flow cytometry (CD25, CD69)	Human T-cell; PBMC	Dasatinib	↘ T-cell activation	(Schade et al. 2008; Blake et al. 2008)
	Flow cytometry (CD25, CD69)	Human T-cell	Nilotinib	↘ T-cell activation	(Chen et al. 2008)

CFSE: carboxyfluorescein succinimidyl ester; CXCL1: (C-X-C motif) ligand 1; ELISA: enzyme-linked immunosorbent assay; ELISPOT: enzyme-linked immunospot; IFN: interferon; IL: interleukin; MCP: monocyte chemoattractant protein-1; MIP-1: macrophage inflammatory protein 1; NO: nitric oxide; PBMC: peripheral blood mononuclear cell; qPCR: quantitative polymerase chain reaction; SDF-1: stromal cell-derived factor 1; TNF: tumor necrosis factor

impact on SMC apoptosis whereas others indicate increased SMC death.(Vallieres, Petitclerc, and Laroche 2009; Chen et al. 2006; Nakamura et al. 2012; Li et al. 2006; Rocha, Azevedo, and Soares 2007) Imatinib also affects VSMC functions and decreases their migration and LDL binding, inducing decreased LDL retention by the sub-endothelium.(Chen et al. 2006; Ballinger et al. 2010) Imatinib also exerts negative effect on the synthesis of major extracellular matrix components (type I collagen and fibronectin A) by fibroblasts, correlating to decreased extracellular matrix accumulation *in vivo*.(Distler et al. 2007) The impact of imatinib on SMCs is thought to be mediated by PDGFR inhibition (Nakamura et al. 2012) which is involved in several VSMCs functions including VSMC survival and plasticity.(Chen et al. 2006) Subsequent to the hypothesis that imatinib inhibits PDGFR signaling, prevents abundant SMC and fibroblast proliferation and inhibits abundant extracellular matrix accumulation; imatinib has been tested for the management of several fibrotic diseases (e.g. dermal and liver pulmonary fibrosis, systemic sclerosis).(Ma et al. 2012; Distler et al. 2007; Yoshiji et al. 2005) Imatinib successfully acts on pulmonary fibrosis and pulmonary arterial hypertension (i.e. a disease involving vascular remodeling mediated by pulmonary SMC proliferation),(Nakamura et al. 2012; Frost et al. 2015) and has beneficial activity in sclerotic chronic graft-versus-host disease.(Salhotra et al. 2015) Finally, imatinib was tested *in vivo* for the prevention of cardiovascular diseases and demonstrates efficacy for the treatment of myocardial fibrosis by reducing extracellular matrix component synthesis (i.e. procollagen I and III).(Ma et al. 2012) In a rat model, imatinib successfully inhibits stenosis after balloon injury and presents interest in intimal hyperplasia and stenosis after bypass grafts.(Rocha, Azevedo, and Soares 2007; Makiyama et al. 2008; Park et al. 2013; Leppanen et al. 2004; Li et al. 2006) Imatinib also successfully prevents arterial thrombosis following microvascular surgery in rabbits.(Sevim et al. 2012) Imatinib was also encompassed in a stent but do not demonstrate efficacy in restenosis prevention.(Hacker et al. 2007)


ECM degradation
MMP
Necrotic core
ECM deposition
Collagen
SMC migration
Dividing SMC
SMC phenotypic switch
Intima
Media
Contractile SMC

◄ **Figure 3.6** | **Fibrous cap thickness and plaque rupture**
ECM: extracellular matrix; MMP: matrix metalloproteinase; SMC: smooth muscle cell

Box 3.3 Fibrous cap thickness and plaque rupture

Atherosclerosis is considered as a fibroproliferative disease. Indeed, during atherogenesis, VSMCs undergo phenotypic switch from a contractile phenotype to a migratory and secretion phenotype. They acquire the capacity to migrate and produce extracellular matrix proteins, additionally to a greater capacity to proliferate.(Bennett, Sinha, and Owens 2016) The stability of atherosclerotic plaque correlates to the thickness of its fibrous cap, which is composed of SMC in a collagenous-proteoglycan matrix. Different mechanisms may contribute to fibrous cap thickening, notably a high MMP concentration (**Figure 3.6**) or high SMC mortality that directly reduce extracellular matrix content in the fibrous cap. Therefore, the content of VSMCs within the plaque and their capacity to secrete extracellular matrix components directly correlate with the thickness of the fibrous cap and therefore play a major role to differentiate rupture-prone plaque from stable plaque.(Bennett, Sinha, and Owens 2016) Additionally, VSMCs are involved in plaque repair in case of rupture through their capacity to proliferate and synthesize the extracellular matrix. Thus, the presence of VSMCs in advanced lesions is beneficial and the destabilization of atherosclerotic plaques might be due to alteration of the balance between VSMC proliferation and migration versus VSMC death and senescence that might promote atherogenesis and plaque instability.(Bennett, Sinha, and Owens 2016)

Table 3.4 | *In vitro* and *in vivo* studies on effects of BCR-ABL TKIs on proliferation, survival and major functions of smooth muscle cells and fibroblasts.

Endpoints	Methods	Models	TKIs	Findings	Ref
Proliferation /survival	Resazurin assay; immunofluorescence; ^{3}H-thymidine incorporation; BrdU incorporation; MTT assay	HVSMC; BAoSMC; PASMC; ASMC; VSMC; HAoSMC; HCASMC; Rabbit	Imatinib	↘ SMC proliferation	(Leppanen et al. 2004; Vallieres, Petitclerc, and Laroche 2009; Nakamura et al. 2012; Li et al. 2006; Rocha, Azevedo, and Soares 2007; Hacker et al. 2007; Perros et al. 2008)
	Caspase assay; PARP (Western blot); JC-1 dye; Annexin V staining	BAoSMC; Dermal fibroblast; PASMC	Imatinib	= SMC/fibroblast apoptosis	(Vallieres, Petitclerc, and Laroche 2009; Distler et al. 2007; Perros et al. 2008)
	TUNEL; caspase assay	PASMC; HAoSMC; Rabbit	Imatinib	↗ SMC apoptosis (PDGF-stimulated)	(Nakamura et al. 2012; Rocha, Azevedo, and Soares 2007; Leppanen et al. 2004)
	Trypan blue exclusion	HCASMC; A10 cell line	Imatinib	= SMC viability	(Hacker et al. 2007)
	Cell counting; Propidium iodide staining	A10 cell line, HAoSMC	Dasatinib	↘ SMC proliferation	(Chen et al. 2006; Wu, Chen, and Bhalla 2014)
Migration	Transwell cell migration assay	HAoSMC; PASMC; HCASMC; A10 cell	Imatinib	↘ SMC migration	(Rocha, Azevedo, and Soares 2007; Perros et al. 2008; Hacker et al. 2007)
	Transwell cell migration assay	HAoSMC; A10 cell	Dasatinib	↘ SMC migration	(Chen et al. 2006; Wu, Chen, and Bhalla 2014)
Secretion/ synthesis	Radiolabel incorporation	Human VSMC	Imatinib	↘ proteoglycan synthesis	(Ballinger et al. 2010)

	RT-PCR; Western blot; Sircol collagen assay	Dermal fibroblast	Imatinib	↘ COL1A1, COL1A2, fibronectin 1 synthesis ↘ collagen synthesis	(Distler et al. 2007)
	RT-PCR	Dermal fibroblast	Imatinib	= MMP-1, MMP-2, TIMP-1, TIMP-2, TIMP-3 and TIMP-4	(Distler et al. 2007)
	qRT-PCR	Human fibroblast	Nilotinib	Decreases COL1A1 and COL1A2 synthesis	(Marinelli Busilacchi et al. 2016)
Fibrosis	Sirius red staining	Rat	Imatinib	↘ myocardial fibrosis, liver fibrosis	(Ma et al. 2012; Yoshiji et al. 2005)
	Intima/media ratio	Rat (Balloon injury model)	Imatinib	↘ stenosis	(Makiyama et al. 2008; Park et al. 2013)
	Intima/media ratio	Rabbit	Imatinib	↘ intimal thickness	(Sevim et al. 2012)
	Hydroxyproline, collagen content	Rat liver	Imatinib	↘ hydroxyproline and collagen content	(Shiha et al. 2014)
	Hydroxyproline, collagen content	Rat liver	Nilotinib	↘ hydroxyproline and collagen content	(Shiha et al. 2014)
	Sirius red staining	Rat liver	Nilotinib	↘ liver fibrosis	(Shiha et al. 2014)

ASMC: arterial smooth muscle cell; BAoSMC: Bovine aortic smooth muscle cell; BrdU: bromodésoxyuridine; COL: collagen; HaOSMC: Human aortic smooth muscle cell; HCASMC: Human coronary artery smooth muscle cell; HVSMC: Human vascular smooth muscle cell; MMP: matrix metalloproteinase; PARP: poly(ADP-ribose) polymérase; PASMC: Pulmonary smooth muscle cell; PDGF: Platelet-derived growth factor; qRT-PCR: quantitative reverse transcription polymerase chain reaction; SMC: smooth muscle cell; TIMP: tissue inhibitor of metalloproteinase; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling; VSMC: Vascular smooth muscle cell

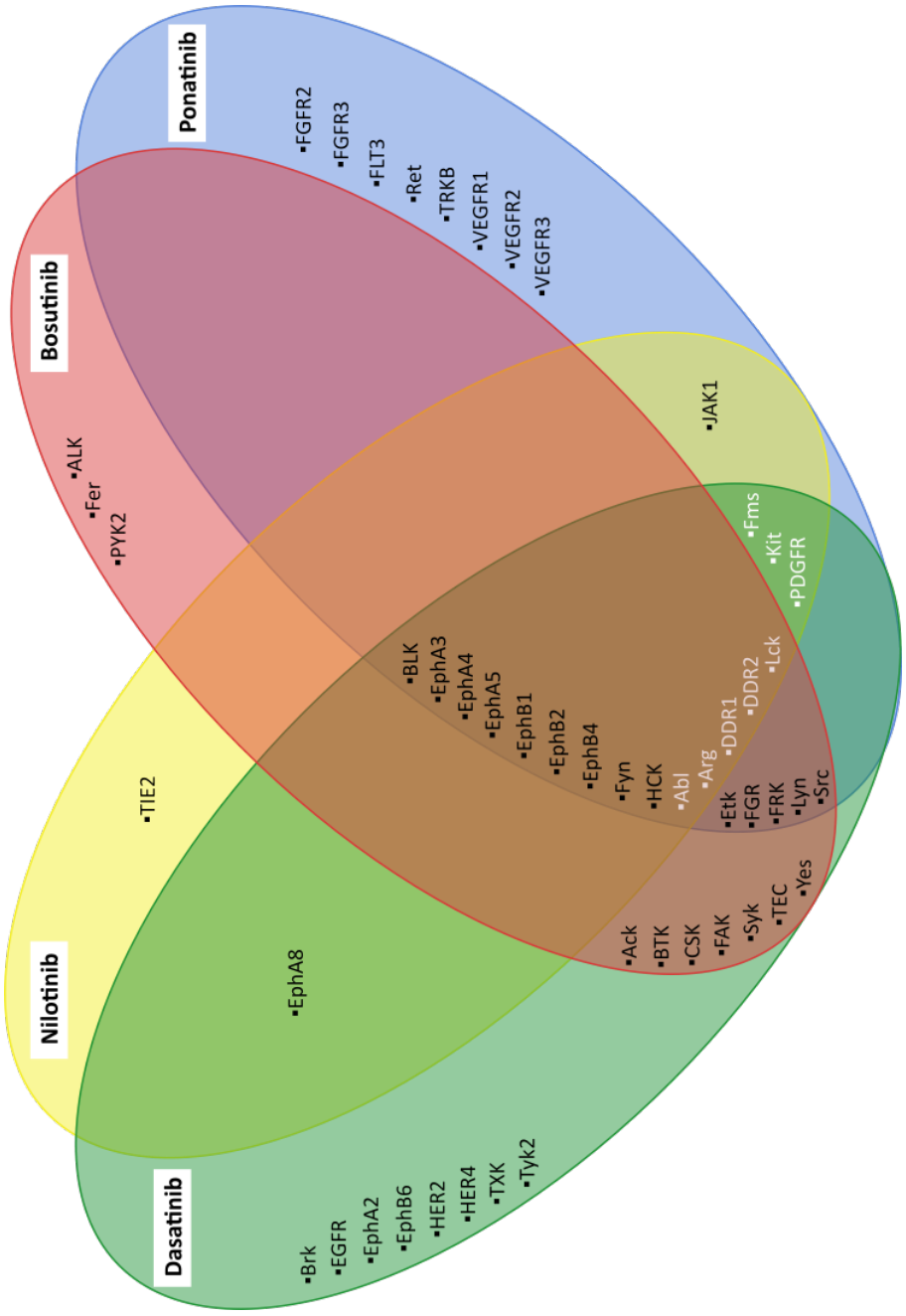
Impact of new generation TKIs on fibrosis was less studied but demonstrate similar inhibitory effect on VSMCs and fibroblasts. Indeed, dasatinib inhibits PDGFR more potently than imatinib,(Chen et al. 2006) and the hypothesis that dasatinib prevents restenosis similarly to imatinib was emitted. Therefore, a patent has been filed claiming the use of dasatinib for the prevention of stenosis and restenosis.(Wu, Chen, and Bhalla 2014) Compared with imatinib, dasatinib has additional off-targets and is able to inhibit Src, a kinase involved in dermal fibrosis in addition to PDGFR.(Sanchez-Ortega et al. 2012) Therefore, dasatinib was tested in patients with scleroderma-like chronic graft-versus-host disease, a disease resulting from inflammation and progressive fibrosis of the dermis and subcutaneous tissues, and first results are encouraging.(Sanchez-Ortega et al. 2012) Nilotinib also appears to be clinically efficient in scleroderma-like graft-versus-host disease by reducing collagen expression.(Marinelli Busilacchi et al. 2016) Finally, nilotinib was tested *in vivo* for the treatment of liver fibrosis and demonstrates decreased fibrotic markers and inflammatory cytokines (IL-1α, IL-1β, IFN-γ, IL-6).(Shiha et al. 2014) However, only low-dose nilotinib was found to be efficient against fibrosis and normalized collagen content.(Shiha et al. 2014) This lack of anti-fibrotic effect at higher doses might be explained by inhibition of additional off-targets by nilotinib that affects the benefit of low-dose nilotinib against fibrosis. Arterial thrombosis occurring with dasatinib and nilotinib are probably not the consequence of VSMC impairment but investigations should be performed on VSMCs rather than fibroblasts. Additional investigations are warranted to complete impact of BCR-ABL TKIs on VSMC functions (e.g. VSMC apoptosis, proliferation and migration) and confirm their safety toward VSMCs.

Off-Targets

BCR-ABL TKIs bind the highly conserved ATP binding site and are therefore not very specific to BCR-ABL and possess multiple cellular targets (kinases and non-kinase proteins).(Giles, O'Dwyer, and Swords 2009; Rix et al. 2007) This allowed the possibility to exploit them in other indications (e.g. PDGFR

inhibition by imatinib is used in BCR-ABL negative chronic myeloid disorders),(Pardanani and Tefferi 2004) but this may also induce toxicity and side effects.(Giles, O'Dwyer, and Swords 2009) The development of arterial thrombotic events with new generation BCR-ABL TKIs is likely to be related to inhibition of off-target receptors and kinases, as described throughout this review. **Figure 3.7** describes inhibitory profiles of imatinib, dasatinib, nilotinib, bosutinib and ponatinib. Globally, imatinib is the most selective BCR-ABL TKIs whereas dasatinib and ponatinib inhibit numerous off-targets.

However, inhibitory profiles are difficult to determine and several research published discrepancies. For conflicting results, a conservative approach has been applied in **Figure 3.7** but supplementary information (**Table 3.6**) describes the tyrosine kinase selectivity profile of the five BCR-ABL TKIs and indicate divergences between studies.(Rivera et al. 2014; Bantscheff et al. 2007; Rix et al. 2007; Weisberg et al. 2005; Buchdunger et al. 2000) These discrepancies can be explained by the difference in drug concentration and methodologies. To date, several methods have been used to determine the inhibitory profile of BCR-ABL TKIs including *in vitro* kinase assay (Weisberg et al. 2005; Buchdunger et al. 2000; Manley et al. 2010) kinase expression in bacteriophages (Fabian et al. 2005) and affinity purification methods combined with mass spectrophotometry.(Bantscheff et al. 2007; Rix et al. 2007) However, all these methods suffer from caveats, including the incompatibility to perform live-cell studies. A cell-permeable kinase probe was developed to figure out this problem, but this assay is still limited by the number of off-target tested (i.e. it requires to predefine tested off-targets) and therefore, the missing of targets is possible.(Shi et al. 2012) For this reason, the inhibitory activity of each TKI has not been tested toward all tyrosine kinases and **Figure 3.7** only includes off-targets for which at least one of the five BCR-ABL TKI has been tested. Thus, inhibitory profiles need to be carefully considered and it has to keep in mind that BCR-ABL TKI metabolites may possess activity against supplemental off-targets.


Nilotinib
Bosutinib
Ponatinib
Dasatinib
TIE2
ALK
Fer
PYK2
FGFR2
FGFR3
FLT3
Ret
TRKB
VEGFR1
VEGFR2
VEGFR3
JAK1
EphA8
BLK
EphA3
EphA4
EphA5
EphB1
EphB2
EphB4
Fyn
HCK
Abl
Arg
DDR1
DDR2
Lck
Fms
Kit
PDGFR
Ack
BTK
CSK
FAK
Syk
TEC
Yes
Etk
FGR
FRK
Lyn
Src
Brk
EGFR
EphA2
EphB6
HER2
HER4
TXK
Tyk2

▲ **Figure 3.7** | **Specificity of imatinib, dasatinib, nilotinib and ponatinib toward tyrosine kinases.** Green, yellow, red and blue circles contain tyrosine kinase inhibited by dasatinib, nilotinib, bosutinib and ponatinib respectively. Tyrosine kinases in white represent imatinib off-targets. This figure summarizes results from 13 experiments.(Rivera et al. 2014; Bantscheff et al. 2007; Rix et al. 2007; Weisberg et al. 2005; Buchdunger et al. 2000; Albrecht-Schgoer et al. 2013; Canning et al. 2014; Hantschel, Rix, and Superti-Furga 2008; Manley et al. 2010; Fabian et al. 2005; Shi et al. 2012; Remsing Rix et al. 2009; Green, Newton, and Fancher 2016) In case of conflictual results between studies, a conservative approach has been applied. Additional information is provided in supplementary files.

As described over this review, PDGF signaling has countless effects on several cells and tissues and is involved into several pro-atherogenic mechanisms (e.g. adipogenesis, vascular leakage, VSMC viability and functions) and vascular homeostasis, which led to the suggestion of its implication in the potential beneficial cardiovascular effect of imatinib.(Leppanen et al. 2004; Liu et al. 2011; Rocha, Azevedo, and Soares 2007) However, dasatinib, nilotinib and ponatinib also inhibit PDGFR but increase the risk of arterial occlusive events. This difference of clinical outcome might be explained by the concentration of BCR-ABL TKIs necessary to obtain a same degree of PDGFR inhibition.(Rivera et al. 2014) Indeed, Rivera et al. reported that when adjusted to the maximum serum concentration, imatinib inhibits more profoundly PDGFR than dasatinib, nilotinib and ponatinib.(Rivera et al. 2014) Therefore, at effective concentration, it is probable that the degree of PDGFR inhibition is too low with dasatinib, nilotinib and ponatinib to obtain the beneficial effect of PDGFR inhibition on atherosclerosis. Another possible hypothesis concerns the less conclusive specificity of new generation TKIs which leads to inhibition of additional off-targets that might counterbalance the positive effect of PDGFR inhibition.

Other tyrosine kinases have been incriminated in the occurrence of arterial thrombosis with new generation TKIs. DDR-1 possesses functions in vascular homeostasis, atherogenesis and is expressed in pancreatic islet cells. However, and similarly to PDGFR, it is inhibited by all BCR-ABL TKIs.(Gora-Tybor, Medras, Calbecka, Kolkowska-Lesniak, et al. 2015; Aichberger et al. 2011a) Other hypotheses include impairment of VEGF signaling by ponatinib(Rivera et al. 2014; Hadzijusufovic et al. 2016) or the inhibition of several ephrin receptors by new generation TKIs but not by imatinib which might inhibit monocyte recruitment.(Sakamoto et al. 2011) Finally, it has been suggested that the inhibition of c-Abl itself is implicated in the increase of the cardiovascular risk. Indeed, imatinib possesses lower inhibitory effect on c-Abl that new generation TKIs, which might further explain the difference in cardiovascular safety.(Rivera et al. 2014) Additionally, c-Abl modulates Tie-2, a tyrosine kinase that possesses an important effect on endothelial cell

function, angiogenesis and inflammation.(Khan et al. 2014; Chislock, Ring, and Pendergast 2013)

Perspectives and Conclusions

This review summarizes the data underlying the potential preventive effect of imatinib on the occurrence of arterial thrombosis. Globally, *in vitro* and *in vivo* experiments demonstrate that imatinib possesses anti-platelet activity, hypolipidemic and hypoglycemic effects and inhibits inflammation and atherosclerosis development in several cell types (i.e. decreases of inflammatory cell and VSMC functions and increased vascular permeability). These benefits were largely attributed to PDGFR inhibition. It is currently unknown why new generation TKIs that also inhibit PDGFR present opposite cardiovascular safety profile and this point need to be elucidated.

New generation BCR-ABL TKIs increase the risk of arterial thromboembolism with different clinical features (e.g. time-to-event and absolute rate) and are associated with different safety profiles, suggesting that different pathways explain the pathophysiology. The safety profile of nilotinib is mostly characterized by impaired glucose and lipid metabolism. However, both the molecular mechanisms of these alterations and their impact on the occurrence of arterial thrombosis are unknown. Both dasatinib and ponatinib exhibit anti-platelet effect whereas it was recently suggested that nilotinib potentially induces pro thrombotic phenotype of platelets. Based on the clinical characteristics and case-reports, atherosclerosis appears the most plausible mechanisms by which new generation TKIs induce arterial thrombosis. However, *in vitro* and *in vivo* studies of viability and functions of SMCs and inflammatory cells demonstrate reassuring impact of dasatinib and nilotinib, even if additional studies are required to complete this evaluation. However, first experiments indicate that dasatinib, nilotinib and ponatinib influence endothelial cell survival and/or endothelium integrity, suggesting a reasonable hypothesis by which new generation TKIs induce atherosclerosis development and subsequently, arterial thrombosis. Additional studies on

the shedding of functional extracellular vesicles by endothelial cells might be interesting regarding their important role in coronary artery diseases.(Boulanger et al. 2017) Finally, the impact of new generation TKIs on human blood coagulation and fibrinolysis has never been studied and should be addressed.

To conclude, new generation TKIs increase the risk of arterial thrombosis in patients with CML, whereas imatinib, the first generation TKI might prevent the development of cardiovascular events. To date, the cellular events and signaling pathways by which these events occurred are unknown and research are extremely limited, focusing mainly on imatinib and nilotinib. Research needs to be extended to all new generation BCR-ABL TKIs (i.e. dasatinib, bosutinib and ponatinib). The understanding of the mechanisms by which new generation BCR-ABL TKIs induce or promote arterial occlusive events will improve the clinical uses of these therapies. To date, only general risk minimization measures have been proposed (e.g. management of dyslipidaemia, diabetes, arterial hypertension following standard of care).(Haguet et al. 2017; Douxfils, Haguet, Mullier, Chatelain, Graux, and Dogné 2016; Moslehi and Deininger 2015; Aghel, Delgado, and Lipton 2017; Damrongwatanasuk and Fradley 2017; Valent et al. 2017; Garcia-Gutierrez et al. 2016) The understanding of the pathophysiology is required to implement the most appropriate risk minimization strategies for thrombotic events and to select patients to whom the prescription of these drugs should be avoided when applicable. Finally, the understanding of the pathophysiology will help in the design of new BCR-ABL inhibitors sparing the toxic targets.

Review criteria

Relevant articles published from the database inception to July 11, 2017 were identified from an electronic database (PubMed) using the keywords “vascular”, “thrombosis”, “atherosclerosis”, “arteriosclerosis”, “venous”, “arterial”, “hemostasis”, “metabolic”, “metabolism”, “glycemia”, “glycaemia”, “cholesterol”, “triglycerides” and “platelet” combined with the 5 approved BCR-ABL TKIs. The search strategy is presented in

supplementary files. Articles published in languages other than English were excluded from the analysis. Primary criteria were pathophysiological explanation of arterial thrombotic events. Abstracts and full-text articles were reviewed with a focus on atherogenesis, plaque rupture, platelet functions and their link with the development of arterial thrombosis with BCR-ABL TKIs. The reference section of identified articles was also examined.

Author contributions: HH was responsible for the first draft of the manuscript. FM, CC and CG, JMD and JD contributed to the final draft of the manuscript.

Supplemental materials

Clinical Characteristics of Vascular Occlusive Events with BCR-ABL TKIs

Table 3.5 summarizes time and location characteristics of arterial occlusive events associated with BCR-ABL TKIs. A link exists between location of the obstructed artery and the clinical presentation. With dasatinib, nilotinib and ponatinib, arterial occlusive events occurred preferentially in the cardiovascular circulation leading to myocardial infarction and angina pectoris. (Dahlen et al. 2016; Sam et al. 2016; Hochhaus et al. 2016; Mauro et al. 2016; Gugliotta et al. 2015; Stève-Dumont et al. 2015; Fossard et al. 2016) Though, numerous patients also developed ischemic events in both the peripheral and cerebral circulation. To our knowledge, the etiology of these events is unknown. Peripheral and cerebral occlusive events might be the result of embolism or might be the consequence of on-site atherosclerotic lesion rupture/erosion.(Lyaker et al. 2013) The differentiation between the two is difficult to establish because both of them shared similar clinical pictures. It has to be noted that arterial emboli can arise both from aortic atherosclerotic lesions and from atrial fibrillation. Nilotinib and ponatinib have been both associated with common occurrence of atrial fibrillation whereas these events occurred rarely with imatinib.(European Medicines Agency 2017c, 2017b, 2017a) Nevertheless, no causal relationship have been established and the incidence of drug-induced atrial fibrillation is too low to be responsible of the majority of arterial occlusive events with these two treatments. Remarkably, numerous patients taking nilotinib developed peripheral arterial occlusive disease with preferential tropism for lower limbs and small-vessel, indicating a pathology close to those found in diabetic patients, and potentially linking nilotinib-induced diabetes to the development of peripheral occlusive events.(Kim et al. 2013; Aichberger et al. 2011b) This indicate that peripheral arterial occlusive disease occurring with nilotinib might be the result of atheroemboli that come from atherosclerotic lesions and are formed by small cholesterol crystal particles, rather than arterial emboli.(Lyaker et al. 2013)

Table 3.5 | Clinical features of vascular occlusive events with new generations BCR-ABL TKIs.

	Dasatinib	Nilotinib	Bosutinib	Ponatinib
Relative risk (OR; 95%CI)				
VOEs (Douxfils et al. 2016)	2.91 (1.43-5.94)	3.48 (2.21-5.49)	1.77 (0.54-5.83)	3.47 (1.23-9.78)
VTEs (Haguet et al. 2017)	2.24 (0.68-7.42)	1.80 (0.45-7.15)	Unknown	7.29 (0.15-367.61)
AOEs (Haguet et al. 2017)	3.32 (1.37-8.01)	3.69 (2.29-5.95)	1.77 (0.54-5.83)	3.26 (1.12-9.50)
Absolute risk (major AOEs) (/100p-y)	1.1 (Chai-Adisaksopha et al. 2016)	2.8 (Chai-Adisaksopha et al. 2016)	Unknown	10.6 (Chai-Adisaksopha et al. 2016) First year: 15.1 (Cortes et al. 2014)
Arterial location	Coronary > PAOD > Cerebral (Sam et al. 2016) Coronary > Cerebral > PAOD (Cortes et al. 2016)	Coronary > Cerebral > PAOD (Sam et al. 2016) Coronary > PAOD > Cerebral (Gugliotta et al. 2015) Coronary > Cerebral = PAOD (Hochhaus et al. 2016)	Unknown	Coronary > PAOD = Cerebral (Sam et al. 2016; Lipton et al. 2016; Cortes et al. 2014) Coronary > PAOD > Cerebral (Jabbour et al. 2016; Mauro et al. 2016)
Time to event (months)*	19-22 (Gora-Tybor, Medras, Calbecka, Kolkowska-Lesniak, et al. 2015)	42-47 (Fossard et al. 2016; Gugliotta et al. 2015)	Unknown	
Risk factors associated with VOE	Unknown	Age >65 years (Gugliotta et al. 2015) Dyslipidemia (Fossard et al. 2016) Gender: male (Fossard et al. 2016) High dose (800mg) (Fossard et al. 2016; Hochhaus et al. 2016) High homocysteinemia (Fossard et al. 2016) Cardiovascular risk factor at baseline (Gugliotta et al. 2015) High glycated hemoglobin (Fossard et al. 2016)	Unknown	Age >60 years (Cortes et al. 2014) Prior VOE (Cortes et al. 2014) Arterial hypertension (Cortes et al. 2014) Diabetes (Cortes et al. 2014) High dose ponatinib (Cortes et al. 2014)

* Times to event for arterial occlusive disease

AOE: arterial occlusive event; CI: confidence interval; OR: odd ratio; PAOD: peripheral arterial occlusive disease; p-y: patient-year; VOE: vascular occlusive events; VTE: venous thromboembolism

However, even if the three new generations TKIs are close regarding the location of arterial thrombosis, there are discrepancies regarding the delay between the initiation of therapy and the development of cardiovascular events (**Table 3.5**). For ponatinib, arterial occlusive events developed quickly and are more frequent during the first and the second year of treatment (14.5% and 14.1% respectively) than after (10.5% during the 3rd year and 7.2% after 3 years).(Cortes et al. 2014) However, it appears unclear if this reduction over time is real or is an artifact due to ponatinib dose reduction (i.e. recommendations were provided by authorities to decrease ponatinib doses in clinical trials to minimize the risk of blood clots).(Cortes, Pinilla-Ibarz, et al. 2016) Nilotinib associated cardiovascular events developed less quickly and the rate of these events is constant over time with gradual increase of cardiovascular events and no plateau obtain after 100 months of treatment.(Fossard et al. 2016) It takes longer in young patients and in patients without prior risk factors to develop an arterial thrombotic events with nilotinib,(Bondon-Guitton et al. 2016) fostering the hypothesis of progressive development of stenosis or progression of pre-existing atherosclerotic lesions. For imatinib, dasatinib and bosutinib, few data are available. One report described a short time-to-event with dasatinib,(Gora-Tybor, Medras, Calbecka, Kolkowska-Leśniak, et al. 2015) whereas the rare arterial occlusive events in patients treated with imatinib occur very lately.(Fossard et al. 2016)

Table 3.5 also reports risk factors linked to the risk of arterial thromboembolism with new generation BCR-ABL TKIs. Cardiovascular risk factors are difficult to highlight due to the small size of the population in clinical trials and the selection bias (e.g. older patients are usually excluded from clinical trials). However, several analyses demonstrated that arterial occlusive events occurred preferentially in nilotinib treated patients with pre-existing atherosclerotic risk factor(s) (i.e. arterial hypertension, overweight, smoking, hypercholesterolemia, diabetes mellitus) rather than in patients with no comorbidities, but that neither antiplatelet drugs nor lipid-lowering treatments seem to prevent the occurrence of these events.(Bondon-Guitton et al. 2016; Stève-Dumont et al. 2015; Brauchli et al. 2010; Fossard et al. 2016)

Similarly, arterothrombotic events in ponatinib treated patients also occurred preferentially in patients with cardiovascular risk factors. In correlation with the occurrence of arterial thrombotic events mostly in high-risk patients, case reports raised the concern that peripheral arterial occlusive disease occurred preferentially on pathological arteries. Indeed, several case reports described nilotinib associated-peripheral arterial occlusive disease resistant to surgical and medical treatment (i.e. stent implantation, aspirin), suggesting a negative impact of nilotinib on pre-existing atherosclerotic lesions, and possibly on vascular endothelium compliance.(Maurizot, Beressi, Manéglier, et al. 2014; Gautier et al. 2015; Tefferi 2013; Tefferi and Letendre 2011a) Symptoms of peripheral arterial occlusive disease usually improve when nilotinib is discontinued, without surgery.(Mirault et al. 2015) An autopsy of a nilotinib treated patient subsequently to myocardial infarction also revealed obliterating artery disease (i.e. obliterating coronary sclerosis and atherosclerosis of the infra-renal aorta).(Brauchli et al. 2010) Yet, it is important to note that with both nilotinib and ponatinib, arterial thromboembolism also occurred in young patients, without prior risk factors or without atherosclerotic lesions prior to TKI initiation.(Bondon-Guitton et al. 2016; Stève-Dumont et al. 2015; Mirault et al. 2015; Tefferi and Letendre 2011a) For dasatinib and bosutinib, too low arterial occlusive events were reported to correlate risk factors and arterial occlusive events.(Cortes et al. 2014) For the future, it will be interesting to obtain more data on these cardiovascular events that may occur during dasatinib and bosutinib treatments to try to identify patients at risk and develop predictive scores similarly than those existing with nilotinib.(Breccia et al. 2015a)

Method

Literature search was performed on PubMed on April 4, 2016 and updated on **July 11, 2017.** Articles published in languages other than English were excluded from the analysis. Primary criteria were pathophysiological explanation of arterial thrombotic events. Abstracts and full-text articles were reviewed with a focus on atherogenesis, plaque rupture, platelet functions and their link with the development of arterial thrombosis with BCR-ABL TKIs. The reference section of identified articles was also examined.

Keywords Searched in Titles and Abstracts

- Vascular
- Thrombosis
- Atherosclerosis
- Arteriosclerosis
- Cardiovascular
- Venous
- Arterial
- Hemostasis
- Metabolic
- Metabolism
- Glycemia
- Glycaemia
- Cholesterol
- Triglyceride
- Platelet

Search ((((((((((vascular[Title/Abstract]) OR thrombosis[Title/Abstract]) OR atherosclerosis[Title/Abstract]) OR arteriosclerosis[Title/Abstract]) OR cardiovascular[Title/Abstract]) OR venous[Title/Abstract]) OR arterial[Title/Abstract]) OR hemostasis[Title/Abstract]) OR metabolic[Title/Abstract]) OR metabolism[Title/Abstract]) OR glycemia[Title/Abstract]) OR glycaemia[Title/Abstract]) OR cholesterol[Title/Abstract]) OR triglyceride[Title/Abstract]) OR platelet[Title/Abstract]

Imatinib

Search (((imatinib[Title/Abstract]) OR imatinib mesylate[Title/Abstract]) OR STI-571[Title/Abstract]) OR STI571[Title/Abstract]

Total: Search (((((imatinib[Title/Abstract]) OR imatinib mesylate[Title/Abstract]) OR STI-571[Title/Abstract]) OR STI571[Title/Abstract])) AND ((((((((((((((((((vascular[Title/Abstract]) OR thrombosis[Title/Abstract]) OR atherosclerosis[Title/Abstract]) OR arteriosclerosis[Title/Abstract]) OR cardiovascular[Title/Abstract]) OR venous[Title/Abstract]) OR arterial[Title/Abstract]) OR hemostasis[Title/Abstract]) OR metabolic[Title/Abstract]) OR metabolism[Title/Abstract]) OR glycemia[Title/Abstract]) OR glycaemia[Title/Abstract]) OR cholesterol[Title/Abstract]) OR triglyceride[Title/Abstract]) OR platelet[Title/Abstract]))))) *(1726 abstracts)*

Dasatinib

Search ((dasatinib[Title/Abstract]) OR BMS-354825[Title/Abstract]) OR BMS354825[Title/Abstract]

Total: Search ((((dasatinib[Title/Abstract]) OR BMS-354825[Title/Abstract]) OR BMS354825[Title/Abstract])) AND ((((((((((((((((((vascular[Title/Abstract]) OR thrombosis[Title/Abstract]) OR atherosclerosis[Title/Abstract]) OR arteriosclerosis[Title/Abstract]) OR cardiovascular[Title/Abstract]) OR venous[Title/Abstract]) OR arterial[Title/Abstract]) OR hemostasis[Title/Abstract]) OR metabolic[Title/Abstract]) OR metabolism[Title/Abstract]) OR glycemia[Title/Abstract]) OR glycaemia[Title/Abstract]) OR cholesterol[Title/Abstract]) OR triglyceride[Title/Abstract]) OR platelet[Title/Abstract]))))) *(247 abstracts)*

Nilotinib

Search ((nilotinib[Title/Abstract]) OR AMN107[Title/Abstract]) OR AMN-107[Title/Abstract]

Total: Search ((((nilotinib[Title/Abstract]) OR AMN107[Title/Abstract]) OR AMN-107[Title/Abstract])) AND ((((((((((((((((((vascular[Title/Abstract]) OR thrombosis[Title/Abstract]) OR atherosclerosis[Title/Abstract]) OR arteriosclerosis[Title/Abstract]) OR cardiovascular[Title/Abstract]) OR venous[Title/Abstract]) OR arterial[Title/Abstract]) OR hemostasis[Title/Abstract]) OR metabolic[Title/Abstract]) OR metabolism[Title/Abstract]) OR glycemia[Title/Abstract]) OR glycaemia[Title/Abstract]) OR cholesterol[Title/Abstract]) OR triglyceride[Title/Abstract]) OR platelet[Title/Abstract]))))) *(193 abstracts)*

Bosutinib

Search (((bosutinib[Title/Abstract]) OR SKI-606[Title/Abstract]) OR SKI606[Title/Abstract]

Total: Search ((((bosutinib[Title/Abstract]) OR SKI-606[Title/Abstract]) OR SKI606[Title/Abstract])) AND ((((((((((((((((((vascular[Title/Abstract]) OR thrombosis[Title/Abstract]) OR atherosclerosis[Title/Abstract]) OR arteriosclerosis[Title/Abstract]) OR cardiovascular[Title/Abstract]) OR venous[Title/Abstract]) OR arterial[Title/Abstract]) OR hemostasis[Title/Abstract]) OR metabolic[Title/Abstract]) OR metabolism[Title/Abstract]) OR glycemia[Title/Abstract]) OR glycaemia[Title/Abstract]) OR cholesterol[Title/Abstract]) OR triglyceride[Title/Abstract]) OR platelet[Title/Abstract]))))) *(50 abstracts)*

Ponatinib

Search (Ponatinib[Title/Abstract]) OR AP24534[Title/Abstract]

Total: Search (((Ponatinib[Title/Abstract]) OR AP24534[Title/Abstract])) AND ((((((((((((((((((vascular[Title/Abstract]) OR thrombosis[Title/Abstract]) OR atherosclerosis[Title/Abstract]) OR arteriosclerosis[Title/Abstract]) OR cardiovascular[Title/Abstract]) OR venous[Title/Abstract]) OR arterial[Title/Abstract]) OR hemostasis[Title/Abstract]) OR metabolic[Title/Abstract]) OR metabolism[Title/Abstract]) OR glycemia[Title/Abstract]) OR glycaemia[Title/Abstract]) OR cholesterol[Title/Abstract]) OR triglyceride[Title/Abstract]) OR platelet[Title/Abstract]))))) *(66 abstracts)*

BCR-ABLTKIs

Total: Search (BCR-ABL[Title/Abstract]) AND ((((((((((((((((((vascular[Title/Abstract]) OR thrombosis[Title/Abstract]) OR atherosclerosis[Title/Abstract]) OR arteriosclerosis[Title/Abstract]) OR cardiovascular[Title/Abstract]) OR venous[Title/Abstract]) OR arterial[Title/Abstract]) OR hemostasis[Title/Abstract]) OR metabolic[Title/Abstract]) OR metabolism[Title/Abstract]) OR glycemia[Title/Abstract]) OR glycaemia[Title/Abstract]) OR cholesterol[Title/Abstract]) OR triglyceride[Title/Abstract]) OR platelet[Title/Abstract]))))) *(596 abstracts)*

Total: 2878 abstracts

Without duplicates: 2175 abstracts

Table 3.6 | **Inhibitory profiles of BCR-ABL TKIs on tyrosine kinases.** Gray boxes indicate unknown effect of BCR-ABL TKIs on the tyrosine kinase. Red and green boxes indicate respectively no inhibition and inhibition of the tyrosine kinase by the TKI. Seconds columns indicate references. Similarly, numbers in red indicate no inhibition of the tyrosine kinase by the TKI whereas numbers in green indicate inhibition.

	Imatinib		Dasatinib		Nilotinib		Bosutinib		Ponatinib	
Lmr1										
Lmr2										
Lmr3										
Tyk2	O	2, 3	X*	2, 3, 8, 10, 12	O	3, 10	O	2, 10, 12		
JAK1	O	1, 2, 6	O	1, 2	X*	1, 6	O	1, 2	X	1
JAK2	O	4, 5			O	4			X	13
JAK3										
Ack	O	2	X	2, 8			X	2		
Tnk1	O	2	O	2			O	2		
HER3										
EGFR	O	13	X	8, 11, 12	O	13	X	12	O	13
HER2	O	13			O	9, 13			O	13
HER4			X	12			X	12		
TIE1										
TIE2 = Tek	O	5, 6			X	6, 9				
Ret	O	1, 4	X*	1, 8	O	1, 4	O	1, 13	X	1, 13
FGFR1	O	2, 4	O	2	O	4	X*	2, 12		
FGFR2	O	1	X*	1, 12	O	1	O	1, 12	X	1
FGFR3	O	1	O	1	O	1	O	1	X	1
FGFR4										
FLT1 = VEGFR1	O	1, 5	O	1, 12	O	1	X*	1, 12	X	1
KDR = VEGFR2	O	1, 4, 5	X*	1, 10	O	1, 9, 10	O	1, 10	X	1
FLT4 = VEGFR3	O	1	O	1	O	1	O	1	X	1
Fms/CSFR	X*	1, 5	X	1, 12	X	1, 9	X*	1, 12	X	1
Kit	X*	1, 2, 4, 5, 8	X*	1, 2, 8, 13	X	1, 4, 8, 9	O	1, 2	X	1, 13
FLT3	O	1, 4, 5	X*	1, 10	O	1, 4, 10	O	1, 10	X	1, 13
PDGFRα	X	1, 4, 5, 8	X*	1, 8, 12	X	1, 4, 8, 9	O	1, 12	X	1
PDGFRβ	X	1, 4, 5, 8	X	1, 8, 10, 12	X*	1, 4, 8, 9, 10	O	1, 10, 12	X	1
CCK4/PTK7										
LTK										
ALK	O	2	O	2			X	2		
Ros										
InsR	O	4			O	4				
IGF1R	O	4			O	4				
IRR										
DDR1	X*	2, 3, 7, 8	X*	2, 3, 8	X	3, 8, 9	X*	2, 12	X	7

DDR2	X	1, 7	X	1, 8, 12	X*	1, 9	X	1, 12	X	1, 7
MuSK										
TRKA	O	2	X*	2, 12			X*	2, 12		
TRKB	O	1	X*	1, 12	O	1	X*	1, 12	X	1
TRKC	O	1	O	1	O	1	O	1	O	1
ROR1	X	1	X	1	X	1	O	1	X	1
ROR2										
Ron										
Met	O	1, 4, 5	O	1	O	1, 4	O	1	O	1, 13
Axl	O	1	O	1, 12	O	1	X*	1, 12	O	1
Mer	O	1, 2	O	1, 2	O	1	X*	1, 2	O	1
Tyro3/Sky	O	1	O	1, 12	O	1	X*	1, 12	O	1
RYK										
Fer	O	2	O	2, 10	O	10	X	2, 10, 12		
Fes			O	10	O	10	X	10, 12		
Abl	X	1, 3, 8	X	1, 3, 8, 10, 12	X	1, 3, 8, 10	X	1, 10, 12	X	1, 13
Arg	X	1, 2, 8	X	1, 2, 8, 10, 12	X*	1, 4, 8, 9, 10	X	1, 2, 10, 12	X	1, 13
CSK	O	2, 3	X	2, 3, 8, 10, 11	X*	3, 9, 10	X	2, 10, 12		
CTK										
Etk/BMX	O	1, 3	X	1, 3, 8, 12	O	1, 3	X	1, 12	X	1
BTK	O	2, 3	X	2, 3, 8, 10, 11	X*	3, 10	X	2, 10		
TXK			X	8, 12			X	12		
TEC	O	2, 3	X	2, 3, 8, 10, 12	O	3, 10	X*	2, 10, 12		
ITK										
Brk			X	8, 12			X	12		
Srm										
FRK	O	1, 2, 3	X*	1, 2, 3, 8, 10, 12	X*	1, 3, 9, 10	X	1, 2, 10, 12	X	1
BLK	O	1	X	1, 8, 10, 12	X*	1, 9, 10	X	1, 10, 12	X	1
Lck	O	1, 3	X	1, 3, 8, 10, 12	X*	1, 3, 9, 10	X	1, 10, 12	X	1
HCK	O	2, 3	X	2, 3, 8, 10, 11	X*	3, 9, 10	X	2, 10	X	1
Lyn	O	1, 2, 3, 13	X	1, 2, 3, 8, 10, 11	X*	1, 3, 9, 10	X	1, 2, 10	X	1, 13
Fgr	O	1, 3	X	1, 3, 8, 10, 12	O	1, 3, 9	X	1, 12	X	1
Fyn	O	1, 2, 3	X	1, 2, 3, 8, 10, 11	X*	1, 3, 9, 10	X	1, 2, 10	X	1
Src	O	1, 2, 3, 5	X	1, 2, 3, 8, 10, 11	O	1, 3, 9, 10	X	1, 2, 10	X	1
Yes	O	3	X	3, 8, 10, 11, 12	O	3, 9, 10	X	10, 12	X	13
EphA1			X	12	X	9	X	12		
EphA2			X	8, 12	X	9	X	12		
EphA3	O	1	X	1, 8, 12	X*	1, 9	X	1, 12	X	1
EphA4	O	1	X	1, 8, 12	X	1, 9	X	1, 12	X	1
EphA5	O	1	X	1, 8, 12	X	1	X	1, 12	X	1
EphA6										
EphA7										
EphA8			X	8, 12	X	9	X	12		
EphA10										

EphB1	O	1, 2	X*	1, 2, 8, 12	X	1	X	1, 2, 12	X	1
EphB2	O	1, 2, 3, 6	X	1, 2, 3, 8, 12	X	1, 3, 6	X*	1, 2, 12	X	1
EphB3			X	10, 12	O	10	X	10, 12		
EphB4	O	1, 2, 3	X	1, 2, 3, 8, 10, 12	X*	1, 3, 9, 10	X	1, 2, 10, 12	X	1, 13
EphB6	O	3	X	3, 8, 12	X*	3, 9				
FAK	O	2	X*	2, 8, 10	O	10	X	2, 10		
PYK2	O	2	O	2, 10	X	10	X	2, 10		
Syk	O	2, 3	X	2, 3, 8, 10	O	3	X	2		
ZAP70										
JAK1~b										
Tyk2~b										
JAK2~b										
JAK3~b										
SuRTK106										

* Discordant results between the experiments.

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Chapter 4: The Risk of Arterial Thrombosis in Patients With Chronic Myeloid Leukemia Treated With Second and Third Generation BCR-ABL TKIs May Be Explained by Their Impact on Endothelial Cells: An In vitro Study

Clinical experience and preliminary investigations indicate that mechanistic research should focus on atherosclerosis and platelets. The short-time-to-event of vascular occlusive events with ponatinib and the clinical presentation of TKI-induced vascular occlusive events suggested that new generation TKIs might induce atherosclerosis plaque rupture. The stability of atherosclerotic plaque correlates to the thickness of its fibrous cap which is degraded by matrix metalloproteinases (MMP). Thus, we investigated the *in vitro* effect of BCR-ABL TKIs on the production of 2 MMPs (MMP-2 and MMP-9) by a macrophage cell line (THP-1 cells). However, none of the TKI disturbed the macrophage viability nor the MMP production and activity after 24 and 48 hours of treatment, suggesting that vascular occlusive events are not the results of an impact on macrophages (personal data).

Therefore, as BCR-ABL TKIs demonstrated reassuring effects on inflammatory and fibrotic cells, we focused our research on the impact of BCR-ABL TKIs first on endothelial cells. Since the review (**chapter 2**), and in parallel of our experiments, additional studies have been performed and reported that nilotinib and ponatinib affect the endothelium but the mechanisms by which they exert their toxic effects are unknown (Gover-Proaktor et al. 2019; Hadzijusufovic et al. 2017). The impact of dasatinib on the endothelium has been poorly investigated. Additionally, evidence of the role of nilotinib- and ponatinib in some endothelial dysfunction has been established through several investigations. However, the lack of homogeneity in the *in vitro* studies precluded direct comparison of the effects of TKIs on endothelial cells. In this chapter, we aim to provide a homogeneous

assessment of the effect of BCR-ABL TKIs on endothelial cells viability and major functions *in vitro*, with a special focus on the type of cell death, and a particular consideration to dasatinib and bosutinib.

Original article in Frontiers in pharmacology 11: 1007.

The risk of arterial thrombosis in patients with chronic myeloid leukemia treated with second and third generation BCR-ABL TKIs may be explained by their impact on endothelial cells: an *in vitro* study.

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Summary

BCR-ABL tyrosine kinase inhibitors (TKIs) revolutionized the treatment of chronic myeloid leukemia, inducing deep molecular responses, largely improving patient survival and rendering treatment-free remission possible. However, three of the five BCR-ABL TKIs, dasatinib, nilotinib and ponatinib, increase the risk of developing arterial thrombosis. Prior investigations reported that nilotinib and ponatinib affect the endothelium, but the mechanisms by which they exert their toxic effects are still unclear. The impact of dasatinib and bosutinib on endothelial cells has been poorly investigated.

Here, we aimed to provide an in vitro homogenous evaluation of the effects of BCR-ABL TKIs on the endothelium, with a special focus on the type of cell death to elucidate the mechanisms responsible for the potential cytotoxic effects of BCR-ABL TKIs nilotinib and ponatinib on endothelial cells.

We tested the five BCR-ABL TKIs at three concentrations on human umbilical venous endothelial cells (HUVECs).

This study highlights the endothelial toxicity of ponatinib and provides insights about the mechanisms by which it affects endothelial cell viability. Ponatinib induced apoptosis and necrosis of HUVECs after 72 h. Dasatinib affected endothelial cells in vitro by inhibiting their proliferation and decreased wound closure as soon as 24 h of treatment and even at infra-therapeutic dose (0.005 μM). Comparatively, imatinib, nilotinib and bosutinib had little impact on endothelial cells at therapeutic concentrations. They did not induce apoptosis nor necrosis, even after 72 h of treatment, but they inhibited HUVEC proliferation. Overall, this study reports various effects of

BCR-ABL TKIs on endothelial cells and suggests that ponatinib and dasatinib induce arterial thrombosis through endothelial dysfunction.

Keywords: *BCR-ABL tyrosine kinase inhibitor, chronic myeloid leukemia, endothelial cells, atherosclerosis, cardiovascular*

Introduction

Since the approval in 2001 of imatinib, the first tyrosine kinase inhibitor (TKI) targeting BCR-ABL, the interest and use of BCR-ABL TKIs have continued to grow (Bhamidipati et al. 2013). The discovery of imatinib led to a revolution in the treatment of chronic myeloid leukemia (CML), providing strong and durable molecular responses in numerous patients and improving patient survival close to that of the general population (Iqbal and Iqbal 2014). However, intolerance and resistance to imatinib arose, leading to the development and approval of dasatinib, nilotinib and bosutinib, three second-generation BCR-ABL TKIs that are nevertheless less selective for BCR-ABL than imatinib (Gorre et al. 2001; Bixby and Talpaz 2009). These three drugs were approved for second-line treatment at the time of their approval. However, because of their efficacy in inducing deeper molecular remission, an essential criterion for treatment cessation (*i.e.*, *BCR-ABL1* transcript below 0.01% for at least 2 years), they have been approved for the treatment of chronic phase CML in first line (Cortes, Saglio, et al. 2016; Hochhaus et al. 2016; Brummendorf et al. 2015; National Comprehensive Cancer Network 2019). Ponatinib was developed as a fifth BCR-ABL TKI (Cortes et al. 2012). Because of its efficacy against T315I-mutated BCR-ABL, a mutation that confers resistance to all other BCR-ABL TKIs (Cortes et al. 2012), this third generation drug is used to treat CML resistant to first-line treatments. Altogether, the five drugs are the mainstay of CML treatment (National Comprehensive Cancer Network 2019).

Although these treatments are highly effective for CML in chronic phase, three of them (dasatinib, nilotinib and ponatinib) increase the risk of adverse vascular events, particularly the risk of arterial thrombosis associated to myocardial infarction, stroke and peripheral artery disease (Cortes et al. 2015; Pasvolsky et al. 2015; Douxfils, Haguet, Mullier, Chatelain, Graux, and Dogné 2016; Haguet et al. 2017). However, each BCR-ABL TKI exhibits a different clinical vascular safety profile, suggesting different mechanisms of pathogenesis. The most common adverse vascular events associated to

dasatinib are pleural effusion and pulmonary arterial hypertension (Cortes, Jimenez, et al. 2017), whereas arterial thrombosis occurs less frequently (Saglio et al. 2017; Valent et al. 2017). Nilotinib causes hyperglycemia and hypercholesterolemia in addition to arterial thrombosis (Rea et al. 2014; Racil et al. 2013), whereas ponatinib is the TKI associated with the highest arterial thrombotic risk (Haguet et al. 2018). Interestingly, the time-to-event (*i.e.* the time between the initiation of the treatment and the occurrence of arterial thrombosis) differs between molecules. Ponatinib is associated with a rapid development of vascular events (median time-to-event: 8.5 to 14.1 months), compared to nilotinib (median time-to-event: 19.0 to 47.0 months), whereas little is known concerning arterial thrombosis induced by dasatinib (Minson et al. 2019; Cortes et al. 2015; Fossard et al. 2016; Gagnieu et al. 2013). Even if adverse vascular events occur more frequently in patients with prior cardiovascular risk factors, they also affect patients with no attributable cardiovascular risk factors (Aichberger et al. 2011a; Tefferi and Letendre 2011b). Conversely, it has been proposed that imatinib prevents the occurrence of such events (Kadowaki and Kubota 2004), whereas bosutinib apparently presents a safer vascular profile than the other second-generation TKIs (Valent et al. 2017).

Because of the wide implementation of second generation TKIs for chronic patient treatment, their long-term safety is of particular interest. Understanding the pathophysiology of adverse vascular events induced by dasatinib, nilotinib and ponatinib could help to define suitable risk minimization measures and help in the selection of the first-line treatment for CML patients. To date, the mechanisms underlying vascular adverse events are not fully characterized. Several hypotheses have been set forward based on the clinical characteristics of the vascular events. In particular, the predominance of arterial events raised concerns about the impact of BCR-ABL TKIs on platelet functions, endothelial cells and atherosclerosis, and excluded pro thrombotic states to be responsible for these events (Fossard et al. 2016). However, prior investigation indicated that BCR-ABL TKIs have no or anti-aggregation effects on platelets, suggesting a low probability of the

implication of platelets in the occurrence of vascular thrombosis (Haguet et al. 2018). Therefore, research focused on endothelial cells and atherosclerosis.

Previous mechanistic studies interrogating the effect of BCR-ABL TKIs on the vasculature demonstrated that some of these treatments affect the endothelium. For ponatinib and nilotinib, an increasing amount of evidence indicates that their vascular toxicity is a consequence, at least partially, of their effect on endothelial cells. *In vitro*, they both decreased endothelial cell viability and promoted the expression of molecular patterns related to apoptosis and angiogenesis (Gover-Proaktor et al. 2019; Hadzijusufovic et al. 2017). For these reasons, it has been hypothesized that vascular endothelial cells could be at the very origin of vasculopathies induced by ponatinib and nilotinib, which would be responsible for the direct endothelial dysfunction (Hadzijusufovic et al. 2017). Dasatinib has been tested essentially on pulmonary endothelial cells in order to elucidate the mechanism behind dasatinib-induced pulmonary hypertension. It induced apoptosis and increased ROS production by pulmonary endothelial cells (Guignabert et al. 2016). Comparatively, the viability of dasatinib-treated endothelial cells other than pulmonary was unchanged (Gover-Proaktor et al. 2019). It has already been hypothesized that the vascular toxicity of BCR-ABL TKI is due to their lack of specificity towards BCR-ABL. Indeed, all BCR-ABL TKIs also inhibit numerous kinases (Talbert et al. 2015; Rix et al. 2007).

There is a lack of homogeneity in the *in vitro* studies performed to investigate the effects of nilotinib and ponatinib on endothelial cells. Overall, the methods, the tested concentrations and the cell line used often differed, making arduous the comparison between studies and resulting in conflicting reports (Katgi et al. 2015; Vrekoussis et al. 2006; Venalis et al. 2009). Here, we aimed to provide a homogenous evaluation and comparison of the viability of endothelial cells exposed to each of the five commercially available BCR-ABL TKIs. Major endothelial functions, such as ROS generation, the expression of adhesion molecules and migration, have also been assessed, given their implication in vascular homeostasis and atherosclerosis. A

particular consideration has been paid to results with dasatinib and bosutinib, as their impact on endothelial cells had previously been barely investigated, with the exception of dasatinib on pulmonary endothelial cells. In addition, even if the impact of nilotinib and ponatinib on endothelial cells is now well considered, there is still a shadow on the mechanism(s) by which they exert their cytotoxic effects. To contribute to a better knowledge of the pathophysiology, our study further aimed to clarify the mechanism(s) by which BCR-ABL TKIs impair endothelial cell survival *in vitro*.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Verviers, Belgium). Cells were cultured in endothelial cell growth basal medium (Lonza, CC-3121, Verviers, Belgium) supplemented with SingleQuots™ (Lonza, CC-4133, Verviers, Belgium) and maintained at 37°C in a humidified atmosphere containing 5% CO_2. HUVECs were used at passages 2 to 5.

Drugs

Imatinib mesylate, dasatinib, nilotinib, bosutinib and ponatinib were from Absource Diagnostic (Munich, Germany). All experiments were carried out using clinically relevant TKI concentrations (50 nM, 500 nM and 5 μM for imatinib, 5 nM, 50 nM and 500 nM for dasatinib and ponatinib, and 20 nM, 200 nM and 2 μM for nilotinib and bosutinib). These concentrations were selected to consider the binding of TKIs to plasma proteins. Thus, these concentrations reflect the free concentration of TKIs in patients taking 400 mg OD of imatinib, 100 mg OD of dasatinib, 400 mg BID of nilotinib, 500 mg OD of bosutinib and 45 mg OD of ponatinib (**Table 4.1**) (Rivera et al. 2014).

All experiments were performed in 10% dialyzed FBS from Thermo Fischer Scientific (Waltham, MA, USA) in order to minimize interactions with serum-associated proteins, unless indicated otherwise.

Viability

Cell metabolic integrity and viability were assayed using MTS (Promega, Fitchburg, MA, USA) and LDH (Roche, Basel, Switzerland) kits, respectively. In brief, HUVECs were seeded in 96-well plates at a density of 2,500 cells/cm^2. Four days after plating, cells were treated with a TKI or 0.2% DMSO (control) diluted in a medium containing 10% dialyzed FBS. Cell metabolic integrity and viability were assessed after 24 h and 72 h of treatment using MTS and LDH assays according to manufacturers' instructions. The absorbance at 490 nm was measured with a SpectraMax ID3 (Molecular Devices, San Jose, CA, USA).

Apoptosis

To assess apoptosis induced by BCR-ABL TKIs, HUVECs were stained with Annexin V-FITC (staining of phosphatidylserine) and 7-AAD (DNA staining) in accordance with the manufacturer's instructions (Abcam, Cambridge, UK). Briefly, HUVECs were plated in 12-well plates and exposed to a TKI or 0.2% DMSO (control) for 24 h or 72 h. Cells were then stained with Annexin V-FITC and 7-AAD in binding buffer. Stained HUVECs were analyzed on a FACS verse flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA). Early apoptotic (*i.e.* cells with externalized phosphatidylserine but with preserved membrane integrity) and late apoptotic/necrotic cells (*i.e.* cells with disrupted plasma membrane integrity) were counted based on the relative number of Annexin V-FITC$^+$/7-AAD$^-$ and 7-AAD$^+$ cells, respectively, using the BD FACSuite® software.

Proliferation

Cell proliferation was assessed using the Click-it 5-ethynyl-2'-deoxyuridine (EdU) flow kit (Thermo Fisher Scientific). In addition to the staining of EdU, a DNA stain was added (FxCycle Violet stain, Thermo Fisher Scientific). Aphidicolin at 10 μM was used as a positive control (Sigma-Aldrich, Saint-Louis, MO, USA). Briefly, HUVECs were plated in 12-well plates and exposed

to a TKI, 0.2% DMSO (control) or aphidicolin. After 24 h or 72 h of incubation, EdU was added for 2 h. Cells were then fixed, permeabilized, and the EdU detection mix was added for 30 min at room temperature. Finally, DNA was stained by incubating cells with FxCycle Violet stain for 30 min. HUVECs were then analyzed by flow cytometry (BD FACSverse), based on the fact that cells in S-phase are those that incorporate EdU ($FITC^+$) and cells in G0/G1 phase and in G2/M phase do not incorporate EdU but are $FxCycle^-$ and $FxCycle^+$, respectively. Data were analyzed using the BD FACSuite® software.

Reactive Oxygen Species Concentrations

After the exposure of HUVECs to one of the 5 TKIs or to 0.2% DMSO (control) for 72 h, ROS content was quantified using CM-H2DCFDA (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. The oxidized fluorescent product retained inside cells was quantified using the FACSverse flow cytometer. Data were analyzed using the BD FACSuite® software. SIN-1 hydrochloride (Sigma-Aldrich) was used a positive control.

Endothelial Cell Migration

Endothelial cell migration was evaluated by a scratch assay that monitors the ability of cells to migrate in a wound. HUVECs were cultured in 24-well plates until confluence, and were then pretreated with a given TKI or 0.2% DMSO (control) in a medium containing 1% FBS (to minimize the contribution of cell proliferation on scratch closure) or 10% FBS for 24 h. The confluent monolayer was scratched with a pipette tip, and wound closure dynamics were tracked using an inverted Leica DMi1 microscope (Weltzar, Germany). Pictures were captured at baseline and after 2 h, 4 h, 6 h and 24 h of incubation, and analyzed using the Leica Application Suite software version 4.7 to quantify the extent of cell migration in the cell-free scratch.

On-Cell ELISA

The expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-/P-selectin was measured by on-cell

enzyme-linked immunosorbent assay (ELISA). Briefly, HUVECs were cultured in 96-well plates and exposed to a TKI or 0.2% DMSO (control) for 24 h. Cells were then activated with TNF-α (R&D Systems, Minneapolis, MN, USA) at 10 ng/mL for 4 h. After treatment, cells were fixed with 0.025% glutaraldehyde for 10 min, and blocked with PBS-BSA 1% for 2 h. Fixed cells were incubated overnight with monoclonal antibodies targeting human ICAM-1 (recombinant human ICAM-1 clone #14C11, R&D systems), VCAM-1 (recombinant human VCAM-1 clone #HAE-2Z, R&D systems) and E-/P-selectin (CD62E/P, clone #BBIG-E6, R&D systems), and finally incubated with a secondary horseradish peroxidase (HRP)-conjugated antibody for 1 h (mouse IgG HRP-conjugated antibody, R&D systems). After incubation with 100 µL of the HRP substrate (50 µL of hydrogen peroxide and 50µL of tetramethylbenzidine; R&D systems) for 20 min, optical density was measured at 450 nm with an Infinite® M200 PRO (Tecan, Mechelen, Belgium). Results are expressed as the mean of the absorbance values relative to control.

Statistical Analyses

Results are expressed as the means of the differences between the treated value and the control ± standard error of the mean (SEM). All experiments were repeated independently at least 3 times (N = 3). Comparison between different conditions was performed using the Wilcoxon signed rank test for experiments for which a standard distribution could not be defined. For flow cytometry analyses, a one-sample t-test was used. All statistical analyses were performed using the Prism 8 software (GraphPad Software Inc., San Diego, CA, United States). Data that were statistically significant in comparison to controls are indicated with * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$).

Table 4.1 | Summary of the findings of the impact of BCR-ABL TKIs on endothelial cells *in vitro*.
This table summarizes the results obtain in this study. Only statistically significant differences are reported. The number of arrows represents the magnitude of the following of difference: ↘ (from 0 to -24%), ↘↘ (from -25 to -49%), ↘↘↘ (from -50 to -74%) and ↘↘↘↘ (from -75 to -100%) and ↗ (from 0 to 24%), ↗↗ (from 25 to 49%), ↗↗↗ (from 50 to 74%) and ↗↗↗↗ (from 75 to 100%). The concentrations that significantly differed from the control are indicated in brackets.

		Imatinib	**Dasatinib**	**Nilotinib**	**Bosutinib**	**Ponatinib**
	Clinically effective C_{max}*	0.682 µM	0.072 µM	0.171 µM	0.209 µM	0.040 µM
Assay	Measured parameters					
Endothelial cells exposed to BCR-ABL TKIs for 24 h						
MTS assay	Mitochondrial activity	=	=	=	=	↘ (0.5 µM)
LDH assay	LDH release (membrane integrity)	↘ (0.05 µM)	=	=	=	↗ (0.05 µM)
Apoptosis assay	Early apoptosis	=	=	=	=	=
	Late apoptosis/necrosis	=	=	=	=	=
Proliferation assay	Cells in S-phase	↗ (0.05 µM) ↘ (5 µM)	↘↘ (0.05 µM) ↘↘↘ (0.5 µM)	↘ (2 µM)	↘↘↘ (2 µM)	=
Adhesion molecule expression (ELISA)	ICAM-1	=	↘ (0.5 µM)	↘ (2 µM)	↘ (0.02 µM) ↗ (2 µM)	↘ (0.005 µM; 0.05 µM) ↘↘↘ (0.5 µM)
	VCAM-1	↘ (0.5 µM)	↘ (0.5 µM)	↘ (2 µM)	=	↘ (0.005 µM; 0.05 µM) ↘↘↘↘ (0.5 µM)
	E-/P-selectin	=	↘ (0.5 µM)	=	=	↘ (0.5 µM)

Endothelial cells exposed to BCR-ABL TKIs for 72 h						
MTS assay	Mitochondrial activity	↗ (5 µM)	↗ (0.005 µM; 0.5 µM)	=	=	↘↘ (0.5 µM)
LDH assay	LDH release (membrane integrity)	↗↗ (0.05 µM; 0.5 µM)	↗↗ (0.005 µM; 0.05 µM; 0.5 µM)	↗↗ (0.02 µM; 0.2 µM; 2 µM)	↗↗ (0.02 µM; 0.2 µM; 2 µM)	↗↗ (0.005 µM; 0.5 µM) ↗↗↗ (0.05 µM)
Apoptosis assay	Early apoptosis	=	↘↘ (0.005 µM; 0.05 µM; 0.5 µM)	=	↘↘ (2 µM)	↗↗↗↗ (0.5 µM)
	Late apoptosis/necrosis	=	=	=	=	↗↗↗ (0.05 µM)
Proliferation assay	Cells in S-phase	↘ (0.05 µM)	=	↘ (0.02 µM) ↘↘ (2 µM)	↘↘ (0.2 µM)	=
ROS assay	ROS levels	=	=	=	=	=

* Clinically effective C_{max} represents the C_{max} corrected for the functional effects of protein binding.(Rivera et al. 2014)
C_{max}: maximum serum concentration

Results

Impact of BCR-ABL TKIs on endothelial cell viability

To test the cytotoxicity of BCR-ABL TKIs, HUVECs were exposed to 3 different concentrations of imatinib mesylate, dasatinib, nilotinib, bosutinib or ponatinib for 24 h or 72 h. Cell metabolic activity was tested using an MTS assay that reports on the activity of mitochondrial reductases[5] (Buttke, McCubrey, and Owen 1993). Ponatinib exposure decreased cell metabolism after 24 h and 72 h of treatment, whereas the other BCR-ABL TKIs did not (**Figure 4.1A** and **Figure 4.7A**). Rather, HUVECs exposed to imatinib at 5 μM and dasatinib at 5 nM and 50 nM increased their metabolism. To link these results to cell viability, we performed a LDH assay that measures the leakage of LDH, an intracellular enzyme that is released from cells upon the cell membrane damaged. After 24 h of treatment, TKIs did not induce more LDH release than control, to the exception of ponatinib at 0.05 μM that increased LDH release, suggesting that BCR-ABL TKIs do not induce early cell membrane damage (**Figure 4.7B**). However, after 72 h of treatment, HUVEC membrane integrity was impacted, as shown by increased LDH release with all BCR-ABL TKIs (**Figure 4.1B**). Surprisingly, this alteration did not depend on the TKI concentration in the range that we tested.

Because MTS and LDH assays do not fully discriminate the different cell death modes and to clarify the mechanism(s) by which BCR-ABL TKIs impair endothelial cell survival, we next tested apoptosis and late apoptosis/necrosis using specific FACS assays. After 24 h of incubation, TKIs did not induce apoptosis or late apoptosis/necrosis (**Figure 4.8**). However, after 72 h, ponatinib dose-dependently induced HUVEC apoptosis and necrosis (**Figure 4.2**). Interestingly, dasatinib at all concentrations and bosutinib at 2 μM reduced the number of apoptotic cells. The other BCR-ABL TKIs did not significantly modulate HUVEC apoptosis nor necrosis.

[5] MTS assay assesses mitochondrial deshydrogenase capacity to produce $NADH,H^+$. A main limitation of this assay is that for drugs that affects only mitochondria, an increase in the MTS reduction could be observed due to an extra-mitochondrial metabolic compensation (i.e. glycolysis stimulation).(Huet et al. 1992)

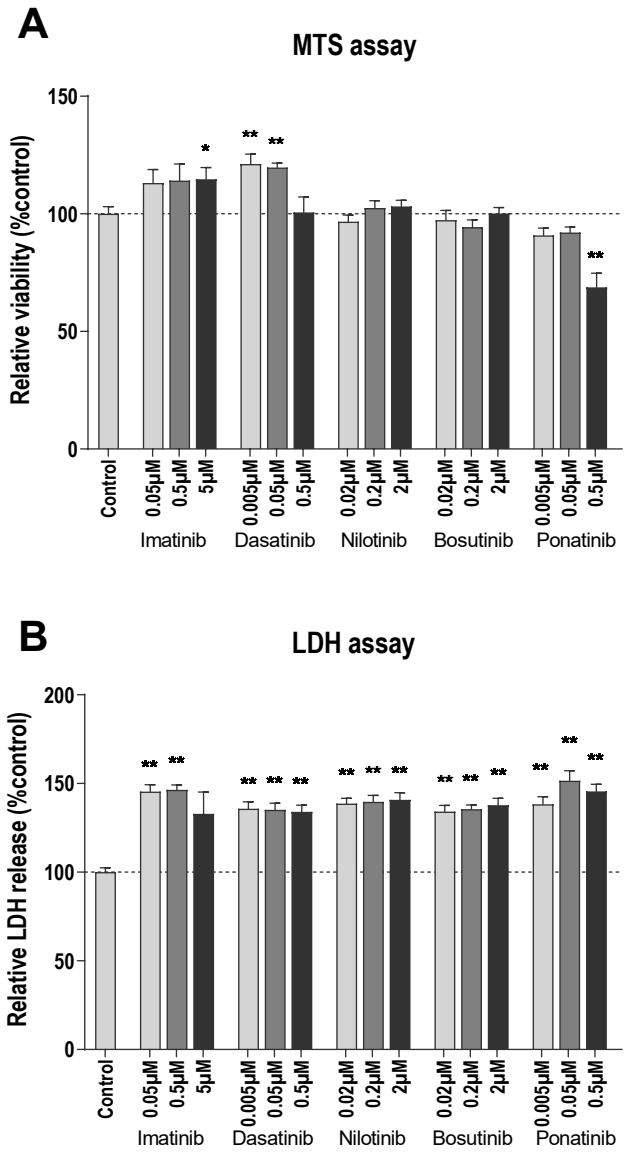


Figure 4.1 | **All BCR-ABL TKIs induce HUVEC membrane damage after 72 hours.** MTS (**A**) and LDH (**B**) assays were performed on HUVECs exposed for 72 h to the indicated BCR-ABL TKI in medium with 10% dialyzed FBS. Data are presented as means ± SEM of n = 9 of three independent experiments (N = 3). Results are expressed relative to control (DMSO 0.2%). Differences between conditions were tested using the Wilcoxon signed rank test that compared the effect of each TKI condition *versus* control. *p < 0.05 and **p < 0.01.

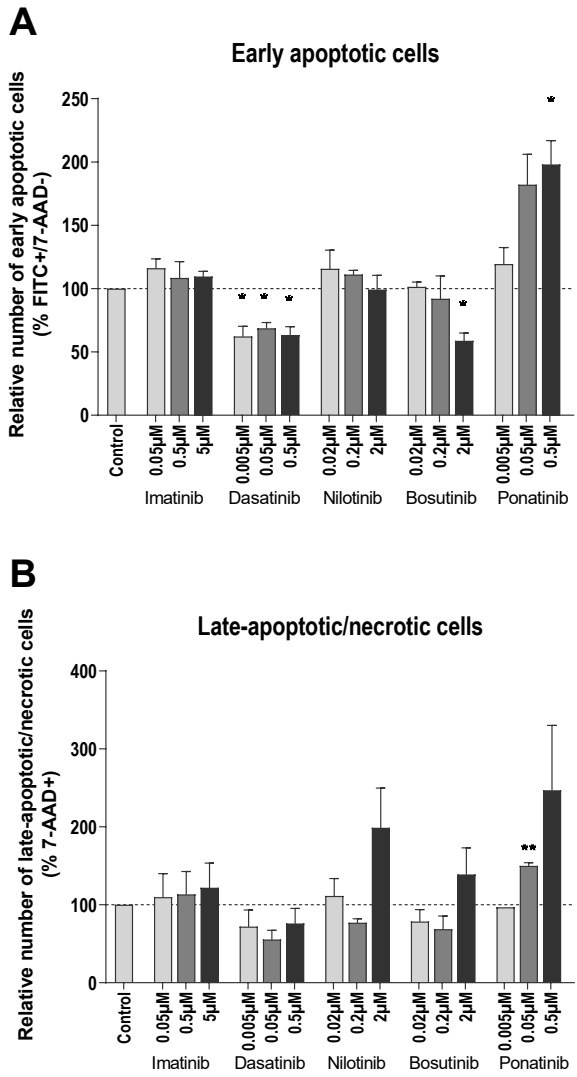


Figure 4.2 | **Ponatinib induces HUVEC apoptosis and necrosis.** HUVECs were labeled with Annexin V^{FITC} and 7-AAD after exposure to BCR-ABL TKIs for 72 h in medium with 10% dialyzed FBS. The percentage of Annexin V^+/7-AAD^- and 7-AAD^+ cells revealed early apoptotic (*i.e.* cells exposing phosphatidylserine but with preserved membrane integrity) (**A**) and late apoptotic/necrotic (*i.e.* cells with disrupted membrane integrity) (**B**) HUVECs, respectively. Bars represent the means of three experiments ± SEM. Results are expressed relative to control (DMSO 0.2%). Differences between conditions were tested using a one sample t-test that compared each TKI condition *versus* control. *$p < 0.05$ and **$p < 0.01$.

For what concerns cell cycling, all BCR-ABL TKIs influenced HUVECs as early as 24 h after treatment, as shown by a decreased of the number of cells in the S-phase and an increased number of cells in the G0-G1 phase (**Figure 4.3**), suggesting that these drugs block HUVEC progression from G1 to S. Responses were dose-dependent, but they were seen only at the highest, supra-therapeutic concentrations of imatinib, nilotinib and bosutinib. Bosutinib differed from the other TKIs in that, at the highest concentration, it also increased cells in the G2-M phase (**Figure 4.3**). Imatinib had the slightest effect on endothelial cell proliferation. After 72 h of treatment, inhibition of proliferation was still detected in HUVECs exposed to imatinib, nilotinib and bosutinib (**Figure 4.9**).

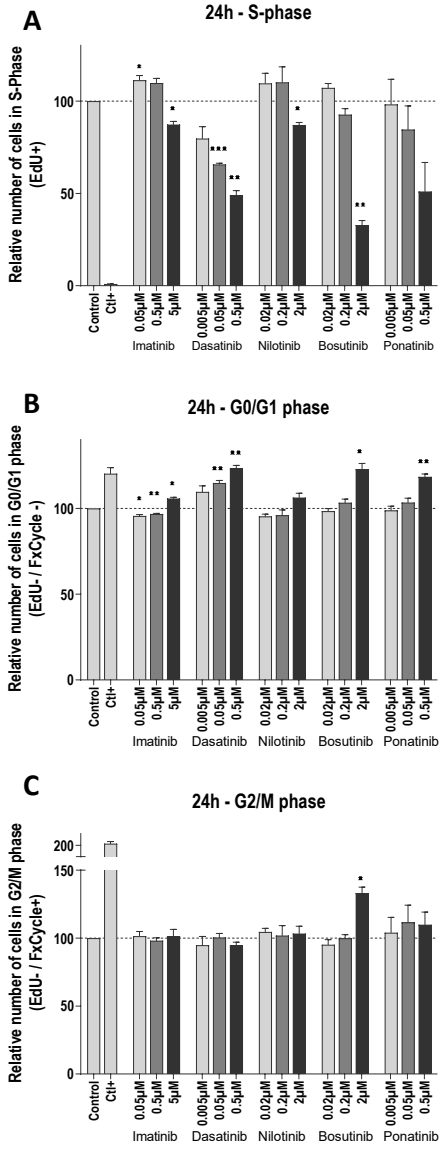
To complete the investigation of the mechanism by which BCR-ABL TKIs impact endothelial cell viability, we evaluated their impact on ROS levels. ROS concentration in HUVECs was not significantly modified by BCR-ABL TKIs (**Figure 4.4**).

Endothelial Cell Migration

As endothelial cell migration is essential for wound healing and tissue regeneration, the ability of endothelial cells to migrate after treatment with the TKIs was evaluated by scratch assays. Assays were first performed in media containing 1% FBS to minimize the effects of cell proliferation on wound closure. In these conditions, there was no statistically significant difference between HUVECs treated with a TKI compared to control (**Figure 4.10**). The absence of effect was possibly due to the high variability of the measurements due to the fact that scratch closure was slow with reduced cell proliferation. When we repeated the assay in media containing 10% FBS, dasatinib, even at infra-therapeutic concentration (0.005 μM) and bosutinib at high-dose (2 μM) inhibited scratch closure, whereas nilotinib facilitated wound healing (**Figure 4.5; Figure 4.11**).

Adhesion Molecule Expression

Leukocyte recruitment is an important process in atherogenesis (Galkina and Ley 2007). It requires the expression of adhesion molecules by activated endothelial cells. Here, we assessed in HUVECs the expression of ICAM-1, VCAM-1 and



◄ **Figure 4.3** | **All BCR-ABL TKIs inhibit HUVEC proliferation.** Cell cycle analysis was performed on HUVECs exposed to BCR-ABL TKIs for 24 h in medium with 10% dialyzed FBS by measuring EdU incorporation and DNA content (FxCycle). The histograms represent cells in S-phase (**A**), G0/G1 phase (**B**) and G2/M phase (**C**). Bars represent the means of the three experiments ± SEM. Three concentrations were tested for each TKI. Results are expressed relative to control (DMSO 0.2%). Differences between conditions were tested using a one sample t-test that compared each TKI condition *versus* control. *$p < 0.01$ and **$p < 0.01$.

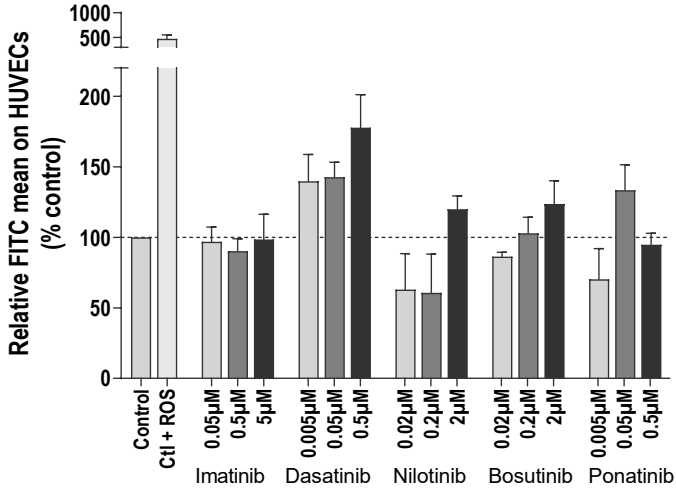


Figure 4.4 | **BCR-ABL TKIs do not increase the ROS levels in HUVECs.** ROS levels in HUVECs after treatment with BCR-ABL TKIs for 72 h in medium with 10% dialyzed FBS, expressed as intensity of FITC. Bars represent the means of three experiments ± SEM. Results are expressed relative to control (DMSO 0.2%). Differences between conditions were tested using a one sample t-test that compared each TKI condition *versus* control.

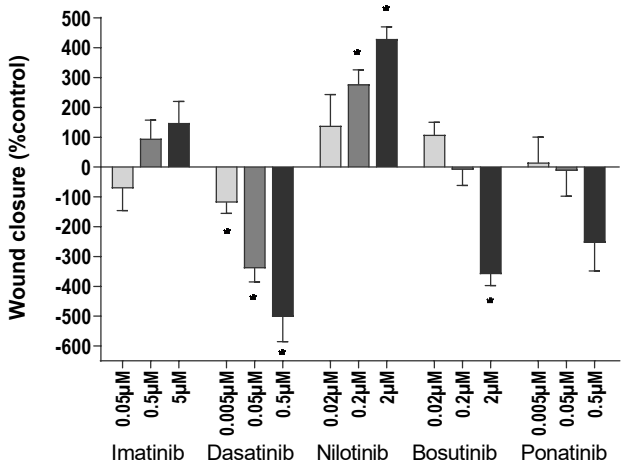


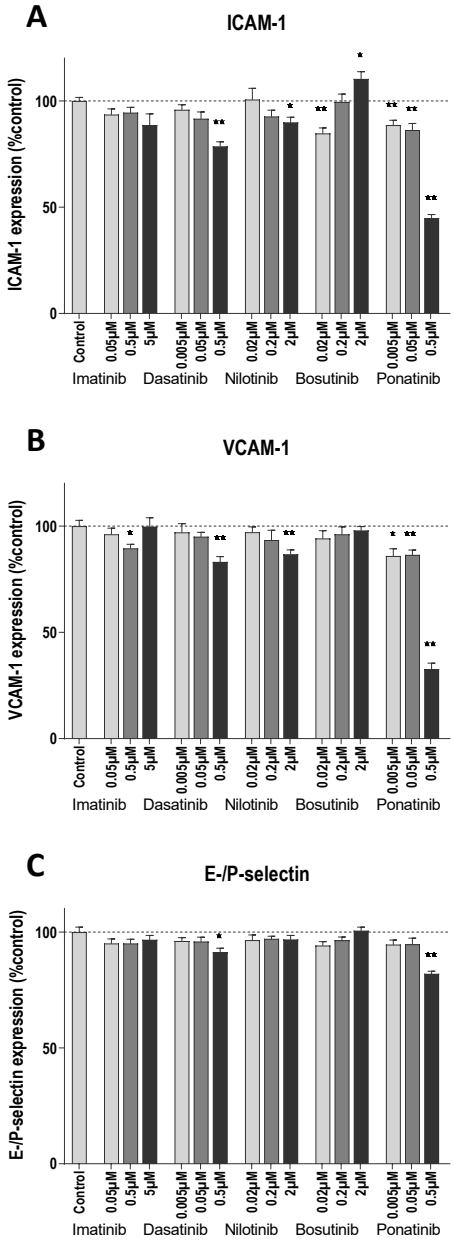
Figure 4.5 | **Dasatinib and bosutinib reduce wound closure.** HUVEC migration was assessed by a scratch test after exposure of the cells to BCR-ABL TKIs for 24 h in 10% FBS media to avoid cell death due to serum-free conditions. The histograms represent wound closure 6 h after the scratch. Bars represent the means ± SEM of n = 6 of three independent experiments (N = 3). Differences between conditions were tested using the Wilcoxon signed rank test that compared each TKI condition *versus* control. *$p < 0.05$.

E-/P-selectin, three adhesion molecules involved in monocyte/macrophage recruitment (Čejková, Králová Lesná, and Poledne 2016). The decreased expression of all three adhesion molecules was observed in HUVECs treated with dasatinib, nilotinib and ponatinib at high concentrations for 24 h (0.5 μM, 2 μM and 0.5 μM, respectively; **Figure 4.6**). Imatinib and bosutinib had no or little impact on adhesion molecule expression.

Discussion

Our study demonstrates that BCR-ABL TKIs impact endothelial cells differently *in vitro* (**Table 4.1**). Ponatinib is the most cytotoxic for endothelial cells and induces apoptosis and necrosis, whereas the other BCR-ABL TKIs inhibited HUVEC proliferation. In addition, dasatinib and bosutinib delayed wound closure. These findings correlate with their clinical vascular safety profile (Aghel, Delgado, and Lipton 2017), and could explain why ponatinib is the BCR-ABL TKI that induces arterial occlusion the most frequently.

To our knowledge, the present study is the first to report that dasatinib affects endothelial cells from a different origin than pulmonary, indicating that the effect of dasatinib is not peculiar to pulmonary endothelial cells (Guignabert et al. 2016). Dasatinib inhibited endothelial cell proliferation at all the doses that we tested, and delayed wound closure. The comparison of the results of the scratch assay in the presence of 1% *versus* 10% FBS supports the hypothesis that delayed wound closure is the consequence of an inhibition of cell proliferation rather than an impact on endothelial cell migration. However, prior experiments suggested that dasatinib inhibits cell migration by altering the organization of the actin cytoskeleton and by inhibiting the formation of intercellular contacts (Kreutzman et al. 2017; Dasgupta et al. 2017). This impact on the endothelial cell cytoskeleton occurs through a disruption of the inhibitory signals from integrins to RhoA, a pathway implicating numerous tyrosine kinases, leading to RhoA/ROCK pathway activation. However, implicated tyrosine kinase(s) has/have not yet been identified. Interestingly, dasatinib did not increase ROS levels in endothelial cells, restraining the number of pathways by which dasatinib affects endothelial cell viability. In addition to its effects on endothelial cell proliferation and



◀ **Figure 4.6 | BCR-ABL TKIs do not increase adhesion molecule expression on HUVECs.** Expression of ICAM-1 (**A**), VCAM-1 (**B**), E-selectin and P-selectin (**C**) by HUVECs after a 4 hours activation by 10 ng/mL of TNF-α followed by a 24 h treatment with BCR-ABL TKI in medium without FBS. Data are presented as means of the absorbance ± SEM of n = 9 of three independent experiments (N = 3). Three concentrations were tested for each TKI. Results are expressed relative to control (DMSO 0.2%). Differences between conditions were tested using the Wilcoxon signed rank test. *$p < 0.05$ and **$p < 0.01$.

Migration, dasatinib increased LDH release after 72 hours of treatment, without inducing necrosis. This suggests that dasatinib does not induce endothelial cell death, but potentially induces cell damage. Interestingly, dasatinib protected cells against apoptosis and increased their metabolism, in accordance with the literature (Gover-Proaktor et al. 2019). This could explain why dasatinib is associated with a lower rate of arterial thrombosis than nilotinib and ponatinib.

Our results confirm the toxicity of ponatinib towards endothelial cells, endorsing the hypothesis that ponatinib would facilitate atherosclerosis and arterial thrombosis through alteration of endothelial cell viability (Gover-Proaktor et al. 2019; Hadzijusufovic et al. 2017; Gover-Proaktor et al. 2017; Katgi et al. 2015). Indeed, cell death within the arterial wall has already been recognized in atherosclerosis. Our study further provides insights about the mechanisms by which ponatinib dose-dependently affects endothelial cell viability. Ponatinib induced necrosis without increasing ROS levels, and might participate in atherosclerosis in this way (Paez-Mayorga et al. 2018). The identification of the type of endothelial cell death induced by ponatinib supports at least two hypotheses regarding the molecular signaling pathways responsible for this effect. One hypothesis would link necrotic cell death to a blockade of the cell cycle by the TKIs during the progression from G1 to S phase. Interestingly, previous *in vitro* investigation reported a cell cycle arrest in the G1 phase in liver cancer cells treated with ponatinib, and identified that this cell cycle blockade was mediated by a reduction in the function of the CDK4/CDK6/Cyclin D1 complex (Liu et al. 2019). This complex is regulated by the pro-survival PI3K/Akt pathway, known to be impacted by some BCR-ABL TKIs, including ponatinib (Talbert et al. 2015). Therefore, future research should analyze the effects of BCR-ABL TKIs on CDK4, CDK6 and Cyclin D1 levels/activities, and identify the implicated signaling pathways. A second hypothesis would link ponatinib-induced cell necrosis to Akt inhibition. Indeed, Akt is also highly implicated in the regulation of apoptosis signaling and mediates the responses of a majority of growth factors (Somanath et al. 2006), and ponatinib is known to inhibit Akt in cardiomyocytes (Singh et al. 2019). These observations highlight the need for a further thorough evaluation of this pathway in the endothelial response to ponatinib.

Interestingly, nilotinib had distinct impact on HUVECs. It increased LDH release after 72 h, but did not induce necrosis nor apoptosis at the tested concentrations, suggesting induction of nonfatal cell damage. It decreased HUVEC proliferation, but without affecting wound closure. These results are in line with the literature, and suggest a different etiology of vascular events. Other mechanisms than endothelial dysfunction with nilotinib should be explored. Because of its association with the development of hyperglycemia and hypercholesterolemia, accelerated atherosclerosis has been proposed as a potential mechanism of vascular thrombosis (Rea et al. 2014; Damrongwatanasuk and Fradley 2017). However, it is currently unknown if the induction of these metabolic disorders alone is sufficient to explain the vascular complications associated with nilotinib. Another hypothesis is the induction of coronary artery spasms (Fiets et al. 2018).

Our work confirmed the little impact of imatinib on endothelial cells, in accordance with the literature (Venalis et al. 2009; Gover-Proaktor et al. 2017; Hacker et al. 2007; Vrekoussis et al. 2006; Guignabert et al. 2016; Sukegawa et al. 2017). Even if imatinib did not induce apoptosis nor necrosis, it increased the release of LDH after 72 h of treatment, suggesting the induction of nonfatal cell damage, similarly than with dasatinib and nilotinib. Bosutinib had little impact on endothelial cells. Yet, similarly to imatinib, it induced LDH release without inducing cell death, also suggesting the induction of cell damage. Interestingly, at high concentration (2 μM), it presented a profile close to that of dasatinib, as it inhibited cell proliferation, retarded wound closure and decreased apoptosis. Understanding the molecular pathways involved in endothelial cell death with therapy is important to develop more specific therapies. To anticipate the occurrence of such events and bring sufficient care to the vascular safety of new TKIs in clinical trials, *in vitro* endothelial testing of new drugs should be a requirement. Non-clinical testing should include *in vitro* methods for assessing cell death, because global assays (such as the MTS and the LDH assays) may generate false-positive results and are not able to discriminate between cytotoxic and antiproliferative effects (Mery et al. 2017). The dose-dependent response seen here in most of the tests also suggests that the choice of the dose is important for

in vitro experiments. This is one of the parameters that varied the most between studies, explaining most of the discrepancies between them. Of further notes, numerous studies evaluated BCR-ABL TKIs *in vitro* using non-clinically relevant concentrations, thus leading to results that are not pertinent to clinical settings. One example is the report by Hadzijusufovic et al. that showed increased ICAM-1, VCAM-1 and E-selectin expression in HUVECs upon a 7.5 μM nilotinib treatment (Hadzijusufovic et al. 2017), whereas doses of nilotinib from 0.02 μM to 2 μM reduced the expression of the same protein (this study). Another important parameter that should be considered when designing the testing of compounds on endothelial functions *in vitro* is their binding to serum proteins, which would directly affect their biodisponibility.

Perspectives and Conclusions

This study demonstrates that the responses of endothelial cells are different according to the TKI, corroborating the difference of their clinical vascular safety profiles. There are limitations to our study in that the findings are limited to one cell line (HUVECs). Even if this cell type is a good model for atherosclerotic studies (Onat et al. 2011), key observations should be confirmed on endothelial cells from other origin (*e.g.* HCAEC) and using more elaborated models (*e.g.* models involving vascular functions). BCR-ABL TKIs should also be tested on other cell lines than endothelial cells to assess if they modulated other physiological functions. In addition, different times of TKI treatment should be tested, particularly for the assessment of adhesion molecule expression as Hadzijusufovic et al. reported increased in ICAM-1 and VCAM-1 cell surface levels after 4 h of nilotinib treatment (Hadzijusufovic et al. 2017).

To ascertain the relevance of these findings to humans, it will be interesting to evaluate endothelial biomarkers in patients treated with BCR-ABL TKIs and to address their predictability. Circulating endothelial cells, endothelial progenitor cells and endothelial microparticles are good candidate biomarkers. They are measurable by blood sampling and have already been associated with damaged endothelium, as well as to predict the outcome of ischemic vascular diseases (Thomson A and Garbuzova-Davis S 2016; Sabatier et al. 2009). In this context,

Gover-Proaktor et al. suggested that endothelial progenitor cells may be more sensitive to ponatinib than mature endothelial cells, possibly because of their stronger dependence on pro-survival factors and/or on the different penetration of ponatinib within the cells (Gover-Proaktor et al. 2017).

Our *in vitro*-based study analyzed the effects of BCR-ABL TKIs on endothelial cells with a special focus on cell death. It reports various effects of BCR-ABL TKIs on endothelial cells, suggesting a possible multifactorial cause and the possible implication of several off-targets in the development of cardiovascular events. Future research should focus on the study of the molecular mechanisms that are responsible for endothelial cell death or impaired cell proliferation with BCR-ABL TKIs. Among the five commercialized BCR-ABL TKIs, ponatinib showed the most effects on endothelial cells. It reduced endothelial cell viability by inducing apoptosis and necrosis, which possibly facilitates the development of atherosclerosis through impaired endothelium permeability, enabling cell migration and the trapping of lipoproteins in the intima. Additionally, we found that dasatinib inhibits endothelial cell proliferation and can reduce endothelial cell migration, which might contribute to arterial thrombosis formation.

Because of the chronic use of these treatments, long-term safety is an important issue, and understanding the impact of these treatments may help in the design of new therapies. It may be of interest in the conception of strategies aiming to minimize the risk of adverse events, *i.e.* by avoiding the use of certain therapies in patients with pre-existing impairments in pathways disturbed by these therapeutic agents.

Author Contributions: HH and JD were responsible for the conception and design of the study. HH and EM were responsible for the acquisition, analysis and interpretation of data. HH was responsible for drafting the manuscript. CB, ASD, AW, PS and JMD contributed to the final draft of the manuscript. All authors agree to be accountable for the content of the work.

Acknowledgments: The authors thank the "Unité de recherche en Biologie des Micro-organismes" (URBM) and the Namur Nanosafety Center (NNC) of the University of Namur for providing logistic support.

Supplemental files

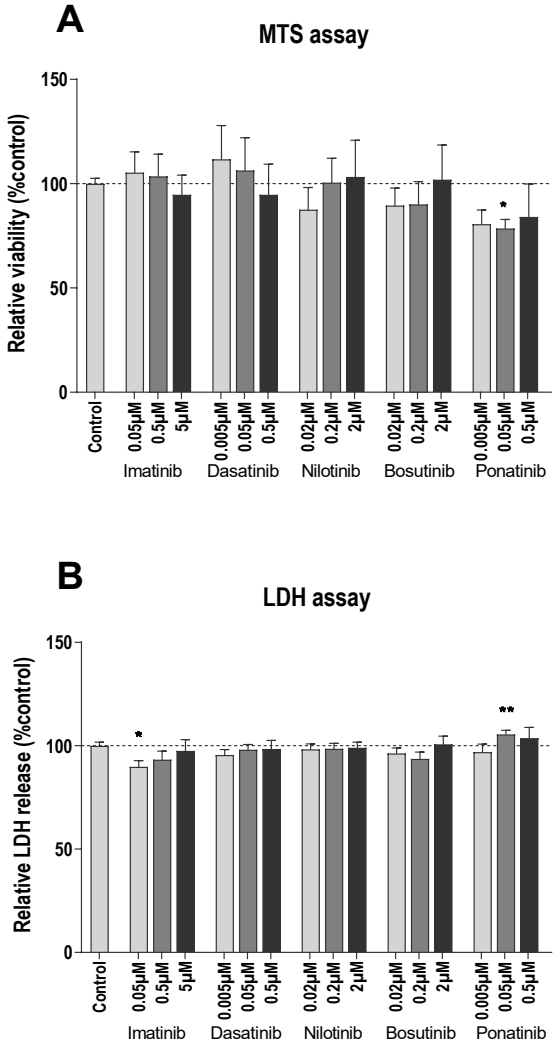


Figure 4.7 | **Ponatinib affects endothelial cell viability after 24 hours.** MTS (**A**) and LDH (**B**) assays were performed on HUVECs exposed for 24 h to the indicated BCR-ABL TKI in medium with 10% dialyzed FBS. Data are presented as means ± SEM of n = 9 of three independent experiments (N = 3). Results are expressed relative to control (DMSO 0.2%). Differences between conditions were tested using the Wilcoxon signed rank test that compared the effect of each TKI condition *versus* control. *p < 0.05 and **p < 0.01.

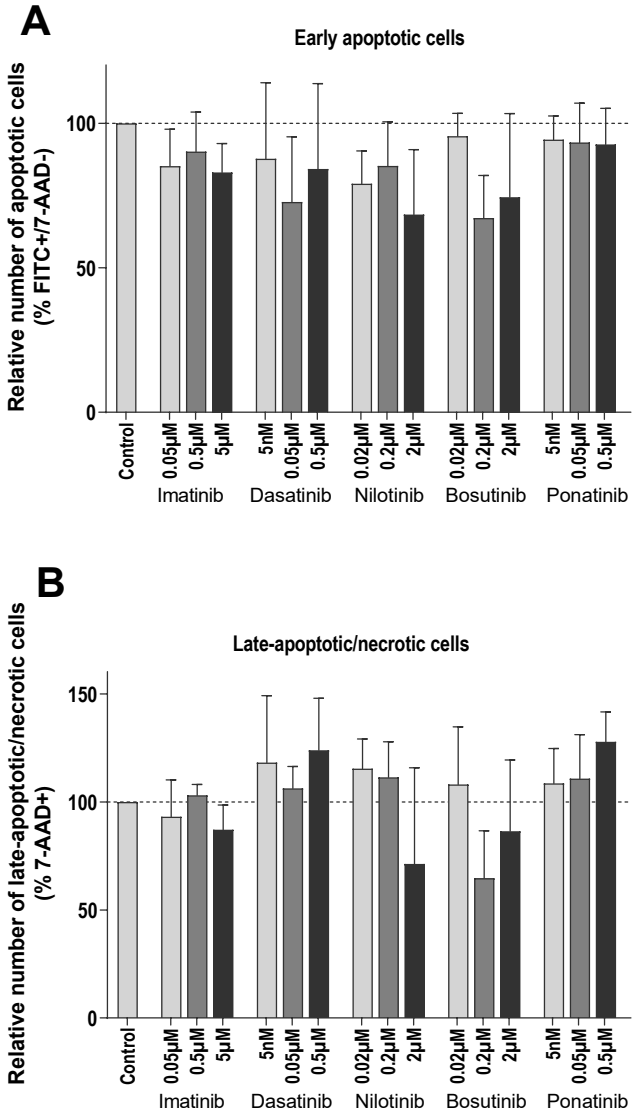
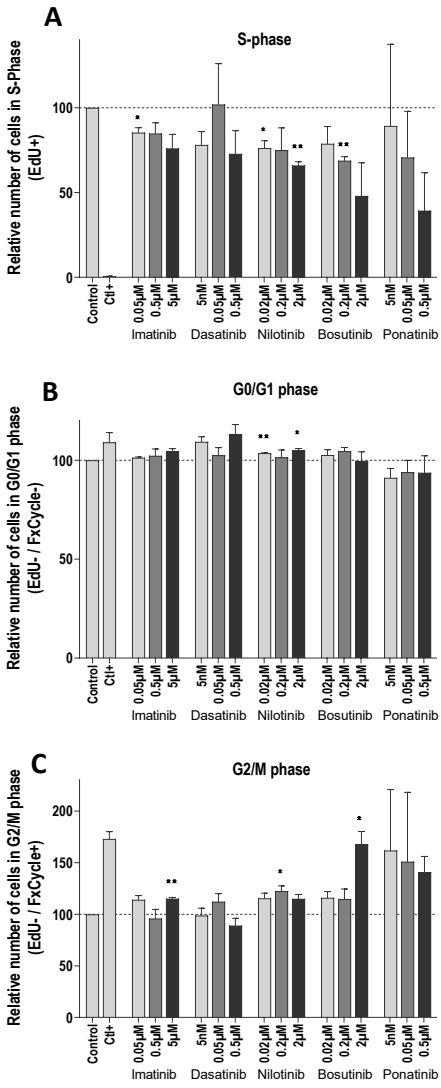


Figure 4.8 | **BCR-ABL TKIs did not increase HUVEC apoptosis or late apoptosis/necrosis after 24 hours.** HUVECs were labeled with Annexin V^{FITC} and 7-AAD after exposure to BCR-ABL TKIs for 24 hours in medium with 10% dialyzed FBS. The percentage of Annexin V^+/7-AAD^- and 7-AAD^+ cells revealed early apoptotic (**A**) and late apoptotic/necrotic (**B**) HUVECs, respectively. Bars represent the means of the three experiments ± SEM. Results are expressed relative to control (DMSO 0.2%). Differences between conditions were tested using a one sample t-test that compared each TKI condition *versus* control.



◄ **Figure 4.9 | Imatinib, nilotinib and bosutinib inhibit HUVEC proliferation after 72 hours.** Cell cycle analysis was performed on HUVECs exposed to BCR-ABL TKI for 72 hours in medium with 10% dialyzed FBS by measuring EdU incorporation and DNA content (FxCycle). The histograms represent cells in S-phase (**A**), G0/G1 phase (**B**) and G2/M phase (**C**). Bars represent the means of three experiments ± SEM. Three concentrations were tested for each TKI. Results are expressed relative to control (DMSO 0.2%). Differences between conditions were tested using a one sample t-test that compares each TKI condition *versus* control. *p<0.05 and **p<0.01.

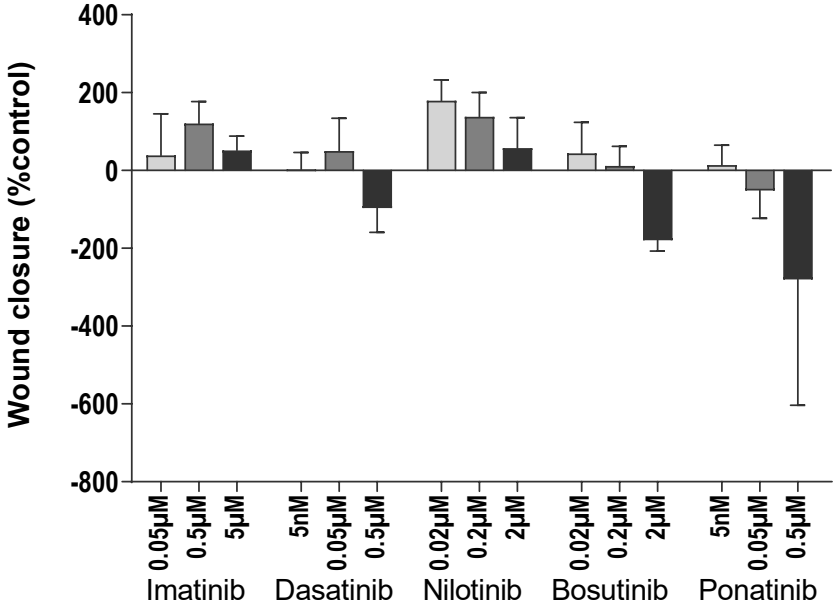
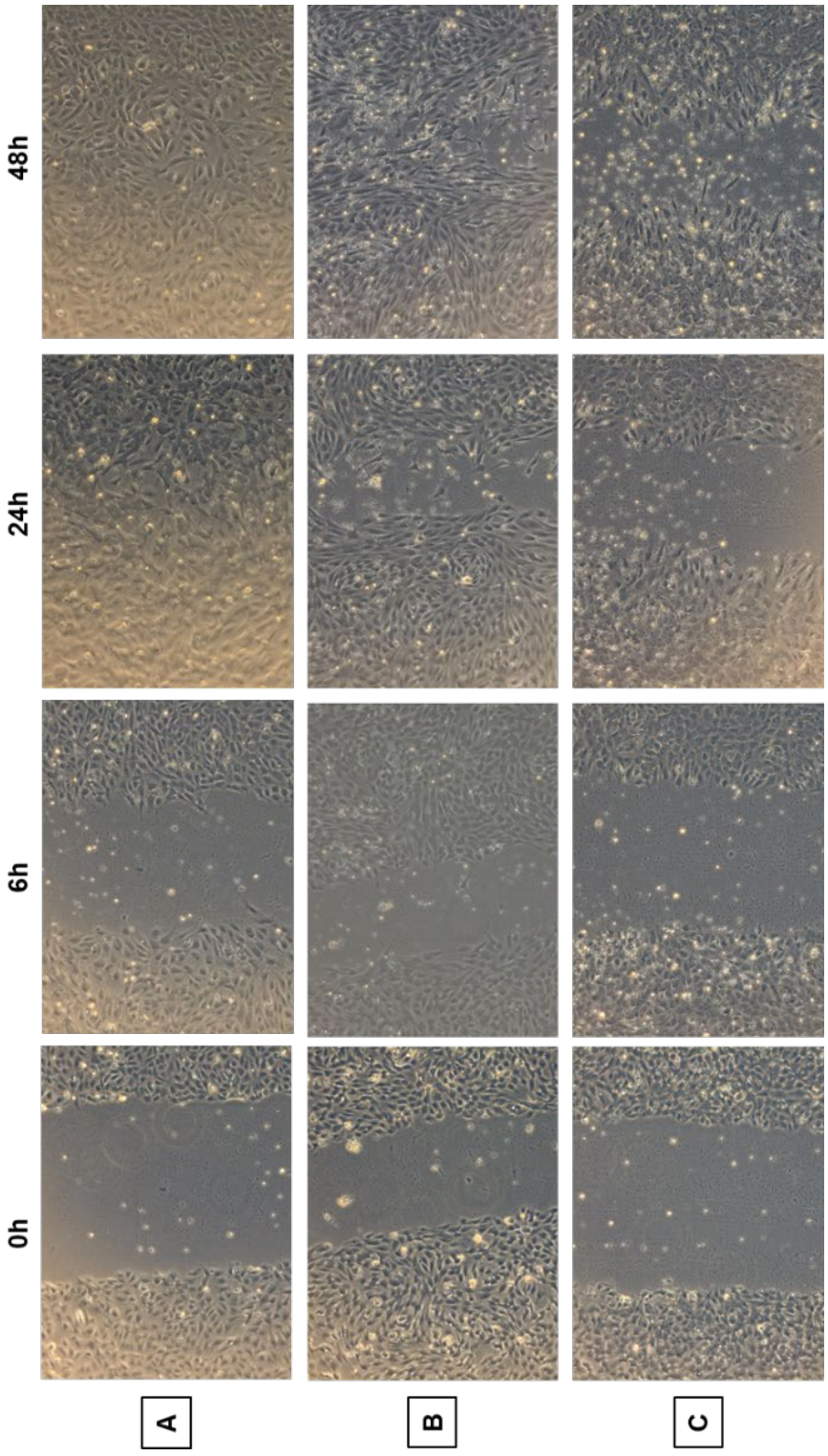


Figure 4.10 | **BCR-ABL TKIs do not affect scratch closure in media containing 1% FBS.** Endothelial cell migration was assessed by a scratch assay after exposure to BCR-ABL TKIs for 24 hours. The scratch assay was performed in 1% FBS media to minimize the impact of cell proliferation. The histogram represents wound closure 6 hours after the scratch. Bars represent the means ± SEM of n = 6 of three independent experiments (N = 3). Differences between conditions were tested using the Wilcoxon signed rank test that compared each TKI condition *versus* control.


48h
24h
6h
0h
A
B
C

▲ **Figure 4.11** | **Dasatinib and bosutinib inhibited wound closure.** Endothelial cell migration was assessed by a scratch assay after exposure to BCR-ABL TKIs for 24 hours. The scratch assay was performed in 10% FBS media. Pictures are representative images of scratch closure with control (0.2% DMSO) (**A**), dasatinib 0.5µM (**B**) and bosutinib 2µM (**C**) at the time of the scratch (0h), after 6 hours, 24 hours and 48 hours.

Perspectives and Conclusions

Chapter 5: Perspectives and Conclusions

Mechanistic Studies Interrogating Vascular Effects: State-of-the-art and Clinical Perspectives

Through a series of meta-analyses (**chapter 2**), we identified that 3 out of 4 second and third generation BCR-ABL TKIs induce higher risk of arterial occlusive events than imatinib. At the time of the meta-analysis, this suggested a possible class effect, with ponatinib being the TKI that induces the most important risk of arterial occlusive events. A deep literature review (**chapter 3**) allowed to emit the hypotheses that the pathophysiology may differ between TKIs and can be multifactorial. These hypotheses were based on the clinical profile and results from the first experimental studies that demonstrate different impact of BCR-ABL TKIs on main actors of atherosclerosis and arterial thrombosis. In addition, differences between TKI off-target spectrum support the diversity of arterial occlusive event pathophysiology. The literature review also indicates that atherosclerosis is the most plausible mechanism underlying arterial occlusive diseases with dasatinib, nilotinib and ponatinib. Impact on platelets, a cell type that is usually involved in arterial thrombosis is less probable regarding the first investigations, except for nilotinib, for which a study demonstrates a pro-thrombotic phenotype of platelets (i.e. increase of platelet secretion, adhesion and activation).(Alhawiti et al. 2016) Endothelial cells being key actors in atherosclerosis, many studies were performed in order to assess the effect of nilotinib and to a lesser extent ponatinib on endothelial cells but data were sparse for dasatinib and bosutinib. Consequently, we performed *in vitro* investigations (**chapter 4**) to assess and compare the impact of BCR-ABL TKIs on endothelial cells. These studies confirm different impact of BCR-ABL TKIs on this cell type, supporting different etiologies of arterial occlusive events with these therapies. We will address in the next paragraphs a state-of-the-art of the pathophysiology of arterial occlusive events induced by BCR-ABL TKIs.

Ponatinib

Both clinical data and first mechanistic investigations pointed toward endothelial cells as the main culprit of ponatinib induced-arterial occlusive events. The *in vitro* investigations on endothelial cells (**chapter 4**) demonstrate that ponatinib is the BCR-ABL TKI that impacts the most endothelial cells *in vitro* by altering their viability through the induction of necrosis and apoptosis.(Haguet, Bouvy, et al. 2020) We therefore hypothesized that ponatinib promotes atherosclerosis through defections in maintenance of endothelium integrity. However, the precise underlying mechanism is not yet fully elucidated. Interestingly, in addition to their principal implication in the atherosclerotic process, endothelial cells are also a main culprit in the pathophysiology of acute arterial thrombosis (i.e. thrombosis that occurred by superficial erosion of the endothelial monolayer, without atherosclerotic lesions) (**Figure 5.1A**).(Herrmann 2020) Therefore, by affecting endothelial cell viability, ponatinib may induce arterial occlusive events through the development of acute arterial thrombosis and/or by accelerating atherosclerosis and/or by facilitating the erosion of atherosclerotic plaque.

Clinical and *in vitro* data generated on endothelial cell models and the increasing knowledge on the pathophysiology of arterial occlusive events permit the generation of several hypotheses to specify the pathophysiology of ponatinib induced endothelial cell damage. Ponatinib induced-arterial occlusive events are characterized by a short time-to-event (i.e. rapid occurrence of arterial occlusive events after ponatinib initiation). However, after treatment cessation, the risk of arterial occlusive events returned to baseline value. Based on these characteristics, acute arterial thrombosis is a likely mechanism (Herrmann 2020) and it could be hypothesized that by altering endothelial cell viability, ponatinib induces endothelium injury and provokes the exposition of subendothelium vWF, inducing platelet activation and aggregation (**Figure 5.1A**). In addition to its direct cytotoxic effect on endothelial cells, ponatinib also inhibits proliferation of this cell type impacting the regeneration of the endothelium which may in definitive

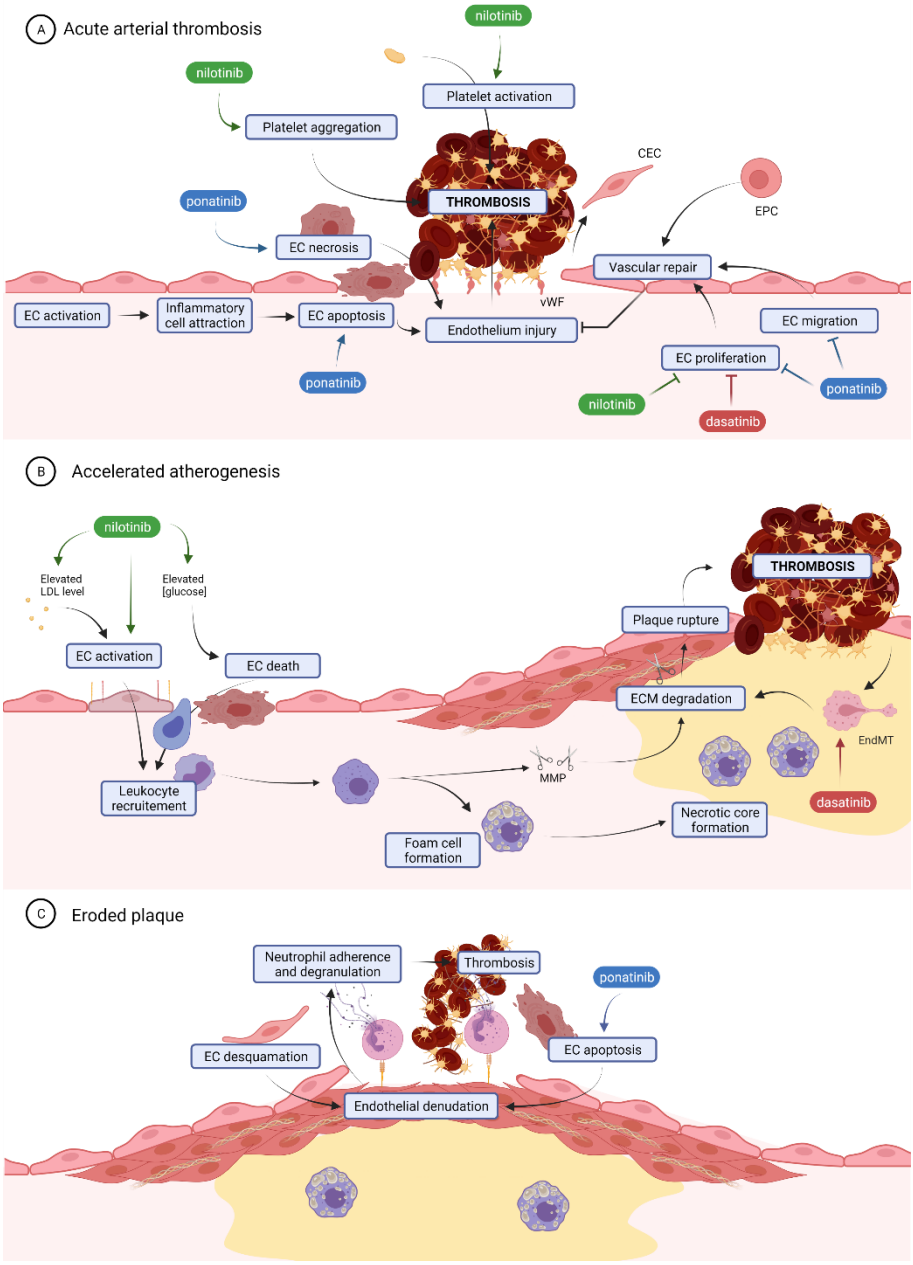


Figure 5.1 | Role of endothelial cells in arterial occlusive diseases and proposed hypotheses of the pathophysiology of arterial occlusive events associated with dasatinib, nilotinib and ponatinib. CEC: circulating endothelial cell; EC: endothelial cell; EndMT: endothelial to mesenchymal transition; EPC: endothelial progenitor cell; LDL: low-density lipoprotein; MMP: matrix metalloproteinase; vWF: von Willebrand factor.

favor the occurence of acute arterial thrombosis.(McDonald et al. 2018) A point that remains unknown is whether ponatinib impacts endothelial progenitor cells, a cell type also involves in vascular repair.(Zhang, Malik, and Rehman 2014) Potential inhibitory or cytotoxic effects on this cell type may accentuate the impact of ponatinib on endothelium injury and deserves further investigations. Interestingly, this mechanism could explain the occurrence of arterial occlusive events in relatively young patients treated with ponatinib even in the absence of additional cardiovascular risk factors.

The viability of endothelial cells also has a prominent role in the erosion of the atherosclerotic plaque (**Figure 5.1C**). Therefore, it could be hypothesized that by inducing endothelial cell apoptosis, ponatinib led to endothelial denudation at the atherosclerotic plaque level and contribute to its erosion. This endothelial denudation induces neutrophil adherence, activation and degranulation leading to the formation of neutrophil extracellular traps (NETs). This can generate a highly thrombogenic environment resulting in the formation of arterial occlusive events.(Quillard et al. 2017)

Finally, the hypothesis that ponatinib induces arterial occlusive events through accelerated atherosclerosis development is unlikely, mostly because of the short event onset of arterial occlusive events. In addition, such hypothesis is not supported by *in vivo* data since a study realized in a mice model even demonstrated a decrease of the atherosclerotic burden following ponatinib therapy. The risk of atherosclerotic plaque rupture was not increased as well, strengthening the low probability of this hypothesis.(Pouwer et al. 2018)

As endothelial injury is a major contributor of both accelerated plaque erosion and acute arterial thrombosis and that the clinical features of these two pathological mechanisms are overlapping, it is difficult to determine the exact contribution of each of these underlying mechanisms in the occurrence of arterial occlusive events with ponatinib. To further elaborate on these preliminary observations, additional clinical investigations should be done to determine the percentage of arterial occlusive events that occurred on an

atherosclerotic context and if these events are the result of a plaque erosion. One proposition could be to characterize arterial occlusive events that occurred during ponatinib treatment by optical coherence tomography, an intravascular imaging technique making possible the visualization of microscopic features of an atherosclerotic plaque permitting the diagnosis of plaque erosion.(Vergallo, Jang, and Crea 2021) The quantification of systemic myeloperoxidase level could also be interesting as higher levels of systemic myeloperoxidase have already been reported in patients with acute coronary syndrome presenting with eroded plaque [6].(Ferrante et al. 2010)

Nilotinib

Compared to ponatinib, nilotinib did not affect drastically endothelial cell viability and the proposed pathological mechanism thus differs. Evidence based on clinical and experimental data suggests that atherosclerotic lesions contribute to arterial occlusive events, whereas acute arterial thrombosis appears less probable.(Tefferi 2013; Sukegawa et al. 2017; Kakadia et al. 2020) Numerous reports demonstrated obliterating artery disease secondary to atherosclerosis (Coon et al. 2013; Aichberger et al. 2011a; Tefferi and Letendre 2011b) and preclinical investigations demonstrated increased atherosclerosis burden in mice undergoing nilotinib therapy.(Hadzijusufovic et al. 2017; Detriche et al. 2019) Arterial occlusion occurred more frequently in patients with cardiovascular risk factors and general prediction models of the risk of atherosclerotic cardiovascular disease (ASCVD) are able to stratify patients undergoing nilotinib therapy.(Breccia et al. 2015b) Finally, the fact that the time-to-event is counted in years (i.e. long delay between treatment initiation and occurrence of the event) and that arterial occlusive events occurred later

6 Myeloperoxidase in plaque erosion is released from macrophages and promote the formation of hypochlorous acid that contribute to plaque erosion by inducing local endothelial loss through oxidative stress.(Niccoli, Dato, and Crea 2010)

after treatment initiation in patients without cardiovascular risk factors supports the hypothesis of accelerated atherosclerosis in the development of nilotinib induced-arterial occlusive events.

At least 3 mechanistic hypotheses can be emitted regarding the increased atherosclerotic burden with nilotinib. (1) Nilotinib facilitates atherosclerosis by inducing vascular permeability and facilitating inflammatory cell recruitment within the arterial wall (**Figure 5.1B**). (2) Nilotinib accelerates atherosclerosis indirectly by inducing endothelial cell damage through increased blood glucose level and/or (3) by increasing neointima formation due to increased blood LDL level.(Alhawiti et al. 2016; Ito et al. 2013) These last 2 hypotheses could provide explanations regarding the underestimated effect of nilotinib on endothelial cells observed during the experiments presented in **chapter 4**. Clinical data support the second hypothesis as arterial occlusive events occurring with nilotinib have a tropism for lower limbs and small vessels, a phenotype resembling to vascular events occurring in diabetic patients.(Aichberger et al. 2011a)

In addition to its effect on the atherosclerotic burden, nilotinib also possesses different effects on platelets that may contribute to arterial occlusive events. Until recently, numerous reviews judged that platelets do not play a major role in the pathogenesis of arterial occlusive events with BCR-ABL TKIs in light of the mechanistic investigations demonstrating diminished platelet function with TKIs.(Herrmann 2016) However, it has been shown that nilotinib increases platelet secretion, adhesion and activation, numerous actions that can contribute to arterial occlusive events (**chapter 3**).(Haguet et al. 2018; Hadzijusufovic et al. 2017) Interestingly, Deb *et al.* recently specified a marked interindividual variation in platelet activation markers measured by flow cytometry following TKI therapy.(Deb et al. 2020) Based on their results, they developed a TKI sensitivity map relying on the assessment of the changes in the platelet activation markers with different combinations of agonists in presence of the different TKIs. This sensitivity map could help in

the decision of frontline treatment for patients with chronic phase CML.(Deb et al. 2020)

Nilotinib is the BCR-ABL TKIs for which the effect on the cardiovascular system was the most studied. The pathophysiology of these occlusive events is the most documented and revealed an impact on the metabolism on the recruitment of inflammatory cells by the endothelium and a pro-thrombotic impact on platelets (i.e. increased platelet secretion, adhesion and activation). However, as for ponatinib, the implication of each of these underlying mechanisms is not clearly stated. Therefore, future research should focus on the contribution of these different mechanisms on the occurrence of arterial occlusive events with nilotinib. The molecular mechanisms underlying this pathophysiology should also be elucidated. It remains uncertain whether the vascular toxicity induced by ponatinib is the consequence of the inhibition of off-targets or ABL itself. Indeed, ABL has an important role in ECs, including the regulation of their survival and a barrier protective role. (Chislock, Ring, and Pendergast 2013; Rizzo, Aman, et al. 2015) Therefore, the inhibition of ABL by itself could participate to the BCR-ABL TKI-related toxicity.

Dasatinib

The pathophysiology of dasatinib-induced arterial occlusive events was often understudied because clinical data were often disputed and research focused mainly on the pulmonary vascular toxicity of dasatinib. However, both our meta-analysis and data from the literature reported an increased risk of arterial occlusive events in patients treated with dasatinib, even if these events occurred less frequently than with ponatinib and nilotinib.(Herrmann 2020) The toxicity of dasatinib on pulmonary endothelium has been well established but to date, really few data are available on the toxicity of dasatinib on endothelial cells from other origins. In the **chapter 4** of this thesis, we demonstrated that the toxic effect of dasatinib is not specific to pulmonary endothelial cells and that dasatinib inhibits proliferation and impairs wound healing. This impairment might participate to the pathophysiology of arterial

occlusive events by impacting vascular repairs and inducing acute arterial thrombosis (**Figure 5.1A**). This mechanism is partially similar to one of the mechanisms proposed with ponatinib, with the difference that dasatinib did not induce endothelial cell apoptosis nor necrosis which is in line with the lower risk of arterial occlusive events of dasatinib. The rare data on arterial occlusive events with dasatinib indicate an early onset after dasatinib's induction, supporting the hypothesis of acute arterial thrombosis due to impaired vascular repairs.

Interestingly, a recent *in vitro* study reports that dasatinib induces changes in human vascular endothelial cells and promotes a mesenchymal transformation, a phenomenon linked to accelerated atherosclerosis formation.(Alkebsi et al. 2020) It has already been hypothesized that this endothelial to mesenchymal transition contributes to the vulnerability of the atherosclerotic plaque by the secretion of high amount of various extracellular matrices degrading proteins by the transitioned cells resulting in atherosclerotic plaque destabilization.(Fledderus et al. 2021) Consequently, future research should focus on the content of the extracellular matrix of atherosclerotic plaques observed after treatment with dasatinib. These investigations should also be extended to ponatinib and nilotinib. Furthermore, additional research is required to clinically define the clinical characteristics of dasatinib-induced arterial occlusive events and to define if the risk of arterial occlusive events arise in an atherosclerotic context or not to guide additional mechanistic investigations.

Are There Other Mechanisms Implicated in the Vascular Toxicity of BCR-ABL TKIs?

Can BCR-ABL TKIs induce cardiovascular diseases by decreasing endogenous fibrinolysis?

The endogenous fibrinolysis capacity is an important regulator of thrombus generation and cardiovascular risk by preventing the spreading of formed thrombi after the dissolution of an initial thrombus. Consequently,

dysregulation of the fibrinolytic system can cause thrombus formation or delay arterial thrombotic dissolution.(Okafor and Gorog 2015) Among the BCR-ABL TKIs investigated in this thesis, it has been hypothesized that ponatinib and nilotinib may affect fibrinolysis by impairing VEGF signaling in endothelial cells, leading to lower levels of urokinase-type plasminogen activator and tissue plasminogen activator, two key enzymes involved in fibrinolysis. The rationale for this hypothesis is that the VEGF signaling pathway (Hadzijusufovic et al. 2017) is a potent activator of endothelial cell mediated fibrinolysis through a regulation of tissue plasminogen activator and urokinase-type plasminogen activator expression by endothelial cells.(Pawlak et al. 2012; Kuenen 2002) In addition, *kit*, a tyrosine kinase also impacted by some BCR-ABL TKIs, is implicated in the release of tissue plasminogen activator through the regulation of growth, survival and migration of mast cells, strengthening the hypothesis that BCR-ABL TKIs may also impact fibrinolysis.(Aichberger et al. 2011a)

To date, the influence of BCR-ABL TKIs on fibrinolysis has been poorly investigated. Only one study found that both ponatinib and nilotinib do not increase mRNA expression of urokinase and tissue plasminogen activator in a murine model.(Pouwer et al. 2018) However, coagulation and fibrinolysis are complex, interconnected, dynamic and multifaceted phenomena and individual levels of isolated fibrinolytic factors failed in numerous studies to be useful to predict cardiovascular diseases.(Okafor and Gorog 2015) Thus, measurements of individual components of the coagulation and thrombolytic pathways may not provide an accurate evaluation of these complex systems.(Okafor and Gorog 2015) For these reasons, additional investigations should be performed using global assays rather than isolated quantification of coagulation and fibrinolytic plasma biomarkers.

Can BCR-ABL TKIs induce cardiovascular toxicity through NETosis?

Neutrophil extracellular traps are web-like structures that comprised cytosolic proteins and nuclear DNA that are extruded from neutrophils.

Supportive evidence from studies on human indicates that NETs are abundant in coronary thrombi and in thrombi related to an ischemic stroke.(Laridan, Martinod, and De Meyer 2019) Different constituents of NETs (e.g. plasma nucleosome, neutrophil elastase and myeloperoxidase (MPO)) have already demonstrated their clinical value to predict coronary artery diseases.(Laridan, Martinod, and De Meyer 2019; Zhang et al. 2001) Interestingly, NETs are involved in both acute arterial thrombosis (i.e. without atherosclerotic plaque) and in atherosclerotic-related arterial thrombosis (**Figure 5.2**). In acute arterial thrombosis, NETs form a scaffold for adhesion of platelets, red blood cells, fibrinogen, vWF and fibronectin.(Laridan, Martinod, and De Meyer 2019) The release of histones by neutrophils induces endothelial cell damage, prevents protein C activation by binding of histones to thrombomodulin and triggers platelet aggregation. This could turn to a vicious circle as activated platelets promote NET formation (NETosis) by means of adhesion molecules and membrane receptors (i.e. HMGB1/RAGE/TLR2/TLR4 and P-selectin/PSGL-1; **Figure 5.2A**).(de Bont, Boelens, and Pruijn 2019) Finally, the release of serine proteases promotes the intrinsic and extrinsic coagulation pathways.(Laridan, Martinod, and De Meyer 2019) In advanced atherosclerotic lesions, NET components such as DNA complexes and nucleosomes may be released by apoptotic endothelial cells.

As protein kinases actively participate in the regulation of various steps of NETosis (Khan et al. 2019; Perez-Figueroa et al. 2021; Azcutia, Parkos, and Brazil 2017) it could be hypothesized that BCR-ABL TKIs impact NETosis by impairing some kinases. For example, Akt and the Raf-MEK-ERK kinase pathways are involved in neutrophil survival regulation and NETosis.

For example, it has been reported that levamisole, an anthelmintic drug, can trigger NET formation by interfering with the Raf-MEK-ERK pathway leading to vascular toxicity.(Carmona-Rivera et al. 2017) In addition, a large screening program assessing drugs from different pharmacological classes

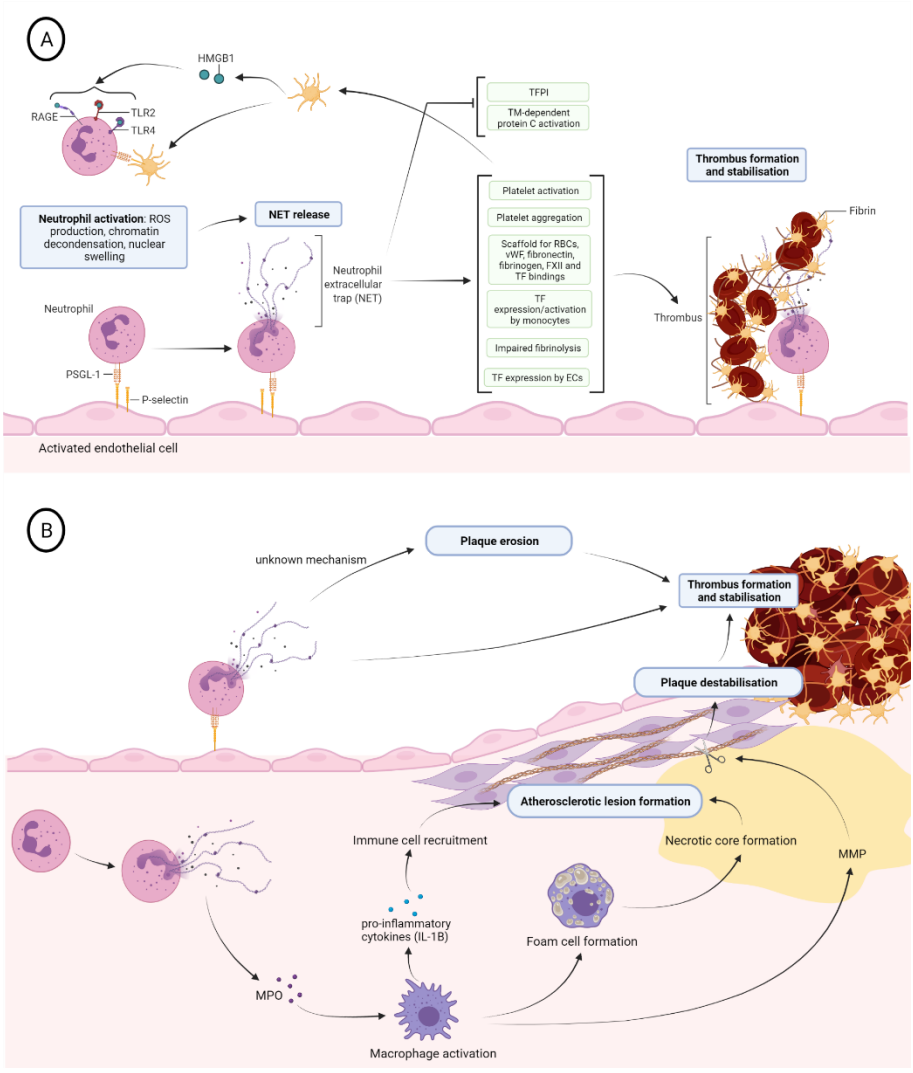


Figure 5.2 | **Role of Neutrophil Extracellular Traps in arterial thrombosis.** This figure represents the implication of NETs in acute arterial thrombosis (A) and in the atherosclerotic process (B). Figure created using BioRender. EC: endothelial cell; FXII: factor XII; HMGB1: high–mobility group box 1; IL: interleukin; MMP: matrix metalloproteinase; MPO: myeloperoxidase; NET: neutrophil extracellular trap; PSGL-1: P-selectin glycoprotein ligand-1; RAGE: receptor for advanced glycation endproducts; ROS: reactive oxygen species; RBC: red blood cell; TF: tissue factor; TFPI: tissue factor pathway inhibitor; TLR: toll-like receptor; TM: thrombomodulin; vWF: von Willebrand factor.

revealed that TKIs[7] impact NETosis.(Khan et al. 2019) Consequently, it would be relevant to investigate the effect of BCR-ABL TKIs on NETosis but using more physiological concentration (i.e. the study of Khan *et al.* was performed at a constant concentration of 20 μM for all the drugs). The first step would be to identify if BCR-ABL TKIs induced NETosis via a NOX-dependent and/or independent mechanism. This could be performed *in vitro* by incubating the BCR-ABL TKIs with human neutrophils isolated over a density gradient and quantifying the NET formation using fluorescent microscopy after neutrophil activation with lipopolysaccharide, phorbol 12-myristate 13-acetate, A23187 or Ionomycin.(Irizarry-Caro et al. 2018) The degradation of NETs by serum nucleases like DNAse could also be studied in such a model. Clinical investigations could also be indicative of the impact of BCR-ABL TKIs on NETosis by quantifying the presence of specific nucleosomes, neutrophil elastase, myeloperoxidase-DNA complexes and citrullinated histones in the patient's blood.(Thalin et al. 2019)

Perspectives in the Management of Patients With CML

The identification of the increased risk of arterial occlusive events with dasatinib, nilotinib and ponatinib permitted to highlight the need for implementing risk minimization measures for patients treated with BCR-ABL TKIs.(Seguro et al. 2021; Douxfils, Haguet, et al. 2016b) However, guidelines from expert societies aiming to provide better care for patients treated with a BCR-ABL TKI only recommend preventive strategies based on those used in the general population. This is mainly due to the lack of appropriate definition of the pathophysiology of TKI-induced arterial occlusive events and the lack of clinical studies correctly designed to identify those cardiovascular risk factors in the CML population.(Moslehi and Deininger 2015)

Identification of patients that will be more susceptible to arterial occlusive events remain challenging. Currently, selection of the frontline therapy for

[7] Information on which TKIs have been tested is not available.

chronic phase CML relies on a CML risk-score, patient comorbidities, toxicity profile of the BCR-ABL TKIs, drug interactions and patient preference.(National Comprehensive Cancer Network 2020; Seguro et al. 2021) Classical scoring systems of cardiovascular risk (e.g. Framingham risk score) have demonstrated promising predictive value in patients treated with nilotinib but required larger validation.(Breccia et al. 2015b; Rea et al. 2015). Ankle-brachial index (ABI) may also be a valuable non-invasive and financialy acceptable tool to guide clinical decision. This method has already been investigated to assess the peripheral arterial occlusive disease status in nilotinib-treated patients.(Manouchehri et al. 2020) However, its predictive value also remains to be confirmed in larger prospective studies.

Based on the experimental data and the increased knowledge about the pathophysiology of BCR-ABL TKI-associated arterial occlusive events, future research should focus on the identification and validation of predictive biomarkers that better reflect the pathophysiology of BCR-ABL TKI-induced arterial occlusive events. As several proposed mechanisms report endothelial cell toxicity, circulating endothelial cells blood levels and released of endothelial procoagulant microparticles should be quantified in the blood of patients treated with a BCR-ABL TKI and experiencing cardiovascular adverse drug reaction in order to ascertain their predictive value. Interestingly, both of these biomarkers have already been associated with damaged endothelium and are predictive of ischemic vascular diseases.(Thomson A and Garbuzova-Davis S 2016; Sabatier et al. 2009) If further investigations confirm the atherosclerotic context of BCR-ABL TKI-related arterial occlusive events, non-invasive imaging techniques such as brachial artery flow-mediated dilation, carotid intima-media thickness and carotid plaques imaging could also be promising predictive tools. They measured early processes of atherosclerosis and could have considerable interest as atherosclerotic markers.(Soulat-Dufour et al. 2012)

Another crucial challenge for next years in the CML landscape will be the validation of biomarkers able to identify CML patients who are more likely to

respond poorly and resist to imatinib.(Apperley 2021) The identification of accurate predictive biomarkers of imatinib response and of BCR-ABL TKI-associated cardiovascular disease could lead to the development of a global decision tool to help clinicians choose frontline therapy for chronic phase CML patients. This decision tool should include the actual factors used for treatment considerations (i.e. CML risk-score, patient comorbidities, TKI toxicity profile, drug interaction and patient preference) combined with the risk of arterial occlusive events and the probability of imatinib-therapy success in order to define which patients will benefit from a 2nd generation BCR-ABL TKIs.

Research should also focus on the development of effective preventive strategies, particularly for ponatinib for which primary prevention is essential as patients usually may not have other therapeutic options since to date this therapy is used after all other failed. To date, guidelines recommend an intensive management of modifiable cardiovascular risk factors (e.g. using the ABCDE approach[8]) and a continuous monitoring of the cardiovascular risk factors after initiation of BCR-ABL TKI therapy.(Seguro et al. 2021; Manouchehri et al. 2020; Santoro et al. 2019) However, most of these recommendations are general and have not been validated in the CML population so their therapeutic benefit is expected but not verified and documented. For example, prospective clinical research should investigate the benefit of lipid-lowering drugs as primary prevention in nilotinib patients since lipid metabolism seems to be the main driver of the atherosclerotic risk observed with this compound. This example exemplifies the reason of better

[8] The "ABCDE" approach is a comprehensive approach for the management of cardiovascular risk factors in daily clinical practice. This approach includes interventions regularly used to reduce the cardiovascular risk, including the assessment of risk, antiplatelet therapy, blood pressure management, cholesterol management, cigarette or tobacco cessation, diet, diabetes prevention and treatment and exercise.(Hsu et al. 2013)

delineation of the underlying mechanisms to propose additional preventive strategies.

Asciminib is a promising new molecule for the management of CML patients. Asciminib is the first-in-class STAMP (Specifically Targeting the ABL Myristoyl Pocket) inhibitor that binds to the myristate binding pocket of BCR-ABL, conferring it better specificity than ATP-binding pocket TKIs like imatinib, dasatinib, bosutinib and ponatinib.(Manley, Barys, and Cowan-Jacob 2020) Because of its higher specificity towards BCR-ABL, it is expected that asciminib overcomes resistance and presents a favorable safety profile by being less likely to interfere with other tyrosine kinases. Preliminary results of a phase 3 study comparing asciminib and bosutinib supports this favorable safety profile and demonstrate good efficacy regarding surrogate outcomes.(Hochhaus, Boquimpani, et al. 2020) Interestingly, asciminib also exhibit a good safety profile in patients heavily pretreated without therapeutic options and mostly intolerant to previous TKIs. More data are expected to evaluate asciminib efficacy, and future research should also assess its vascular safety.

Drug-induced Vascular Toxicity: Could We Identify Them Earlier?

Drug-induced vascular toxicity

Advances in therapies, including the approval of targeted therapies in the beginning of the third millennium, reduced the number of patient deaths due to cancer across a broad range of cancer types. However, the adverse event-related mortality and morbidity increased.(Zamorano et al. 2016) In addition, targeted therapies need to be taken on long-term (i.e. compared to conventional cytotoxic chemotherapy), reinforcing the need for long-term safe treatments. The cardiovascular toxicity became a great concern and a shadow in the achievement of effective and safe targeted therapies.

The cardiotoxicity is discussed in the literature since long (e.g. cardiotoxicity with 5-fluorouracil), but the drug-induced vascular toxicity concern emerged more recently, since the 2000s (**Figure 5.3**).(Herrmann 2020) Vascular toxicity remains a major concern and is currently the second most frequent cause of death in patients with cancer.(Herrmann 2020) First investigations focused on drug-induced venous thromboembolism, with extensive experiments undertaken to elucidate the apparent thrombogenic effects of oral contraceptives (Zbinden 1976) but new cancer therapies highlighted the need to investigate arterial toxicity.(Herrmann 2020)

Focusing on TKIs, since the approval of imatinib in 2001, this pharmacological class has expanded considerably those last 20 years with the approval of more than 50 new active substances. Inherent to their pharmacodynamic profile, the toxicity of TKIs is difficult to avoid since kinases are involved in multiple cellular processes common to normal and cancer cells. For example, sunitinib and sorafenib, two TKIs targeting the VEGF receptor and developed to inhibit angiogenesis, were among the first TKIs associated with cardiovascular diseases. Since then, numerous other TKIs directed against the VEGF receptor have been approved and their vascular safety profile remains similar, increasing the number of patients suffering from arterial hypertension, stable angina and acute coronary syndrome.(Herrmann 2016) The molecular mechanisms underlying these events appears to be common among these molecules and relies on the inhibition of the VEGF signaling pathway.

Namely, by inhibiting VEGF signaling, these TKIs decreased NO synthase activity as a result of decreased PKB/Akt activity (**Table 5.1**).(Herrmann 2016) This increases the systemic vascular tone, inducing arterial hypertension as well as vasospasm. It has also been proposed that VEGF impairment induce accelerated atherosclerosis and destabilization of atherosclerotic plaque through reduction of the viability of neovessels of the atherosclerotic plaque level.(Holm et al. 2009) Another mechanism which is specific to sunitinib has been proposed, relying on its supplemental PDGF inhibition (**Table 5.1**).(Chintalgattu et al. 2013) It has been proposed that sunitinib eliminates

pericytes from the coronary microcirculation, inducing deleterious effects on endothelial cells.(Chintalgattu et al. 2013) However, as described in the **chapter 3**, imatinib also inhibits PDGF signaling without inducing adverse cardiovascular events. The inhibition of this pathway by imatinib was even considered as beneficial and contributing to the safe cardiovascular profile of this TKI.

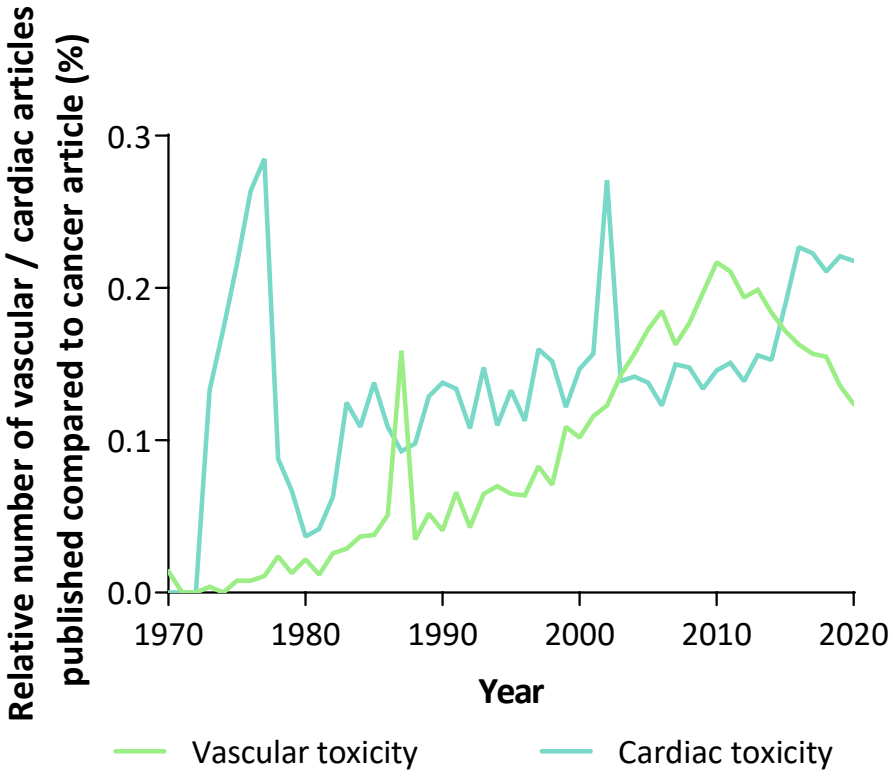


Figure 5.3 | **Overview of the percentage of articles related to vascular and cardiac toxicity associated with cancer therapies.** This figure represents the number of articles published per year on Pubmed related to vascular/cardiac toxicity of cancer therapies normalized by the number of articles on cancer. The literature search has been performed using the following keywords: (1) "cardiac toxicity cancer therapy" or "cardiotoxicity cancer therapy" for the cardiac toxicity, (2) "vascular toxicity cancer therapy" or "vasculotoxicity cancer therapy" for the vascular toxicity and (3) "cancer" for the cancer search.

Table 5.1 | **Vascular toxicity associated with approved-TKIs.** Adapted from Herrmann 2020. Data came from Herrmann 2020 based on data from Micromedex and Lexicomp except for tofacitinib (data from (Mease et al. 2020)).

TKI	Vascular toxicity						Pathophysiology
	Clinical presentation*						
	ATH	Angina	AMI	Stroke	PAOD	DVT/PE	
BCR-ABL inhibitor							
Dasatinib	-	++	-	-	-	+	Acute arterial thrombosis: prolonged arterial injury due to defective vascular repair (working hypothesis). Accelerated atherogenesis: increased ECM degradation following endothelial-mesenchymal transition (working hypothesis).
Nilotinib	++	++	+	++	+++	Unk	Accelerated atherogenesis: subsequent to hyperglycemia and LDL level and EC activation. Pro-aggregatory effect on platelets.
Ponatinib	+++	+++	+++	++	++	++	Acute arterial thrombosis: endothelium injury due to altered EC viability and defective vascular repair (working hypotheses). Atherosclerotic plaque erosion: facilitated by EC apoptosis. (working hypothesis).
VEGFR inhibitor							
All VEGFR inhibitors							Acute vasospasm: ↘ PKB/Akt activity → ↘ NO synthase activity and expression → ↗ systemic vascular tone Atherosclerosis progression and plaque rupture: Destabilization of plaque neovessels (Holm et al. 2009)
Sorafenib	+++	+	++	+	-	+	Acute vasospasm: ↘ *MEK activity in VSMCs* → ↗ *ROCK pathway* → ↗ *Ca^{2+} sensitivity (working hypothesis)* (Naib, Steingart, and Chen 2011)

Sunitinib	+++	+++	+	+	-	++	Acute vasospasm: PDGF inhibition → eliminate pericytes from coronary microcirculation → deleterious effects on ECs (Chintalgattu et al. 2013)
Pazopanib	+++	+++	++	+	-	++	Cf. all VEGFR inhibitors.
Axitinib	+++	+	++	+	-	++	Cf. all VEGFR inhibitors.
Regorafenib	+++	+	+	-	-	++	Cf. all VEGFR inhibitors.
Lenvatinib	+++	-	++	-	-	++	Cf. all VEGFR inhibitors.
Vandetanib	+++	-	-	+	-	++	Cf. all VEGFR inhibitors.
Cabozantinib	+++	-	++	++	-	++	Cf. all VEGFR inhibitors.
EGFR inhibitor							
Erlotinib	-	+++	++	++	-	+++	Unk
Dacomitinib	-	++	-	-	-	-	Unk
ALK inhibitor							
Crizotinib	-	-	-	-	-	++	Unk
Alectinib	-	-	-	-	-	+	Unk
JAK inhibitor							
Tofacitinib	Unk	Unk	+	+	Unk	+	Unk
BRAF inhibitor							
Dabrafenib	+++	-	-	-	-	+	Unk
MEK inhibitor							
Binimetinib	++	-	-	-	-	++	Unk
Trametinib	+++	-	-	-	-	++	Unk

-, not reported; + uncommon (<1%); ++, common (1-10%); +++, very common (>10%)

AMI, acute myocardial infarction; ATH, arterial hypertension; EC, endothelial cell; DVT, deep venous thrombosis; PAOD, peripheral artery disease; PE, pulmonary embolism; TKI, tyrosine kinase inhibitor; Unk, unknown; VSMC, vascular smooth muscle cell

Recently, concerns were raised on the vascular toxicity of TKIs targeting other kinases than BCR-ABL and VEGF receptors.(Bai et al. 2019) Based on data from Micromedex and Lexicomp,[9] Herrmann *et al*. identified vascular toxicity with 18 approved TKIs (**Table 5.1**).(Herrmann 2020) Hypertension and arterial occlusive events, including acute myocardial infarction, angina and stroke were the main physiological expression of this vascular toxicity.(Herrmann 2020) However, for most of them, these data need to be consolidated to confirm the causality of the drug and the underlying pathophysiology need to be investigated.

Assessment of Vascular Toxicity During Drug Discovery and Preclinical Development

The vascular toxicity associated with some of the BCR-ABL TKIs demonstrated the difficulty to highlight drug-induced vascular toxicity during drug development. Indeed, it took some years after dasatinib and nilotinib marketing to recognize that they were associated with vascular toxicity, whereas, at the present time, the potential biological mechanisms explaining this toxicity are not fully elucidated. This situation is nor isolated nor exceptional and it usually takes many years after drug marketing to identify drug-induced vascular toxicity. Most of the vascular adverse events are not detected during drug development and are rather reported through post-marketing observations, indicating possibility inappropriate preclinical and clinical investigations to capture this toxicity.(Laverty et al. 2011; Tu et al. 2021)

Undoubtedly, delaying the identification of these drug-associated vascular toxicity is a multifaceted problem for the patients, the healthcare professionals and the pharmaceutical industries. A delay in the identification of drug-

[9] Lexicomp and Micromedex are applications that combined evidence-based drug content with artificial intelligence in order to deliver reliable answer and evidence-based drug information.

induced vascular toxicity increases the number of patients at risk by preventing adequate patient selection and monitoring. Failure to identify vascular toxicity during preclinical and clinical studies is also a significant cause for drug marketing withdrawal and this represents a major concern for the pharmaceutical industry.(Qureshi et al. 2011) For all these reasons and because vascular toxicity became a concern with many cancer therapies, a more appropriate evaluation of vascular toxicity during drug development should be performed systematically and prospectively before drug approval.

Current Practices and Guidelines for Cardiovascular Safety Investigations

Non-clinical safety evaluations rely on guidelines edited by the International Conference on Harmonization (also known under the acronym, ICH) in order to harmonize the nonclinical requirements among the different regulatory bodies. These guidelines define the general principles and recommendations on the design and conduct of the preclinical safety studies in order to define an initial safe starting dose and to identify parameters for clinical monitoring for potential adverse events. Safety is assessed in the preclinical phases through pharmacological studies which provide cellular and biochemical data based on pharmacodynamic endpoints, and through toxicology studies relying on histopathological endpoints to provide tissue-specific information. Recommendations to perform these studies are provided in the ICH S7A, ICH S7B and ICH M3(R2) guidelines.(International Council for Harmonisation of Technical Requirements for Pharmaceuticals 2001, 2005, 2013)

Regarding cardiovascular safety, the ICH S7A guidelines rely on investigations of the effect of the drug candidate on a battery of tests that are representative of the vital functions. In this regard, the cardiovascular system is considered a system composed of vital organs that have to be monitored by measuring blood pressure, heart rate and by performing electrocardiograms. Assays investigating repolarization and conductance abnormalities also have to be done to provide information at the cellular level. Investigations of the cardiac output, the ventricular contractility and the vascular resistance should

be selected on a case-by-case basis after considering risk factors, such as existing non-clinical or human data. The conducting of toxicology studies is defined in the ICH M3(R2) guideline. This guideline defines the drug administration exposure schemes and the animal models that should be used to perform acute and repeated dose toxicology studies. These guidelines mainly focus on the cardiotoxicity whereas the investigation of the vascular toxicity is not a requirement.

The preclinical cardiovascular studies for the three BCR-ABL TKIs associated with vascular toxicity are presented in the **Table 5.2**. These studies have been performed in accordance with the ICH guidelines and include human Ether-à-go-go-Related Gene (hERG) *in vitro* studies, electrophysiological *in vivo* investigations as well as histopathological assessment of the heart in single-dose and repeat-dose toxicity studies. Main findings were that dasatinib may induce QT prolongation, increased blood pressure and cause histopathological abnormalities which are related to heart damage. The heart was defined as a target organ of nilotinib toxicity, which has demonstrated proarrythmic potential. In addition, nilotinib increased cholesterol levels *in vivo* and prolonged activated partial thromboplastin time (aPTT) in some monkeys. For ponatinib, it was concluded that it has a similar profile as other TKIs. In conclusion, for these three TKIs, preclinical safety assessments indicate a potential risk of cardiovascular toxicity for dasatinib and nilotinib by possibly increasing blood pressure and cholesterol levels respectively, but the major concerns remain the cardiac toxicity which has been linked to QT prolongation and arrhythmia.

Strategies to Improve Vascular Preclinical Investigations

Even if up to 70% of toxicity may be approximated and anticipated during the preclinical development (Savoji et al. 2019) the detection of the vascular toxicity remains an issue. As stated above, a major limitation for the detection of vascular toxicity of a new drug candidate is the lack of specific vascular investigations and internationally recognized testing during preclinical development. Given that vascular toxicity emerged as the second most

Table 5.2 | **Summary and comparison of cardiovascular preclinical investigations with dasatinib, nilotinib and ponatinib.** These studies are those reviewed by the FDA for the drug approval. Clinically effective concentration for each TKIs is indicated in brackets (U.S. Food and Drug Administration 2012, 2007, 2006)

	Nilotinib (C_{max}= 0.171 µM)	**Dasatinib (C_{max}= 0.072 µM)**	**Ponatinib (C_{max}= 0.040 µM)**
Safety pharmacology core battery: cardiovascular system	*Effects on cloned hERG channels expressed in mammalian cells.* Inhibition of hERG current (IC_{50}: 0.13 µM). *Effects on hERG tail current in stably transfected HEK293 cells.* Non-significant ↘ of hERG current by a nilotinib metabolite. *Electrophysiological investigations in the isolated rabbit heart.* No pro-arrhythmic potential and QT prolongation. ↘ coronary flow at 0.5 µM that may be due to micro-embolus formation. *Ex-vivo study in human subcutaneous resistance and coronary arteries.* Dose-independent changes in coronary artery tone: vasoconstriction at 3 µM; no change at 10 µM. *23-day cardiovascular toxicity in telemetered dogs.*	*In vitro effects of dasatinib and its metabolites on hERG/Ikr currents (transfected human embryonic kidney cells) and rabbit Purkinje fiber action potential.* Dose-dependent inhibition of hERG currents. ↗ of the action potential duration. Minimal to moderate inhibition on hERG for 3 dasatinib metabolites not considered major metabolites in humans. *In vitro evaluation of effects of dasatinib metabolites on receptors and ion-channel binding and enzyme activity.* *Single-dose oral cardiovascular safety pharmacology study in telemetered monkeys.* ↗ systolic (5-15%) and diastolic (8-21%) BP for ± 2h after dose. No QT prolongation.	*Effects of ponatinib on hERG K+ currents in HEK-293 cells.* Dose-dependent inhibition of hERG current. *Cardiovascular evaluation of ponatinib in telemetered beagle dog.* No effects on cardiac, circulatory functions or ECGs.

	No evidence of cardiotoxicity. No hemodynamic changes.		
Single dose toxicity studies	*Single-dose oral toxicity study in rats.* No cardiovascular impact.	*Single-dose oral toxicity study in rats.* Multifocal myocardial necrosis and hemorrhage. *Single-dose oral toxicity in monkeys.* ↗ systolic and diastolic arterial BP.	*Acute oral toxicity study in mice, rats and monkeys.* Not reviewed in the FDA review.
Repeat-dose toxicity studies	*4-week oral toxicity study in rats.* ↗ heart weight. Minimal cardiomyopathy (for the highest drug doses) resuming at the end of the recovery period. *26-week oral toxicity study in rats.* ↗ total cholesterol and triglycerides. ↗ heart weight. *4-week oral toxicity study in dogs*. ↗ cholesterol. Heart focal mesothelial cell proliferation and fibrosis. *39-week oral toxicity study in monkeys.* ↗ total cholesterol and triglycerides. Hemorrhage in heart (1/6 female). ↗ aPTT in certain males. No remarkable changes in ECG.	*1-month oral toxicity studies in rats.* Cardiac hypertrophy at the end of the one-month treatment period. *6-month oral toxicity study in rat.* Cardiac hypertrophy. Heart fibrosis (few animals). *1-month intermittent dose oral toxicity study in monkeys.* Cardiac hypertrophy and chronic inflammation. No QT prolongation. *9-month oral toxicity study in monkeys.* No clear sign of cardiac toxicity. Vascular mineralization and inflammation in the heart (in 1/4 animal). Hyperplasia/hypertrophy of aorta (1/2). Neutrophilic/lymphohistocytic infiltrate in the heart. Not reviewed in the FDA review:	*28-day oral toxicity study in rats.* Mortality in 11/46, 6/46 and 1/46 animals receiving respectively 6, 3 and 1,5 mg/kg/day. Coagulation parameters: no change. Minimal heart inflammation. *6-month oral toxicity study in rats.* Mortality in 20/68 and 6/68 animals receiving 2 and 0,75 mg/kg/day. ↗ fibrinogen values at 2 mg/kg/day suggestive of inflammation. Correlate with abscesses or granulomatous inflammation in preputial/clitoral glands. ↗ cholesterol. *28-day oral toxicity study in monkeys.* Mortality in 3/10 animals receiving 5 mg/kg/day. Systolic heart murmurs. No effects on ECGs and no cardiology abnormalities.

		2-week oral study in rats and monkeys. *1-month intermittent dose toxicity study in rats.* *10-day oral toxicity study in dogs and in monkeys.*	↗ fibrinogen values in males. Transient ↗ in mean aPTT in females. Normal PT. *6-month oral toxicity study in monkeys.* No unscheduled deaths. Myocardial necrosis in 2/8, 4/8 and 1/8 at 0,25, 0,75 and 2 mg/kg/day respectively. No effects on ECGs and no cardiology abnormalities. No coagulation abnormalities.
Conclusions	Heart is identified as target organs of toxicity. Potential for nilotinib to cause pro-arrhythmia (inhibition of hERG current).	Potential for dasatinib to cause: - QT prolongation. - Increased systolic and diastolic arterial blood pressure. Cardiovascular findings in the toxicology studies: - vascular and cardiac fibrosis, - cardiac hypertrophy, - myocardial necrosis, - hemorrhage of the valves, ventricle and atrium, - cardiac inflammation.	Systolic heart murmurs and evidence of a possible dose-dependent effect on necrosis of myocardial cells may be relevant to the clinically observed myocardial toxicity. Arterial thromboembolic events and hypertension are recognized clinical side effects of VEGF inhibitors. Ponatinib exhibited a similar nonclinical toxicological profile as other TKIs.

aPTT: activated partial thromboplastin time; ECG: electrocardiogram; FDA: Food and Drug Administration; hERG: human Ether-à-go-go-Related Gene; PT: prothrombin time; TKI: tyrosine kinase inhibitor; VEGF: Vascular endothelial growth factor.

common toxicity with cancer therapy, a revision of the ICH guidelines could be considered in order to include the assessment of vascular toxicity as a requirement for drugs that will be given on a long-term period. Nevertheless, such testing should be approved and recognized as appropriate for the evaluation of the cardiovascular toxicity and should cover the different pathways possibly leading to this toxicity.

Interestingly, following the discovery of the cardiotoxicity of many TKIs, initiatives were introduced to increase our knowledge and provide protocols able to predict the cardiotoxic potential of TKIs.(Yang and Papoian 2018) In addition to the improvement of histologic method, additional preclinical approaches were proposed, mainly relying on efforts to increase the knowledge on the drug, its metabolites and drug accumulation.(Yang and Papoian 2018) Similar initiatives should be developed for the detection of vascular toxicity during the preclinical phase of drug development.

As stated above, the detection of the vascular toxicity remains challenging. One reason is the lack of understanding of the mechanisms underlying this vascular toxicity and further investigations of the pathophysiology is required.(Laverty et al. 2011) For example, the role of endothelial dysfunction in coronary artery disorders is poorly understood, as well as our knowledge of drug-induced vascular necrosis.(Laverty et al. 2011) Clinical data may also contribute to the understanding of drug-induced vascular susceptibility. However, clinical data are often scare, and the vascular adverse events are not well characterized in oncology clinical trials. Post-marketing studies and registries aiming as the primary endpoint the detection of adverse cardiovascular events should be encouraged to better define the incidence of these drug reactions and to orientate mechanistic studies based on more accurate and complete clinical data. As suggested by the European Society of Cardiology (ESC) in a position paper: « *Multidisciplinary teams including vascular specialists and radiologists should be included in the diagnosis and management of drug-induced vascular toxicity*.»(Zamorano et al. 2016)

The detection of the vascular toxicity during preclinical development may remain difficult to highlight because of subtler events that only emerge when drugs are administered to human for longer periods of time. Indeed, it has been increasingly recognized that minor cardiovascular changes may be very relevant to long-term clinical outcomes (Laverty et al. 2011) explaining why preclinical safety investigations often failed to predict the vascular toxicity. The challenge is therefore to have validated preclinical tools able to capture such minor changes to predict the vasculotoxic potential of drug candidates.

Promising Tools and Models for Vascular Safety Assessment

Most of the preclinical studies assessed the drug toxicology on 2D *in vitro* and animal models. However, animals and 2D *in vitro* models present important limitations to predict the vascular risk. The pathophysiological particularity encountered in the humans cardiovascular system is difficult to reproduce in animal models, limiting their ability to predict the risk.(Bailey, Thew, and Balls 2014; Laverty et al. 2011) *In vitro* 2D models, as those we have discussed in chapters 3 and 4, revealed that through viability and functional assay on endothelial cells, the toxicity of dasatinib and ponatinib can be identified. However, these models remain oversimplist for a global assessment of the vascular safety. They could remain useful for highthrouput screening during the early drug development stage to discriminate the toxicological potential of hit compounds[10] and/or for investigation of the mechanism behind drug-induced vascular toxicity once identified during its use in human subjects. More generally, 2D cell models possess numerous limitations including the lack of tissue architecture and interactions with other cell lines, making such models not sufficiently physiological and complex.

[10] A hit is a compound that has been demonstrated to have specific activity at the target protein following a compound screening.

Recently, induced pluripotent stem cells (iPSCs) technology was developed to enhance 2D cell models.(Tu et al. 2021) This technique uses pluripotent stem cells to generate all the major cell lineage by *in vitro* differentiation and replacement of primary cell lineages. Among the benefits of this technique, we can cite the improvement of the cell quality and the increasing heterogeneity which aim to mimic the individual predispositions to these various risks (i.e. to reflect the diversity of drug response seen between individuals).(Tu et al. 2021) Recent studies have shown encouraging results to justify the use of these cell models during preclinical investigations of drug-induced vascular toxicity.(Tu et al. 2021) However, the predictive value of such models is still limited by the lack of 3D microenvironment of tissues and organs.(Savoji et al. 2019)

To answer this need, preclinical testing was improved by the development of approaches using isolated organs, engineered tissue, 3D tissue models and body-on-a-chip (i.e. an adaptation of computer microchips to engineer microfluidic culture devices) to complete the armamentarium of dose-repeat toxicology studies.(Yang and Papoian 2018) This recent progress on tissue engineering and body-on-a-chip technologies enable a simulation of the *in vivo* vasculature by representing living human vessels and arteries (e.g. vasculature-on-a-chip, artery-on-a-chip, thrombosis-on-a-chip). These approaches have shown promise for addressing the issues encountered with 2D and animal models. They have also demonstrated their ability to be predictive of TKI cardiotoxic potential, another unexpected toxicity encountered with TKI, making possible the preclinical identification and assessment of TKI cardiotoxicity.(Yang and Papoian 2018) However, these models should be validated and their predictive value for vascular toxicity remains to be defined.

Conclusions

Through meta-analyses and *in vitro* experiments, we highlighted the vascular toxicity of three BCR-ABL TKIs in CML patients and contributed to the understanding of their pathophysiology. These three BCR-ABL TKIs affect the

vasculature differently. We demonstrated that endothelial cells are probably a main culprit in ponatinib-related arterial occlusive events, as ponatinib exerts a highly toxic effect on these cell types, possibly inducing acute arterial thrombosis and favoring atherosclerotic plaque erosion. The effect of dasatinib on endothelial cells is less pronounced but could participate to dasatinib-induced arterial occlusive events by impairing endothelium repairs. Nilotinib possesses a different vascular profile. It deregulates glucose and lipid metabolism and impact platelet functions. Additional investigations are now required on more complex models in order to validate these hypotheses. The identification of the precise mechanisms underlying TKI-induced arterial occlusive events is a central element as it influences the management of cancer therapy-related vascular toxicity by guiding treatment selection, realizing vascular monitoring and anticipating vascular toxicity management. Future research should focus on the validation of predictive biomarkers of vascular toxicity that can guide the selection of patients that will better benefit of 2nd generation TKIs.

The vascular safety of BCR-ABL TKIs is particularly important because of the long-term use of these treatments and the normal expected life span of CML patients. The CML landscape drastically changed these last 20 years with the discovery of imatinib. Because of the effective therapeutic armamentarium, interests for a new drug candidate for CML treatment are now on treatment-free remission and quality-of-life. Therefore, future drugs will need to be safer than the current therapies to be classified as a « game changer » in this therapeutic area. In addition, the vascular toxicity is not restricted to the BCR-ABL TKIs and is becoming one of the major concerns regarding drug safety. All of these issues call for improvement of the prediction of vascular toxicity during drug development.

Lexique

ABC transporter: ATP-binding cassette transporters. ABCG1 and ABCA1 are implicated in macrophage reverse cholesterol transport. Their deficiency leads to foam cell formation and atherosclerosis development.

Adiponectin: protein regulating glucose metabolism. Adiponectin increases peripheral insulin sensitivity by improving glucose uptake.

Apobec1: apolipoprotein B mRNA editing enzyme catalytic subunit 1. Apobec1 introduces a stop codon into ApoB mRNA.

Arg: Abelson-related gene (also known as ABL2). Arg possesses cytoskeletal-remodeling functions.

CETP: cholesteryl ester transfer protein.

CSFR: Colony stimulating factor receptor. CSFR drives growth and development of monocytes.

DDR-1: discoidin domain receptor tyrosine kinase 1. DDR1 is involved in regulation of cell growth, differentiation and metabolism.

Ephrin family: members of this family are involved in platelet spreading, adhesion to fibrinogen and platelet secretion.

GLUT4: Glucose transporter type 4. GLUT4 is an insulin-regulated glucose transporter expressed in peripheral tissues.

HCAEC: Human Coronary Artery Endothelial Cells.

hERG: the human ether-a-go-go related gene encodes the pore-forming subunit of a potassium ion channel. Blockage of that channel causes drug-induced QT prolongation with an increased risk of sudden cardiac arrest.

HMGCoA reductase: 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase. HMGCoA reductase catalysis the conversion of HMG-CoA to mevalonic acid, an essential step in cholesterol synthesis.

HUVEC: Human Umbilical Vein Endothelial Cells.

ICAM-1: Intercellular Adhesion Molecule 1. ICAM-1 stabilizes leukocyte-endothelial cell adhesion and facilitates leukocyte transmigration.

IFN-γ: Interferon gamma. IFN-γ is involved in innate and adaptive immunity and activates macrophages.

IL-6: Interleukin 6. IL-6 is a pro-inflammatory cytokine secreted by T-cells and macrophages to stimulate immune response.

IL-10: Interleukin 10. IL-10 exerts immunoregulation and regulates inflammation.

IL-12: Interleukin 12. IL-12 is involved in T-cell differentiation and functions.

JNK: c-Jun N-terminal kinases. JNK is responsive to stress stimuli and mediates insulin resistance through inhibition of insulin receptor substrate.

Lck: lymphocyte-specific protein tyrosine kinase. Lck is mostly involved in T-cell maturation.

LDLR: low-density lipoprotein receptor. This cell surface receptor mediates LDL endocytosis.

MMP-2 and MMP-9 are two proteases capable of degrading extracellular matrix components. These 2 MMPs are the main proteases involved in atherogenesis.

PAR-1: protease-activated receptor 1. PAR receptors mediate cellular effects of thrombin in platelets and endothelial cells.

RhoA-ROCK: Ras homolog gene family, member A - Rho-associated protein kinase. Rho-kinase regulates cytoskeletal reorganization, cell migration, cell proliferation and survival.

SIK: Salt inducible kinase. SIKs regulate production of anti- and pro-inflammatory cytokines.

Src: protein involved in angiogenesis and cell survival and proliferation

Tie-2: tunica interna endothelial cell kinase. Tie-2 regulates angiogenesis, endothelial cell survival, proliferation, migration, adhesion and cell spreading, cytoskeleton reorganization and vascular quiescence. Tie-2 also possesses anti-inflammatory functions by preventing the leakage of pro-inflammatory mediators and leukocytes.

TNF-α: tumor necrosis factor alpha. This cytokine is mainly involved in systemic inflammation and regulates immune cells.

VCAM-1: Vascular Cell Adhesion Molecule 1. VCAM-1 mediates rolling-type and firm adhesion of leukocytes.

VE-cadherin: vascular endothelial cadherin. VE-cadherin is a cell-cell adhesion molecule implies in endothelial junctions.

VEGFR: vascular endothelial growth factor. This protein plays major roles in vasculogenesis and angiogenesis.

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List of publications

Related to the thesis

1) Haguet H*, Douxfils J*, Mullier F, Chatelain C, Graux C, Dogne JM. Association Between BCR-ABL Tyrosine Kinase Inhibitors for Chronic Myeloid Leukemia and Cardiovascular Events, Major Molecular Response, and Overall Survival: A Systematic Review and Meta-analysis. JAMA Oncol. 2016;2(5):625-632.
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2) Douxfils J, Haguet H, Dogne JM. Multiple Causes of Cardiotoxic Effects in Patients With Chronic Myeloid Leukemia-Reply. *JAMA Oncol.* 2016;2(6):829.

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5) Haguet H, Douxfils J, Chatelain C, Graux C, Mullier F, Dogne JM. BCR-ABL Tyrosine Kinase Inhibitors: Which Mechanism(s) May Explain the Risk of Thrombosis? TH Open. 2018;2(1):e68-e88.

6) Haguet H, Graux C, Mullier F, Dogne JM, Douxfils J. Long-Term Survival, Vascular Occlusive Events and Efficacy Biomarkers of First-Line Treatment of CML: A Meta-Analysis. *Cancers (Basel).* 2020;12(5).

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