UNIVERSITÀ DEGLI STUDI DI MILANO – BICOCCA

Facoltà di Scienze Matematiche, Fisiche e Naturali
Dipartimento di Biotecnologie e Bioscienze

Dottorato di ricerca in Biotecnologie Industriali,
XXIV ciclo

Development and Clinical Validation of a Novel and Non-PCR Based Method for the Detection of the JAK2V617F Mutation in Chronic Myeloproliferative Neoplasms

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Anno Accademico 2010/2011

Dottorato in Biotecnologie Industriali, XXIV ciclo

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RINGRAZIAMENTI

*Il primo e doveroso ringraziamento è per la **Dottoressa Giulia Amicarelli**. Devo a lei parte della mia formazione scientifica e il successo di questo lavoro ma soprattutto la ringrazio per il profondo affetto dimostratomi in tutti questi anni, per il supporto costante nei momenti difficili e per aver condiviso con me i momenti più significativi della mia vita professionale e non solo.*
Un grazie senza fine è per il suo entusiasmo che ha alimentato il mio, per essere stata maestra e compagna di "viaggio", per non aver mai smesso di credere in me e per la stupenda persona che per me rappresenta.

*Un sentito e profondo ringraziamento per la sua solerte guida va al **Dottor Francesco Colotta**. La mia stima per lui è dovuta non solo alla sua grande esperienza scientifica ma soprattutto alla sua immensa disponibilità, alla sua grande umanità con la quale ha saputo incoraggiarmi e guidarmi durante una parte importante del mio percorso, per avermi dedicato parte del suo prezioso tempo con pazienza e dedizione senza mai negarmi il suo supporto, per aver alimentato la mia curiosità e condiviso la passione per la ricerca.*
L'entusiasmo e il grande impegno trovano giustificazione sotto la sua sapiente guida.

*Desidero inoltre ringraziare il **Dr. Fabrizio Bonelli** per i suoi preziosi consigli e per avermi accompagnato in una parte significativa di questo lavoro, il **Dr. Daniel Adlerstein** per avermi scelto e i ragazzi del gruppo di oncologia molecolare DiaSorin, in modo particolare **Elena** e **Riccardo** per aver contribuito ai risultati ottenuti presentati in questa tesi.*

*Intendo poi ringraziare il **Professor Alessandro Rambaldi** sottolineando il suo contributo scientifico e la sua grande disponibilità; tutto lo staff Fondazione Paolo Belli (Ospedali Riuniti di Bergamo) in particolare le **Dott.sse Silvia Salmoiraghi** e **Orietta Spinelli** per la loro collaborazione e per avermi fornito i campioni clinici indispensabili per la realizzazione della tesi.*

*Per ultimo ma non per ordine di importanza, vorrei ringraziare la mia meravigliosa **Famiglia** per avermi donato il privilegio dello studio e **Marco** per avermi supportato e "sopportato"… e semplicemente per esserci sempre stato.*

ABBREVIATIONS

AML= Acute myeloid leukemia

AS-LAMP= Allele Specific Loop mediated Isothermal Amplification

BM = Bone marrow

CHL= Chronic Hypereosinophilic Leukemia

CML= Chronic Myeloid Leukemia

CMML = Chronic Myelomonocytic Leukemia

CMPD = Chronic Myeloproliferative Disorder

CNL= Chronic Neutrophil Leukemia

CNTF = Ciliary Neurotrophic Factor

dNTP= Deoxyribonucleotide Phosphate

EPO= Erythropoietin

ET= Essential Thrombocythemia

G-CSF= Granulocyte Colony Stimulating Factor

GH = Growth Harmone

GM-CSF= Granulocyte Monocyte Colony Stimulating Factor

Hb= Hemoglobin

HCT= Hematocrit

HEL= Human Erythrocytic Leukemia (cell line)

IE= Idiopathic Erythrocytosis

IGF-1= Insulin-Like Growth Factor 1

IL-3= Interleukin-3

IM= Idiopathic Myelofibrosis

IMF= Idiopathic Myelofibrosis

Inv= inversion (chromosomal)

LAMP = Loop mediated Isothermal Amplification

LIF = Leukemia Inhibitory Factor

LOH= Loss Of Heterozygozity

MDS= Myelodysplastic Syndrome

MPL= Thrombopoietin receptor

Mut = mutation

PB= Peripheral Blood

PCR= Polymerase-chain-reaction

PMF= Primary myelofibrosis

PNA= Peptide Nuclei Acid

PRL= prolactin

PV = Polycythemia Vera

qPCR= Quantitative Polymerase-Chain-Reaction

SCF= Stem Cell Factor

SE= Secondary erythrocytosis

sLB= self-annealed loop primer

SOCS= Suppressor of Cytokine Signalling

STAT= Signal Transducers and Activators of Transcription

t= Translocation (chromosomal)

TIA= Transient Ischemic Attack

Tm= Melting Temperature

TPO= Thrombopoietin

WBC= White Blood Cells

WHO = World Health Organisation

Wt = Wild type

HUMAN GENES

JAK2 , Janus Kinase 2

ABL, Abelson gene

RIASSUNTO

Il progetto di dottorato illustrato nella presente tesi tratta del disegno, dello sviluppo e della validazione su campioni clinici di un saggio diagnostico molecolare alternativo rispetto ai metodi basati sulla PCR per la rilevazione della mutazione V617F nel gene JAK2, riscontrata con alta frequenza in disordini mieloproliferativi.

Janus Kinase-2 (JAK2) è una tirosin chinasi citoplasmatica essenziale per la trasduzione dei segnali intracitoplasmatici mediati da alcune citochine. In particolare JAK2 è coinvolta nella proliferazione delle cellule ematopoietiche e gioca un ruolo chiave in numerose malattie mieloproliferative croniche Philadelphia-negative, quali la Policitemia Vera (PV), la Trombocitemia Essenziale (TE) e la Mielofibrosi Primaria (IM).

La tirosin chinasi JAK2 possiede, nella sua struttura primaria, 7 domini JAK omologhi (JH1-JH7). Il dominio C-terminale JH1 corrisponde al dominio chinasico o catalitico. Nelle adiacenze del dominio chinasico è localizzato il dominio JH2 detto anche dominio pseudochinasico, poiché inattivo dal punto vista catalitico (kinase-like domain). Sebbene il dominio pseudochinasico JH2 manchi di attività catalitica, esso possiede un importante ruolo regolatorio, agendo come inibitore sul dominio cataliticamente attivo JH1, in assenza di citochine attivatrici.

Una mutazione puntiforme nel dominio JH2 del gene JAK2, localizzato sul braccio corto del cromosoma 9 (9p24.1), porta alla transversione con sostituzione aminoacidica di una valina in fenilalanina in posizione 617 (V1617F) con conseguente destabilizzazione del ruolo inibitorio di JH2 e attivazione costitutiva della tirosin chinasi JAK2 e dei fattori di trascrizione "Signal Transducers and Activators of Transcription" (STAT). La mutazione JAK2 (V617F) è una mutazione acquisita caratteristica delle cellule mieloidi ed eritroidi ed assente nei linfociti T. Tale mutazione conferisce ai progenitori emopoietici mieloidi un vantaggio in termini

di proliferazione e di sopravvivenza, e provoca la loro espansione clonale selettiva.

La mutazione V617F di JAK2 è presente nel 80-95% delle PV, nel 23-57% delle TE e nel 35-95% dei pazienti con IM, pertanto la valutazione della mutazione è obbligatoria per la diagnosi delle malattie mieloproliferative croniche secondo le linee guida WHO (World Health Organization).

Numerose tecniche di diagnosi molecolare sono oggi utilizzate per rilevare la mutazione V617F, tuttavia ciascuna di esse presenta alcune importanti limitazioni come una bassa sensibilità e l'utilizzo di procedure laboriose e strumentazioni sofisticate che potrebbero non essere presenti in tutti i laboratori ospedalieri.

Lo scopo di questa tesi è stato quello di sviluppare un nuovo saggio diagnostico "non PCR based", caratterizzato da un'alta specificità, sensibilità, efficienza e rapidità, che possa rappresentare una valida alternativa alle metodiche di rilevazione della mutazione fino ad oggi disponibili, superandone le limitazioni e venendo incontro ai bisogni espressi dai clinici.

Negli ultimi anni sono state descritte diverse tecniche di amplificazione genica alternative alla PCR, con prestazioni paragonabili e caratteristiche peculiari che le rendono potenzialmente vantaggiose per determinate applicazioni diagnostiche. Una di queste è la tecnologia "Loop-mediated Isothermal Amplification" (LAMP). LAMP è una metodica isoterma di amplificazione degli acidi nucleici che prevede l'utilizzo di una polimerasi termostabile dotata di attività di "strand displacement" e di un set di 6 primers espressamente disegnati per riconoscere 8 zone distinte del target. Attraverso una specifica dinamica di annealing, estensione e displacement che porta alla formazione di loops dei filamenti neo-sintetizzati è possibile amplificare il materiale genico di partenza con elevata efficienza (fino a 10^{10} volte), mantenendo la temperatura sempre costante attorno

all'optimum di attività della polimerasi, in tempi estremamente brevi (da 15 a 60 minuti) e con elevato grado di specificità.

Il segnale di amplificazione può essere rilevato in real-time mediante turbidimetria o fluorescenza. Il primo metodo è reso possibile dell'elevata efficienza di amplificazione propria della LAMP e consiste nel misurare l'intorbidimento della miscela di reazione causato dalla precipitazione di pirofosfato di magnesio. Questo sale insolubile si forma dall'interazione degli ioni Mg^{2+} presenti nella soluzione di reazione con il pirofosfato inorganico liberato dall'incorporazione dei dNTPs nei filamenti nascenti di DNA. La quantità di pirofosfato di magnesio è quindi proporzionale alla quantità di DNA amplificato ed è misurabile come segnale di trasmittanza sia a fine reazione per una valutazione qualitativa end-point, che in tempo reale per un approccio quantitativo. Per la sua semplicità e praticità la turbidimetria rappresenta quindi la tecnica di rilevazione d'elezione per LAMP. Tuttavia, la rilevazione basata sulla turbidimetria non consente di introdurre in reazione un controllo interno di amplificazione, che rappresenta un valore aggiunto nelle applicazioni di diagnostica clinica. Per questo motivo è preferibile rilevare il segnale di amplificazione utilizzando la fluorescenza emessa da un intercalante del DNA. Quest'ultimo, intercalandosi all'interno del filamento neo sintetizzato, emette fluorescenza rilevabile in tempo reale. La quantità di fluorescenza rilevata è proporzionale alla quantità di DNA amplificato. Poiché l'intercalante non consente di distinguere prodotti di amplificazione differenti, al termine della reazione viene eseguita un'analisi di annealing che consente di visualizzare picchi di annealing distinti, ciascuno caratterizzato da una temperatura di annealing specifica dell'amplicone rispettivamente del target e del controllo.

La tecnologia LAMP rappresenta quindi un interessante campo di ricerca e sviluppo per la progettazione di nuovi saggi diagnostici grazie alle sue caratteristiche di rapidità, specificità, basso costo dei reagenti e della semplice strumentazione richiesta. Essendo una tecnica relativamente recente, lascia spazio a numerose modifiche

volte a plasmare il metodo sulle esigenze di rilevazione più diverse, così come è stato possibile negli anni passati per la PCR (nested-PCR, PCR asimmetrica, Touch-down PCR, cold-PCR, PCR allele specifica, ARMS PCR).

Non essendo descritta in letteratura alcuna applicazione LAMP per la rilevazione delle mutazioni puntiformi somatiche, il primo passo per lo sviluppo del saggio è stato il disegno di molteplici strategie molecolari in grado di rilevare la mutazione puntiforme V617F nel gene JAK2. Le differenti strategie molecolari hanno previsto il disegno di numerosi set di primers in grado di discriminare la presenza della mutazione (target mutato) dal target wild type e produrre amplificato selettivamente a partire dalle molecole mutate. Poiché la regione genica nella quale è presente la mutazione V617F è AT-rich, i primers per l'amplificazione sono stati disegnati manualmente, controllando tuttavia le Tm dei primers con l'aiuto di un programma (VisualOMP).

Sulla base dei risultati ottenuti, la strategia molecolare più promettente è stata scelta per l'ulteriore sviluppo e ottimizzazione del saggio diagnostico in turbidimetria e successivamente in fluorescenza. Il passaggio da una rilevazione dell'amplificazione basata sulla turbidimetria ad una basata sulla fluorescenza mediata da intercalante è stato dettato dalla necessità di poter introdurre, nella stessa reazione, un ulteriore sistema di primers specifici per un gene di controllo, la cui amplificazione permette di ridurre il rischio di risultati falsamente negativi.

La strategia molecolare che ha garantito le performance migliori, nominata Allele Specific LAMP (AS-LAMP) , ha previsto l'introduzione di cambiamenti importanti rispetto alla metodica LAMP base descritta in letteratura (Notomi et al, 2000) ed è per questo stata brevettata.

Il saggio AS-LAMP JAK2 prevede l'introduzione di due elementi molecolari per l'amplificazione selettiva delle sole molecole di DNA mutate, a discapito di quelle wild type. In particolare, è stato

introdotto un primer mutazione specifico (self-annealed loop primer, sLB) disegnato in modo da presentare una struttura "stem-loop" che ne determina una sorta di "autosequestro" qualora in soluzione non siano presenti molecole dalla sequenza mutata. In presenza di target mutato, invece, i legami intramolecolari del sLB si dissociano permettendo il legame del target specifico mutato, con conseguente innesco del processo di amplificazione esponenziale.

Inoltre il saggio AS-LAMP JAK2, prevede l'introduzione in reazione di un blocker specifico per il target wild type (Peptide Nucleic Acid, PNA) che si è mostrato essere particolarmente importante per aumentare le capacità discriminative del saggio.

Lo sviluppo del sistema AS-LAMP JAK2 in fluorescenza ha previsto l'introduzione in reazione di un intercalante del DNA (Yo-Pro 1) in grado di emettere fluorescenza quando incorporato nel doppio filamento di DNA neosintetizzato. Fermo restando il set di primer ottimizzato nel saggio in turbidimetria, questa implementazione ha previsto l'ulteriore ottimizzazione delle condizioni di reazione e l'aggiunta di un ulteriore sistema di primers capace di amplificare simultaneamente il gene di controllo ABL, in un'unica miscela di reazione. Al termine della reazione, i differenti prodotti di amplificazione genica del gene mutato (JAK2V617F) e del gene di controllo (ABL), sono facilmente discriminabili grazie ad un'analisi di annealing.

La valutazione delle prestazioni del saggio è stata effettuata dapprima su dei target modello (mini-geni sintetici), successivamente su DNA genomico proveniente da linee cellulari positive e negative per la mutazione V617F ed in ultimo validata su campioni clinici, sia per il sistema in turbidimetria che per il sistema in fluorescenza. Sono state dunque testate la specificità (capacità di amplificare il target specifico rispetto a quello non specifico), la sensibilità (minima concentrazione di target mutato rilevabile) e selettività (minima concentrazione di target mutato rilevabile in presenza di target wild type). Le reazioni sono state condotte in un blocco termico

appositamente progettato per LAMP e dotato di un rilevatore real-time di torbidità e, successivamente, su un diverso strumento in grado di incubare i campioni a temperatura costante e di rilevare in tempo reale la fluorescenza emessa dall' intercalante con successiva analisi di annealing dei prodotti di amplificazione.

La tecnologia LAMP si è dimostrata essere altamente sensibile e specifica, in grado di rilevare la presenza della mutazione sino a livelli pari allo 0.5% di gene mutato in un background di gene wild type, utilizzando una minima quantità di DNA (25 ng/reazione). Non è stata rilevata nessuna amplificazione aspecifica di target wild type entro trenta minuti di reazione, su un elevato numero di replicati. Questo livello di sensibilità e specificità è molto migliorativo rispetto a quello ottenuto dalle differenti tecniche diagnostiche utilizzate oggigiorno nei laboratori ospedalieri di riferimento, che possiedono un intervallo di sensibilità, in seguito ad un necessario arricchimento cellulare, compreso tra il 20 e 1% di gene mutato e una specificità compresa tra il 95 e 98%.

Per testare poi l'applicabilità del metodo nella pratica clinica, il saggio è stato validato su campioni clinici fornitici dal reparto di ematologia degli Ospedali Riuniti di Bergamo.

Il saggio in turbidimetria è stato validato su 87 campioni clinici negativi e 66 campioni clinici JAK2 V617F positivi, utilizzando DNA estratto da granulociti isolati da sangue intero di pazienti affetti. Il saggio in fluorescenza è stato validato su 27 campioni clinici positivi per la mutazione V617F, di cui 10 utilizzando DNA estratto da granulociti e 17 utilizzando DNA estratto direttamente da sangue intero. Tutti i campioni positivi sono stati correttamente identificati da AS-LAMP. Inoltre, la rilevazione della mutazione direttamente su DNA estratto da sangue intero ha evidenziato un ulteriore vantaggio del sistema AS-LAMP in fluorescenza che non necessita del passaggio di isolamento dei granulociti tipico della metodica PCR di riferimento grazie alla sua superiore sensibilità. La validazione del saggio AS-LAMP in fluorescenza è proseguita con il test di 9

campioni da pazienti affetti da disordini mieloproliferativi (DNA estratto da sangue intero) e 10 campioni di DNA estratto da granulociti di donatori sani, precedentemente valutati negativi per ASO-PCR. Soltanto un campione negativo con PCR è stato trovato chiaramente positivo per AS-LAMP. Questo campione, appartenente ad un paziente affetto da Trombocitemia Essenziale, dopo ulteriori indagini si è confermato debolmente positivo per la mutazione V617F anche attraverso la metodica convenzionale, confermando così la superiore sensibilità del saggio AS-LAMP.

I test hanno inoltre dimostrato la robustezza del saggio, che non è soggetto ad amplificazioni aspecifiche o ad inibizioni dovute a possibili effetti matrice.

Concludendo, la presente tesi illustra lo sviluppo e l'ottimizzazione di un nuovo saggio (AS-LAMP) e la sua applicazione in campo clinico per la rilevazione della mutazione puntiforme V617F nel gene JAK2, strettamente correlata alle Neoplasie Mieloproliferative. AS-LAMP è stata ottimizzata su controlli plasmidici e genomici ed è stata validata su campioni clinici. La tecnologia si è mostrata essere molto sensibile, estremamente specifica, semplice da effettuare, efficacemente rapida e poco costosa, poichè non richiede l'utilizzo di strumenti sofisticati. Infatti l'amplificazione e la rilevazione della mutazione avvengono entro trenta minuti di reazione in una singola provetta e, nella versione in fluorescenza, è stato possibile validare i campioni clinici negativi grazie alla presenza del controllo interno. Alla luce di queste considerazioni, la tecnologia mostrata è proposta come un valido e potente strumento nella pratica clinica per la determinazione della mutazione V617F di JAK2 in pazienti con disordini mieloproliferativi.

INTRODUCTION

Hematopoiesis

Hematopoiesis is a highly orchestrated process of blood cells formation that maintains homeostasis by producing billions of White Blood Cells (WBCs), Red Blood Cells (RBCs) and platelets on a daily basis (Figure 1)[(1)].

In mammals, hematopoiesis is divided into two main systems, the primitive embryonic system and the definitive system. The primitive hematopoiesis involves multiple anatomical sites starts in the yolk sac as early as in the first few weeks of embryonic development, producing primitive blood cells and also adult hematopoietic stem cells. Afterwards, definitive hematopoiesis starts in the intraembryonic aorta-gonad-mesonephros (AGM) region, a major site of the *in situ* generation of definitive hematopoietic stem cells (HSCs). From 6 weeks until 6-7 months of gestation, the fetal liver and spleen become the major sites of hematopoiesis and this activity remains detectable until 2 weeks after birth. Later on, the bone marrow gradually becomes the primary blood forming site and during childhood and adult life the bone marrow is the only source of normal hematopoiesis [(2)].

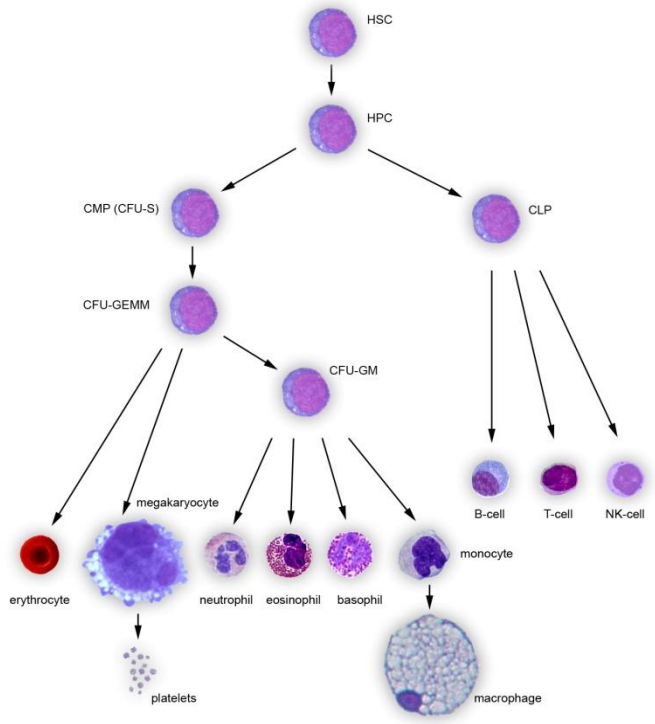

Fig. 1. A common model of Hematopoiesis. HSC, hematopoietic stem cell; CMP, common myeloid progenitors; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythrocyte progenitor; GMP, granulocyte/macrophage progenitor; TNK, T cell natural killer cell progenitor; BCP, B cell progenitor; MkP, megakaryocyte progenitor; EP, erythroid progenitor; GP, granulocyte progenitor; MP, monocyte progenitor; TCP, T cell progenitor; NKP, natural killer cell progenitor.

Regulation of hematopoiesis

In mammals, most blood cells have relatively short life spans, and it is estimated that each day up to 10^{12} fresh blood cells need to be produced in normal adults to maintain the steady-state hematopoiesis. For this reason, HSCs continuously differentiate into multiple lineages of

different blood cell types, simultaneously replicating themselves through self-renewal to prevent depletion of the stem cell pool in the bone marrow [2].

The marrow contains specialized environments that regulate the balance of HSC self-renewal and differentiation and comprise what has been termed the stem cell niche. This highly orchestrated process of blood cell production from HSCs is finely regulated by 3 major mechanisms: the lineage specific transcription factors [4], interaction between hematopoietic cells and the bone marrow niches [5,6] and carefully orchestrated changes in the levels of hematopoietic growth factors (HGFs) [7].

Dysregulation of hematopoietic growth factors underlies a number of disorders such as lymphomas, myeloproliferative disorders and leukemias.

Cytokines and hematopoietin receptor superfamily

The Hematopoietic Growth Factors (HGFs) are a group of acidic glycoproteins that bind to cytokine receptor family members. These cytokines modulate gene expression in diverse cell types. Binding of ligand to receptor triggers receptor conformational changes and transduces extracellular signals inside the cell to instruct cell survival, proliferation, and differentiation.

Common cytokines engaged in normal hematopoiesis include Interleukins (ILs), Interferons (IFNs), Colony-Stimulating Factors (CSFs), Erythropoietin (EPO), Thrombopoietin (TPO) and Leukemia Inhibitory Factor (LIF). All these cytokines have their receptors on the cell surface, called cytokine receptors or hematopoietin receptors. Most hematopoietin receptors belong to type I cytokine receptors[7].

Type I cytokine receptors are transmembrane receptors expressed on the surface of cells with four α-helical strands. This family includes receptors for IL2 (beta-subunit), IL3, IL4, IL5, IL6, IL7, IL9, IL11, IL12, GM-CSF, G-CSF, Epo, LIF, CNTF, and also the receptors for Thrombopoietin (TPO), Prolactin, and Growth hormone[7].

Each of these cytokine receptors is comprised of an intracellular domain containing approximately 100 to 500 amino acids, a transmembrane domain of 20 to 25 residues, and one or two extracellular cytokine-binding domains with several common structural features. The conserved extracellular domain has a length of approximately 200 amino acids, which contains four positionally conserved cysteine residues in the

amino-terminal region and a Trp-Ser-X-Trp-Ser motif (WSXWS motif) located proximal to the transmembrane domain. The four cysteines appear to be critical to the maintenance of the structural and functional integrity of the receptors. The WSXWS consensus sequence is thought to serve as a recognition site for functional protein-protein interaction of cytokine receptors.

The most cytokine receptors are devoid of catalytic activity and they transmit their signals through cellular tyrosine kinases, in most cases via the family of Janus kinases (JAKs).

JAK-STAT signaling in hematopoiesis

Overview of the JAK-STAT activation mechanism

Janus tyrosine kinases play critical roles in cytokine signaling transduction of hematopoietic cells. They are involved in transducing signals from type I receptors (receptors for IL-2-IL-7, IL-13, GM-CSF, GH, PRL, EPO, and TPO) as well as type II cytokine receptors (receptors for IFN-α, β, γ). The Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway is widely exploited by members of cytokine receptor superfamily, including EPO receptor (EPOR), thrombopoietin receptor (MPL), granulocyte colony-stimulating factor receptor (G-CSFR), receptors for interferons and many interleukins.

JAK family proteins and structure

The JAK family proteins (JAKs) are cytoplasmic tyrosine kinases that participate in the JAK-STAT pathway (JAK–Signal Transducers and Activators of Transcription) and consist in four proteins including JAK1, JAK2, JAK3, and TYK2 (tyrosine kinase 2) [(8)].

The JAKs were originally named "Just Another Kinase" [(9)] but , due to their increasing importance with more than 2000 articles published, were renamed, "Janus kinases" in honor of the Roman God of Gates, Janus. The reason is the similarity between the 'active' and 'inactive' domains of JAKs and the God Janus who had the ability to look simultaneously in two directions [(10)].

JAKs, which range from 120 to140 kDa in size, have seven homology regions called Janus homology domains, numbered in descending order from amino-terminal to carboxy-terminal (JH1-7). The main important

domains are JH1 and JH2. JH1, located near the carboxyl terminus of the protein, is the active kinase domain containing the ATP-binding region and critical tyrosine residues, which are phosphorylated when JAKs are activated. The JH2 domain is the pseudokinase domain, which is highly homologous to tyrosine kinase domain, but lacks the catalytic activity due to the absence of residues.

The pseudokinase domain is believed to have autoinhibitory function and regulates both basal activity of the JAK kinases activity and cytokine-induced activation of the catalytic function. Domains JH3-JH7 are defined as JAK homology domains. JH3 and JH4 contain an Src homology region 2 (SH2) motif, although the physiological importance of the motif has not been established. JH5 through JH7 comprise the amino terminal domain, which contains the third critical JAKs domain called FERM_C (Four-point-one, Ezrin, Radixin, Moesin; cd01237) responsible for non-covalent binding to motifs present in the juxtamembrane cytoplasmic region of type I cytokine receptors (Figure. 2) [11-15].

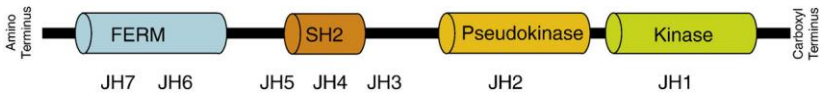

Fig. 2 The molecular structure of JAKs. JH1: active kinase domain with tyrosine residues. JH2: kinase-like domain. JH3 and JH4: Src homology region 2 (SH2) motif. JH5 through JH7: FERM (F for 4.1 protein, E for ezrin, R for radixin, and M for moesin) homology domain.

The JAKs are crucial for normal hematopoiesis: JAK1 is mainly involved in IL-6 signaling (deficient mice showed perinatal lethality and defective lymphoid development); JAK3 plays a non-redundant role in the function of all receptors utilizing the common γ chain (knock out mice showed severe combined immunodeficiency, which affected both T cells and B cells); TYK2 is involved in interferon α/β signaling and mediates activation of interferon-responsive genes; JAK2 (whose gene is located on chromosome 9p24) is activated in response to a variety of cytokines, including EPO, TPO, IL-5, IL-3, and GM-CSF. Fetal liver cells from JAK2-deficient embryos fail to respond to EPO, IL-3, TPO, and mice deficient in JAK2 are embryonic lethal due to the absence of definitive hematopoiesis.[16-18]
All these *in vitro* and *in vivo* experimental data demonstrate that JAK2 plays essential, non-redundant roles in signal transduction induced by critical cytokines regulating hematopoiesis.[19-21]

JAK-STAT signaling pathway

The JAK-STAT system is the crucial step in signaling for numerous cytokines, including erythropoietin (EPO), growth hormone, interferons, prolactin, Thrombopoietin (TPO), granulocyte-colony stimulating factor (G-CSF) , granulocytemacrophage colony stimulating factor (GM-CSF),interleukins (e.g. IL-3 and IL-5), Thrombopoietin (THPO), (Table 1) [19,21,22].

Table 1. Selected list of cytokines and their associated JAK and STAT proteins.

Ligand	Principal JAKs Used	STATs Used
Single-chain family		
Erythropoietin	JAK2	STAT5
Growth hormone	JAK2	STAT5
Prolactin	JAK2	STAT5
Thrombopoietin	JAK2	STAT5
gp130 family		
G-CSF	JAK1, JAK2?	STAT3
Leptin	JAK2	STAT3
LIF	JAK1	STAT3
IL-12	TYK2	STAT4
γC family		
GM-CSF	JAK2	STAT5
IL-3	JAK2	STAT5
IL-5	JAK2	STAT5
IL-2	JAK1, JAK3	STAT5
IL-4	JAK1, JAK3	STAT6
IL-7	JAK1, JAK3	STAT5
IFN family		
IFN, type I (α,β,ω)	JAK1, Tyk2	STAT1, STAT2
IFNγ, type II	JAK1	STAT1
IL-10	JAK1	STAT3

Receptors that use JAKs are known as ‘‘type I’’ cytokine receptors because they lack intracellular TK domains of their own, and are strictly dependent on signaling through the JAKs to phosphorylate downstream targets upon ligand binding [23]. When a ligand binds a receptor, two inactive JAK molecules undergo a conformational change in the receptor that causes a reciprocal phosphorylation [24]. Phosphorylated JAKs acts as an activated tyrosine kinase, phosphorylating the cytoplasmic domain of the type I cytokine receptors, which becomes the docking site of STAT proteins. When STAT proteins are bound to phosphorylated cytoplasmic domains of type I cytokine receptors, they are also phosphorylated by activated JAKs. The activated STATs form dimers and enter the nucleus, where they act as transcription factors to regulate target genes [25-26] (Figure. 3).

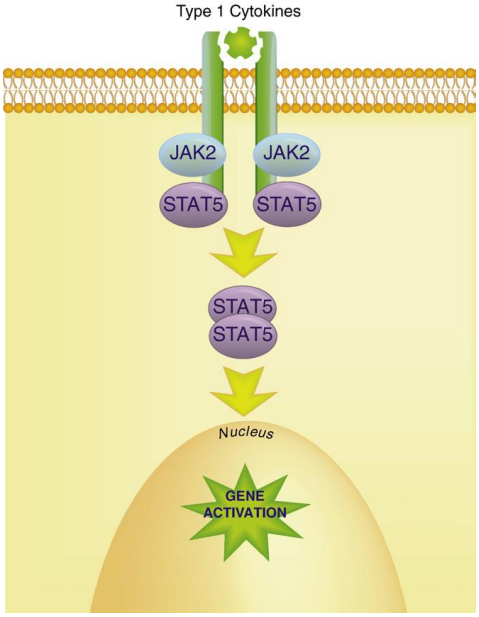

Fig. 3 JAK2 in type I cytokine signaling pathway. The binding of a ligand with type I cytokine receptor induces a conformational change in the receptor, bringing 2 JAK2 proteins close enough to phosphorylate each other. Phosphorylated JAK2 acts as an activated tyrosine kinase, phosphorylating the cytoplasmic domain of the type I cytokine receptors, which becomes the docking site of STAT proteins. When STAT proteins are bound to phosphorylated cytoplasmic domains of type I cytokine receptors, they are also phosphorylated by activated JAK2. The activated STATs form dimers and enter the nucleus, where they act as transcription factors to regulate the target gene.

Mutations or expression changes in JAK-STAT proteins including JAK2 have been associated with a broad range of human diseases [27].

JAK2 mutation and deregulated signaling

In 2005 it emerged that JAK2 plays a very important role in the molecular pathogenesis of MPN, by the finding of a consistent, acquired point mutation, in a highly conserved residue of the autoinhibitory domain of the Janus kinase 2 (JAK2) tyrosine kinase [28–32].

This mutation was isolated in a significant proportion of cases with classical Myeloproliferative disorders. Approximately 80 to 95% of cases with PV and roughly 40 to 60% of cases with ET and MF were found to carry JAK2 V617F, the discovery of which was simultaneously reported in a series of remarkable publications. The specific genetic mutation observed, a single base substitution from Guanine to Thymine at residue 1849 (GenBank accession no. NM_004972), results in substitution of phenylalanine for valine, both hydrophobic nonpolar amino acids, at position 617 of the JAK2 protein (hereafter referred to simply as V617F), within the JH2 pseudokinase domain [33]. Loss of JAK2 autoinhibition results in constitutive activation of the kinase, analogous to other mutations in MPDs and leukemia that aberrantly activate tyrosine kinases [34,35]. (Figure. 4).

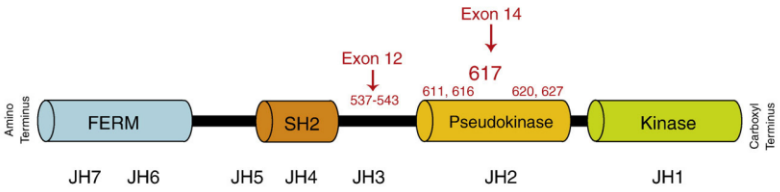

Fig. 4 JAK2 mutations. JAK2 point mutations and deletions implicated in MPNs and acute leukemias are mainly located on exon 14 and 12. Exon 14 is the location of the JAK2V617F mutation and other mutations such as 611, 616, 620, and 627 mutations. Exon 12 mutations are clustered in 537 to 543.

JAK2 V617F mechanism: Structural considerations and animal models

The precise mechanism of aberrant signaling induced by JAK2 V617F in human blood cells remains unknown. Predicted structural modeling suggests that the JH2 domain maintains intimate contact with the activation loop of JH1 through the amino acid cysteine 618 [(36)] (Figure 5).

Therefore, it is possible that V617 normally inhibit movement of the JAK2 activation loop from its inactive to active conformation, indicating that the V617 region plays a direct role in negatively regulating JAK2 signaling V617F substitutes bulkier amino acid for a smaller one, which seems likely to disrupt the normal movement necessary for regulation [(36)], even if protein folding remained intact [(37)].

However, a certain grate of uncertainty remains, since the JAK2 crystal structure has not yet been solved directly [(36)].

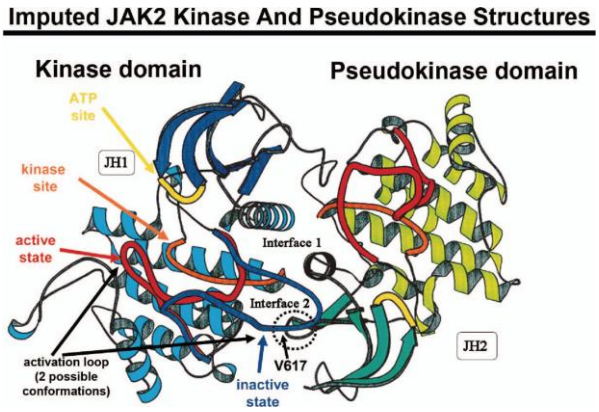

Fig. 5. **Putative JAK2 structure, based on homology with other tyrosine kinases such as fibroblast growth factor, where the crystal structure has been solved.** This ribbon diagram displays the active kinase JH1 domain in blue (left) and the pseudokinase JH2 domain in green (right). The activation loop of JH1 on the left side of the diagram is shown twice, in 2 possible conformations: active (phosphorylated-red) and inactive (non-phosphorylated-navy blue). The JH1 kinase site is shown in orange, and the adenosine triphosphate (ATP) binding site in yellow. Homologous activation loop and kinase domains (non-functional) are shown on JH2 as well. Site of interaction of JH2 and the activation domain of JH1 is shown encircled, along with the location of Val617.

Expression of JAK2 V617F leads to cytokine independent growth of various cytokine dependent cell lines, with constitutive activation of pathways implicated in the control of proliferation, differentiation and cell survival such as STAT5, PI3K/AKT and MAPK. [37,38,39]

Additional insight into the biological effect of JAK2 mutations comes from studies performed using Drosophila melanogaster, where the JAK homologue is encoded by hopscotch (hop). Drosophila mutant with altered hop activity shows a number of developmental abnormalities, including leukemia-like defects [40]. In hopT42 animals, an amino acid substitution (E695K) in the JH2 domain results in increased DNA binding activity of the D-Stat consensus binding element and a leukemic-like disorder of hemocytes. In mice, the same mutation excessively activates STAT5 activation [40].

Consistent with these findings, cell line studies suggest a role for mutant JAK2 in promoting G1/S transition [41]. JAK2 Mutant also appears resistant to inactivation by members of the suppressor of cytokine signalling (SOCS) family, particularly SOCS3 [42].

Taken together, these studies indicate a role for JAK2 mutant in driving cellular proliferation by constitutive activation of signalling pathways, increased entry into cell cycle and resistance to inhibition by negative regulatory proteins.

JAK2 mutation in Myeloproliferative Disorders

Differentiation of haematopoietic cells in the absence of cytokines is one of the hallmark features of the Myeloproliferative Disorders (MPD) [43].

The V617F point mutation in JAK2 is present in the majority of MPD patients, resulting in constitutive phosphorylation of JAK2 and activation of downstream signalling pathways. Retroviral and transgenic mouse models recapitulate many features of the human MPD, including erythrocytosis, thrombocytosis and bone marrow fibrosis [44].

In MPD patients, the JAK2 V617F mutation can be detected in progenitors with B-cell, T-cell, NK-cell and myeloid lineage potential, demonstrating that the mutation arises in a multipotent haematopoietic stem cell (HSC) [45]. However the mutation is detected only rarely and at low levels in peripheral blood Lymphocytes [45].

Of relevance to this observation, the transforming effects of JAK2 V617F are only manifest in cell lines when JAK2 mutant is co-expressed with a type I cytokine receptor [46,47].

Type 1 cytokine receptors, including those for erythropoietin, thrombopoietin and G-CSF, are present in myeloid but not lymphoid cells, and as such lymphocytes harbouring the JAK2 V617F mutation may not have a selective advantage *in vivo*. In keeping with this finding, the JAK2 V617F mutation appears to be exclusive to myeloid neoplasms [48,49].

Expression of JAK2 mutant in progenitor cells produces a bias towards the erythroid lineage, and leads to expansion of erythroid cells at the later stages of differentiation.

These findings suggest that JAK2 mutant is central to the MPD phenotype, and taken together imply a role for *JAK2 V617F* in disease initiation.

Myeloproliferative Disorders: historical overview

The classical myeloproliferative disorders (MPDs), comprising Polycythaemia Vera (PV), Essential Thrombocythaemia (ET) and Idiopathic Myelofibrosis (IMF) are clonal stem cell disorders associated with overproduction of one or more myeloid cell types.

First described in 1879, IMF is characterized by bone marrow fibrosis leading to extramedullary haematopoiesis, splenomegaly and bone marrow failure[50]. Described in 1892, PV is characterized by overproduction of erythroid cells, often accompanied by an increase in neutrophils and/or platelets[51]. Described decades later in 1934 as a distinct syndrome, essential thombocythemia (ET) is characterized by an increased platelet count in the absence of significant erythrocytosis[52]. All three conditions may evolve towards acute myeloid leukaemia (AML) which is generally refractory to therapy.

In 1951, based on observations of a considerable overlap in clinical and laboratory features of these conditions, William Dameshek introduced the concept of the chronic myeloproliferative disorders as a group of closely related diseases featured by excessive proliferation and accumulation of myeloid cells from one or more lineages, sometimes accompanied by myeloid metaplasia[53].

In the nineteen-seventies crucial experiments demonstrated the capacity of erythroid progenitors to proliferate in vitro in the absence of erythropoietin (EPO) in patients with PV – a phenomenon referred to as endogenous erythroid colonies (EEC) [54]. Clonality studies revealed the origin of these diseases in a multipotent hematopoietic stem cell [55-56].

The 2001 WHO classification system groups together as chronic myeloproliferative diseases the three classical myeloproliferative disorders (ET, PV and IMF), Chronic myeloid leukemia (CML), Chronic myelomonocytic leukemia (CMML), Chronic hypereosinophilic leukemia (CHL), chronic neutrophil leukemia (CNL) and a group of unclassifiable diseases [57].

With the advances in our knowledge on cancer molecular biology it became evident that disregulated tyrosine kinase activity of ABL gene, caused by gene fusion products, is a key molecular pathogenetic mechanism causing chronic myeloid diseases as CML, systemic mastocytosis (SM), CMML and CHL[58].

Although CML is molecularly characterized by the t(9;22) resulting in the BCR-ABL fusion gene ET, PV and IMF lack this chromosome translocation and thus have traditionally been referred to as the Philadelphia chromosome negative chronic myeloproliferative disorders (Phnegative CMPD).

The loss of heterozygosity of the short arm of chromosome 9 (9p LOH) was recognized as a rather frequent chromosomal aberration in patients with PV[59]. The recognition of 9p LOH together with the observation that EPO independent colony formation was dependent on molecular signaling through the JAK-STAT signaling pathway [60;61] provided important clues to the understanding of the molecular foundation for the three classical chronic myeloproliferative disorders – PV, ET and IMF.

In spring 2005, four independent research groups identified a somatic point mutation in the gene coding for the Janus Kinase 2 (JAK2) tyrosine kinase. This mutation causes growth factor independent autonomous proliferation of hematopoietic precursors and was found in the majority of patients with PV and approximately half of the patients with ET and IMF[62-65]. The discovery of JAK2 V617F revolutionized, yet also simplified the diagnostic criteria for these diseases in the 4th edition of the WHO classification (2008).

Revisions in the criteria for the classification of MPDs in the 4th edition of the WHO classification were influenced by 2 considerations: 1) the realization that abnormalities of genes encoding tyrosine kinases involved in signal transduction pathways in the MPDs can be used as diagnostic markers, and 2) better characterization of the histologic features that aid in the identification of subtypes of MPDs. Thus in the revised 2008 WHO classification system the phrase 'disease', in MPD, is replaced by 'neoplasm' emphazising the neoplastic nature of MPDs; 'MPD' is now referred to as 'myeloproliferative neoplasm (MPN)'.

The presence of the JAK2V617F is now included as a major criterion in the revised WHO diagnostic criteria for PV, whereas it is considered as a clonal marker for ET and MF **(Table 2).**

2008 WHO diagnostic criteria

	Polycythemia vera[a]	*Essential thrombocythemia*[a]	*Primary myelofibrosis*[a]
Major criteria	1 Hgb $>18.5\,g\,dl^{-1}$ (men) $>16.5\,g\,dl^{-1}$ (women) or Hgb or Hct >99th percentile of reference range for age, sex or altitude of residence or Hgb$>17\,g\,dl^{-1}$ (men), or$>15\,g\,dl^{-1}$ (women) if associated with a sustained increase of $\geqslant 2\,g\,dl^{-1}$ from baseline that cannot be attributed to correction of iron deficiency or Elevated red cell mass >25% above mean normal predicted value	1 Platelet count $\geqslant 450\times10^{9}\,l^{-1}$	1 Megakaryocyte proliferation and atypia[b] accompanied by either reticulin and/or collagen fibrosis, or In the absence of reticulin fibrosis, the megakaryocyte changes must be accompanied by increased marrow cellularity, granulocytic proliferation and often decreased erythropoiesis (i.e. pre-fibrotic PMF).
	2 Presence of *JAK2*V617F or similar mutation	2 Megakaryocyte proliferation with large and mature morphology. No or little granulocyte or erythroid Proliferation.	2 Not meeting WHO criteria for CML, PV, MDS, or other myeloid neoplasm
		3 Not meeting WHO criteria for CML, PV, PMF, MDS or other myeloid neoplasm	3 Demonstration of *JAK2*V617F or other clonal marker or no evidence of reactive marrow fibrosis
		4 Demonstration of *JAK2*V617F or other clonal marker or no evidence of reactive thrombocytosis	
Minor criteria	1 BM trilineage myeloproliferation 2 Subnormal serum Epo level 3 EEC growth		1 Leukoerythroblastosis 2 Increased serum LDH 3 Anemia 4 Palpable splenomegaly

Table 2 The 2008 World Health Organization diagnostic criteria for PV, ET, and PMF.

Therefore, the molecular analysis of this mutation is mandatory in the diagnostic work-up of Ph-neg-CMNs as demonstrated by the wide variety of methodologies that have been described for its detection[(66)]. Considering that the JAK2V617F allele burden at diagnosis is highly variable, the chosen method should be sensitive enough to detect low mutant allele levels, but also specific enough not to produce false positive results.

Clinical phenotypes of the myeloproliferative disorders

Symptoms and clinical characteristics of MPD patients are caused by increased cell proliferation and turn-over of one or more myeloid cell lineages. The dominant symptoms and major cause of morbidity and mortality in PV and ET are due to quantitative and qualitative changes of clonally derived myeloid cells leading to vascular disturbances such as microvascular transient occlusions and major thrombosis in both the venous and arterial systems. The excessive myeloid proliferation may also cause sequestration of myeloid cells in various organs (myeloid metaplasia), most often causing splenomegaly. Although many clinical and biochemical characteristics are shared by the three classical MPDs some manifestations are unique or more pronounced in one of the three distinct disease entities[(66)].

Polycythemia Vera

PV is characterized by myeloid hyperplasia and proliferation[67]. The hallmark is erythrocytosis, reflected by an increased Hb concentration as well as an elevated hematocrit (HCT). In PV, whole blood viscosity increases dramatically when the HCT exceeds 45 %. The elevated HCT has been shown to be a significant risk factor associated with an increased risk of arterial and venous thrombosis, which is the dominant morbidity in patients with PV. Polycythemia vera occurs in about 2 in every 100,000 people. The average age at which the disorder is diagnosed is 60, and it rarely occurs in people younger than 20. More men than women develop polycythemia vera but the cause is not known[68].

Essential Thrombocythemia

ET is characterized by a sustained elevation of platelet number (greater than 600,000/µL), with a tendency for thrombosis and hemorrhage[67]. The clinical picture is dominated by a predisposition to vascular occlusive events (involving the cerebrovascular, coronary and peripheral circulation) and hemorrhages. Some patients with ET are asymptomatic, others may experience vasomotor (headaches, visual disturbances, lightheadedness, atypical chest pain, distal paresthesias, erythromelalgia), thrombotic, or hemorrhagic disturbances. Arterial and venous thromboses, as well as platelet-mediated transient occlusions of the microcirculation and bleeding, represent the main risks for ET patients. Thrombocythemia affects about 2 to 3 of 100,000 people. It usually occurs in people older than 50 and more frequently in women[69].

Idiopathic Myelofibrosis

IMF, also known as Primary Myelofibrosis (PMF) or myelofibrosis with myeloid metaplasia, is characterised by excessive clonal proliferation of megakaryocytes, with a leucoerythroblastic blood profile. Whereas similarities exist between the phenotype and clinical complications of ET and PV, IMF is a distinct clinical syndrome, showing bone marrow fibrosis and extramedullary haematopoiesis which are absent from both ET and PV[70]. However 15-30% of patients with ET or PV eventually develop IMF [71-73].

Symptoms generally arise as a result of marrow fibrosis and disruption of normal blood cell production and include: tiredness (usually as a result of anemia), pain in the left upper area of the abdomen (as a result of an enlarged spleen), fever, night sweats, weight loss, bone

pain, itching (pruritus), bleeding (as a result of low platelets), susceptibility to infections (as a result of low white blood cell count). Myelofibrosis is rare, affecting fewer than 2 of 100,000 people. It occurs with a peak incidence at age 76[66].

Diagnostics techniques and Test Characteristics

Several molecular diagnostic techniques (Table 3.) are currently used to detect JAK2V617F mutation but each of them presents some important limitations, such as a low sensitivity and the requirement of labor intensive procedures performed with expensive specialized equipment that may not always be readily available in clinical laboratories [111].

Table 3. Methods for Detection of JAK2 Mutation

	Method	Specimen	Sensitivity (%)
Baxter et al[1]	Sequencing, ASO-PCR	PB granulocytes	40
Levine et al[2]	Sequencing, MALD1-TOF	PB granulocytes	NR
James et al[3]	Sequencing	PB granulocytes	10
Kralovics et al[4]	Sequencing	PB granulocytes	NR
Jones et al[5]	ARMS-PCR	PB leukocytes	1-2
	Pyrosequencing	PB leukocytes	5
Jelinek et al[6]	Pyrosequencing	PB, Bone marrow	5-10
James et al[10]	Sequencing	PB granulocytes	10
	Melting curve, Taqman ASO	Bone marrow	2-4
Murugesan et al[7]	Melting curve analysis	PB granulocytes; bone marrow	5-10
McClure et al[9]	ASO-PCR capillary electrophoresis	PB leukocytes	0.01-0.1
	Melting curve analysis	PB leukocytes	1-10
Zhao et al[8]	Complementary DNA sequencing	PB leukocytes	15

ASO, allele specific oligonucleotide; ARMS, amplification refractory mutation system; MALD1-TOF. matrix-assisted laser desorption/time-of-flight mass spectrometry; NR, not reported; PB, peripheral blood.

Direct sequencing

In this method, a primer-extension reaction is performed with an amplified DNA template using mixture of dye-labeled dNTPs, and the product is detected by a standard multiwave fluorescence detector (eg, ABI Prism 3100-Avant four capillary system; Applied Biosystems, Foster City, CA) after capillary gel electrophoresis. Although this method provides detailed information and is considered a method of "direct visualization" of sequence information, it has limited sensitivity because of background noise in the generated chromatograms[73].

DNA mixing experiments have demonstrated that for most point mutations, automated sequencing is only sensitive down to about 20% of mutant DNA in a wild-type background[74;75].

This issue is quite relevant to chronic myeloid disorders, where blood and marrow are often composed of a mixture of neoplastic and residual

normal hematopoietic elements. Thus, direct sequencing may not offer the desired or required sensitivity for diagnostic purposes.

Allele-Specific PCR (Amplification Refractory Mutation System [ARMS])

A common method used to detect JAK2V617F mutation is the Amplification Refractory Mutation System (ARMS) [75], whose sensitivity is 1 to 2% [76] mutant DNA in a wild-type background.

The basic principle of this technoques exploits the fact that oligonucleotide primers must be perfectly annealed at their 3' ends for a DNA polymerase to extend these primers during PCR[75].

By designing oligonucleotide primers that match only a specific DNA point mutation, such as that encoding JAK2V617F i.e. primers that do not bind the wild-type allele-ARMS can distinguish between polymorphic alleles. Therefore, these techniques go by the alternative names of "allele-specific PCR" (AS-PCR) or "sequence-specific primer PCR."

The Restriction Fragment Length Polymorphism (RFLP) analysis is possible since the JAK2 1849 G-T mutation abolishes a motif in the wild-type JAK2 sequence that is recognized by the restriction enzyme BsaXI. Although abolition of a restriction site is not as effective as creation of a new restriction site, because a negative enzymatic cleavage reaction could be due either to absence of the mutation or to failure of the digestion procedure, it can be useful as a first pass analysis. Reported proportional sensitivity depends in part on the method used to detect the fragments and is approximately 20% mutant DNA in wild-type background[74;77].

Pyrosequencing

The Pyrosequencing is a method of rapid genotyping that depends on the liberation of pyrophosphate (PPi) whenever a dNTP is incorporated into a growing DNA chain during template-driven DNA polymerization[78].

Pyrosequencing of JAK2 using the automated PSQ HS 96 system (Biotage, Uppsala, Sweden) has been attempted by several groups [78;79] with dilution experiments similar to those described above showing a reported assay sensitivity of 5 to 10% mutant allele in a wild-type background.

Real-Time PCR and DNA-Melting Curve Analysis

Real-time monitoring of PCR product accumulation during thermocycling is a semiquantitative method and DNA-melting curve assays can be used in conjunction with Real-time monitoring of PCR product accumulation as a semiquantitative method. Likewise, James et al. [79] compared fluorescent dye chemistry sequencing with two different real-time PCR based mutation detection systems, one using a LightCycler instrument (Roche Diagnostics) and the other using a Taqman ABI Prism 7500 machine (Applied Biosystems). These real-time PCR techniques detected 0.5 to 1% of HEL cell line DNA diluted in TF-1 cell line DNA and 2 to 4% of homozygously mutated patient DNA diluted in DNA from a healthy person.

Several other mutation detection techniques have been described, including single stranded conformational polymorphism (SSCP) analysis, denaturing gradient gel electrophoresis (DGGE), denaturing high-performance liquid chromatography (DHPLC), single-nucleotide primer extension assays (Pronto), and others. In fact, DHPLC can detect the genomic DNA mutation underlying JAK2 V617F reliably, and it can detect mutations at a proportionality of <1 to 2%. However, DHPLC and the other techniques are either technically challenging or labor-intensive or both. They either do not allow high throughput at a cost suitable for a clinical laboratory (SSCP and DGGE) or require a considerable initial investment for equipment (DHPLC).

Theoretically, protein-based techniques could also be used to detect the JAK2 V617F mutation, but these are generally cumbersome, and access to such resources is limited. Therefore, protein-based assays are usually not preferred if DNA- or RNA-based tests are feasible.

All the methods described are relatively labor intensive and expensive, often requiring specialized equipment that may not always be readily available. Furthermore often an isolation step of granulocytes is required to increase the sensitivity, thus resulting in more than 4 hours time-to-results.

Loop-Mediated Isothermal Amplification

In the last years many different DNA amplification techniques have been developed, showing performance comparable to the polymerase chain reaction (PCR) with certain peculiar characteristics which make them potentially advantageous for molecular diagnostics applications. One of these techniques is the Loop-Mediated Isothermal Amplification (LAMP), first described by Notomi et al in 2000 [(80)].

LAMP principle

LAMP is an isothermal DNA amplification method which relies on the use of a thermostable DNA polymerase with strand displacement activity (*Bst* polymerase from *Bacillus stearothermophilus*) and 4 primers specifically designed to recognize 6 distinct regions on the target gene, consisting in a pair of outer primers (F3 and B3) and a pair of inner primers (FIP and BIP).

The mechanism of the LAMP amplification reaction as illustrated in Figure 6 includes three steps: production of **starting structure**, cycling **amplification** and **elongation**, and **recycling**.

All four primers are operative in the initial steps of the reaction, but in the later cycling steps only the inner primers are required for strand displacement synthesis.

Both FIP and BIP contain two distinct sequences corresponding to the sense and antisense sequences of the target DNA, one for priming in the first step and the other for self-priming in later steps.

At a constant reaction temperature, inner primer FIB hybridizes to F2c in the target DNA and initiates complementary strand synthesis (**structure 1**). Outer primer F3 hybridizes to F3c in the target and initiates strand displacement of the newly DNA chain (**structure 2**), releasing a FIP-linked complementary strand, which forms a looped-out structure at one end (**structure 4**). This single stranded DNA serves as template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis leading to the production of a dumbbell form DNA which is quickly converted to a stem-loop DNA (**structure 6**). The dumbbell serves as the starting structure for LAMP esponential amplification, the second step of the LAMP reaction. The stem-loop DNA structure contains a double stem-loop which is the starting point for the whole LAMP amplification process as it promotes an amplification from its self-annealed 3' terminus and from a newly

annealed internal primer (FIP or BIP) (**structure 7**). During amplification, FIP hybridizes to the loop in the stem-loop DNA and primes strand displacement DNA synthesis, generating as an intermediate one gapped stem loop DNA with an additional inverted copy of the target sequence in the stem, and a loop formed at the opposite end via the BIP sequence (**structure 8**). Subsequent self-primed strand displacement DNA synthesis yields one complementary structure of the original stem-loop DNA and one gap repaired stem-loop DNA(**structure 10**) with a stem elongated to twice as long and a loop at the opposite end (**structure 9**). Both of these products then serve as templates for BIP-primed strand displacement in the subsequent cycles, the elongation and recycling step. Thus, in LAMP the target sequence is amplified 3-fold every half cycle.

Amplification proceeds promoting itself, each strand being displaced by elongation of the new loops formed.

The final product is a mixture of stem-loop DNA with various stem length and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target sequence in the same strand (Figure 7).

Fig.6: **Schematic representation of the mechanism of LAMP**.Steps in the LAMP reaction. This figure shows the process that starts from primer FIP. However, DNA synthesis can also begin from primer BIP.

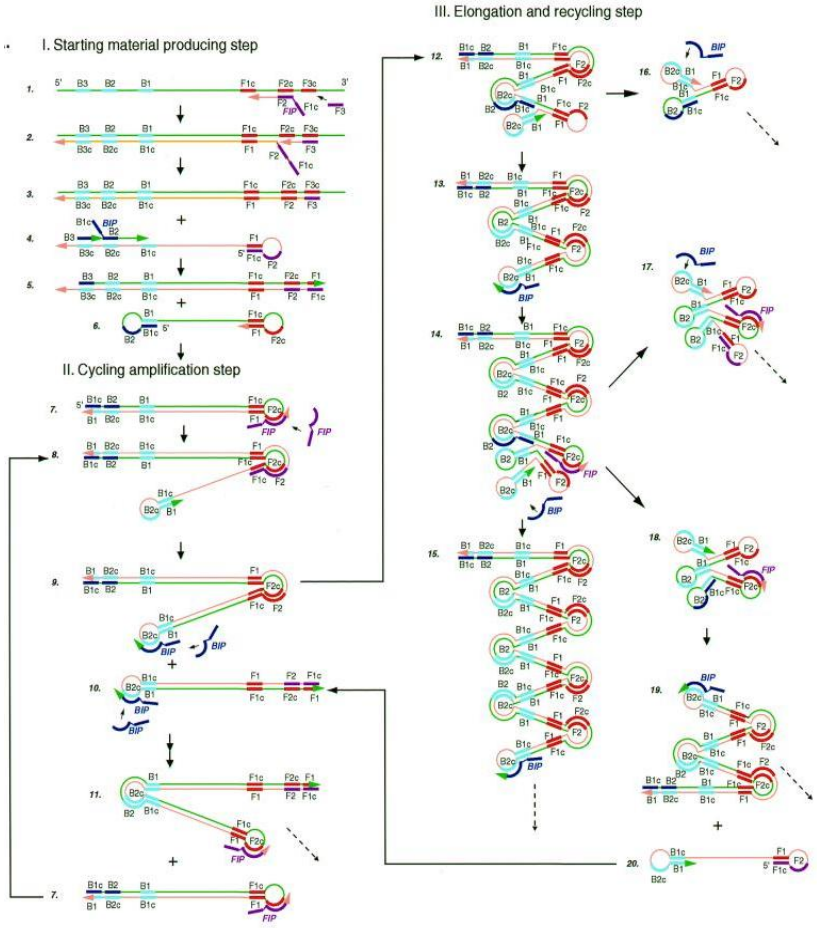

Fig. 7 Reaction products. Inverted repeats concatamers are the main amplification products of a LAMP reaction

Loop primers

In order to accelerate the LAMP reaction, it is possible introduce additional primers, the Loop Primers.

The Loop Primers (either Loop Primer B or Loop Primer F), containing sequences complementary to the single stranded loop region (either between the B1 and B2 regions, or between the F1 and F2 regions) on the 5' end of the dumbbell-like structure, provide an increased number of starting points for DNA synthesis. Loop primers hybridize to the stem-loops, except for the loops that are hybridized by the inner primer, and prime strand displacement DNA synthesis (Figure 8).

Therefore the loop primers act as reaction boosters annealing and extending on single strand regions on the loop regions of the dumbbell structure. Their presence increases the rate of DNA production and the sensitivity of the method and decreases the reaction time.

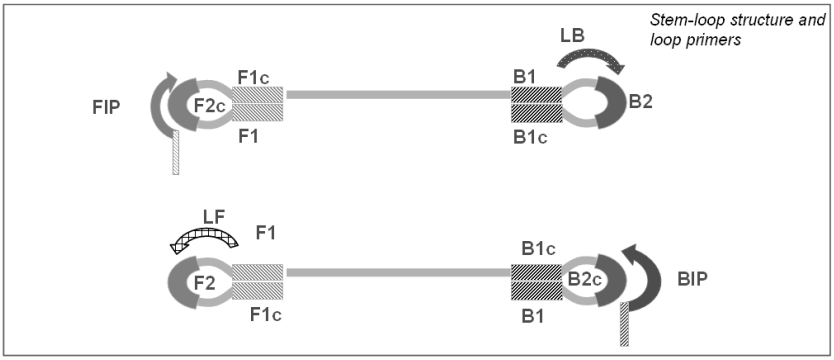

Fig. 8 Stem loop structures. "Dumbbell" structures are generated by FIP, BIP, F3 and B3 activity and they promote the following exponential-like amplification. LF and LB primers anneal on a single stranded region and cooperate to the amplification of DNA.

By these peculiar amplification dynamics and exploting the continuous displacement by Bst polymerase of the already produced DNA strands with no need for denaturation steps, it is possible to amplify the initial DNA amount up to 10^{10} times in less than 60 minutes and with very high specificity, due to the many independent target-recognition events.

Visualisation of LAMP amplification products

Several methods can be used to detect positive LAMP reactions. Reaction results are detectable either by fluorescence or turbidimetry. Many fluorescence approaches are employable, like intercalating dyes or molecular beacons and quenching probes which allow multiplex assays when different fluorophores (specific for distinct targets) are used at the same time.

Turbidimetry detection is made possible by the high amplification efficiency of LAMP reaction [80;81;83]. This detection method consists in the measurement of turbidity of the reaction mix, due to the precipitation of magnesium pyrophosphate. This insoluble salt is formed by the interaction between Mg2+ present in solution and the inorganic pyrophosphate produced by the incorporation of dNTPs in the DNA growing strands.

Thus, the amount of magnesium pyrophosphate is proportional to the amount of amplified DNA. The turbidity is visible by the naked eye (Figure 9) and is measurable on a turbidimeter as a transmittance signal either at end-point for a qualitative analysis or in real-time, allowing quantitative applications.

Turbidimetry is the ideal detection method for LAMP technology, at least for fast clinical applications and point-of-care approaches since it does not require expensive fluorescent reagents and complex detectors.

Fig. 9. **Turbidimetry detection:** End point detection can be accomplished even by visual detection of a white precipitate (magnesium pyrophosphate) in positive samples (A) but not in negative samples (B).

One more possible, very easy, detection approach can rely on **fluorescent dye calcein** and again it exploits the high reaction efficiency of LAMP: this chemical compound, that can be included in the reaction, is complexed with manganese ion and in this state its fluorescence is quenched. When the high amount of inorganic pyrophosphate is produced by the reaction, it complexes the Mn2+ ion instead of Mg2+ and calcein emits fluorescence which can be very easily detected by

irradiating the tubes with a standard transilluminator or even by naked eye (Figure 10).

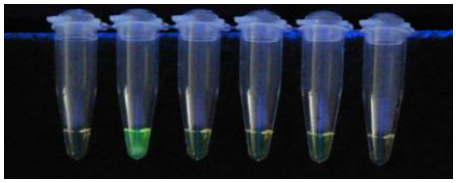

Fig. 10. LAMP reaction revealed by calcein. Calcein included in the reaction mix allows an easy detection by a common UV-transilluminator. Its fluorescence emission is promoted by PPi produced during the amplification.

Alternatively, products of LAMP reaction can be directly visualised in real-time using an **intercalating dye** in the reaction mixture before starting the amplification. The isothermal amplification can be visualized by fluorescence detection of the amplicons and an annealing analysis to confirm the product. This closed-tube system eliminates the need for gel electrophoresis or turbidity.

Advantages of LAMP

The primary characteristic of LAMP is its ability to amplify nucleic acid under isothermal conditions allowing the use of simple cost effective reaction equipments. Both amplification and detection of nucleic acid sequences can be completed in a single step by incubating the mixture of sample, primers, DNA polymerase at a constant temperature[80]. In addition the amplification efficiency of LAMP is very high and the reaction proceeds rapidly as there is no need for initial heat denaturation of the template DNA, and it does not require thermal cycling which makes its application an easy and rapid diagnostic tool in molecular medicine[83].

One of the most important advantages of LAMP is that large amounts of DNA are generated in a short time increasing the concentration of pyrophosphate ions. The produced turbidity observed as a white precipitate enables visual detection of positive LAMP reactions [83;84] and reduces time of post amplification analysis. Another important advantage of the isothermal amplification techniques is their tolerance to some inhibitory materials such as a culture medium and some biological substances that can affect the efficiency of PCR [85]. As LAMP is less affected by the various components of clinical samples than PCR, there is no need for DNA purification [83]. Moreover a Reverse Transcription (RT) LAMP was developed for the detection and amplification

of RNA [86;87] thus moreover expanding the fields of applications for this technique.

LAMP has been explored so far in hundreds of papers for the detection of DNA of a great amount of different organisms, such as bacteria [88;89;90] viruses [91;92;93], fungi [94;95], and parasites [96;97;98], but it seems also suitable for the analysis of human DNA, i.e. for the detection of SNPs and deletions.

Thus LAMP constitutes an attractive alternative to PCR for sequence detection, with a sensitivity and quantitative performance comparable to PCR, with extraordinary specificity, allowing different detection methods, simplex and multiplex reactions applications in a fast, easy and potentially cheap molecular diagnostics platform.

Encouraged by the evidence that the LAMP technology was used successfully in these applications requiring a high specificity, we decided to challenge the potential of the technology for the detection of the point mutation V617F in JAK2 gene present in high frequency in Myeloproliferative Neoplasms.

AIM OF THE THESIS

Myeloproliferative Neoplasms (MPNs) are haematologic disorders of myeloid progenitor cells characterized by the frequent presence of an acquired activating mutation in exon 14 of the Janus kinase 2, consisting in a Valine to Phenilalanine substitution at codon 617 (JAK2V617F). The kinase activity of mutated JAK2 is constitutively activated, inducing uncontrolled cell proliferation and resistance to apoptosis. JAK2V617F is found in 80-95% of Polycythemia Vera (PV), 35-95% of Idiopathic Myelofibrosis (IMF), and 23-57% of Essential Thrombocythemia (ET), the three diseases belonging to MPNs, as described in the World Health Organization (WHO) classification. Thus, the JAK2V617F mutation represents the key clonal marker for diagnosis of MPNs. The identification of the JAK2V617F is mandatory in the diagnostic work up of PV, ET, and IMF [(66)].

Several molecular techniques are currently employed to detect JAK2V617F, but each of them presents several limitations such as a low sensitivity, low specificity, the requirement of labour intensive procedures performed by specialized equipments, high costs of the test and long reaction time[(111)].

Sensitivity is particularly relevant because the mutation is somatically acquired and must be selectively detected in a large amount of unmutated wild type cells. To overcome this limitation, it's often necessary to enrich the portion of cells that could be affected by the mutation prior to perform the molecular diagnostic test, for example by extracting the DNA from granulocytes isolated from whole blood.

The aim of this thesis was to improve the molecular diagnosis of MPNs by the development of a novel method based on the Loop mediated isothermal AMPlification (LAMP) technique that presents several peculiarities that make it intrinsically different and superior compared to PCR.

LAMP is an outstanding gene amplification procedure, in which the reaction can be processed at a constant temperature thanks to the employment of a strand-displacement polymerase and characterized by the use of 4–6 different primers specifically designed to recognize 6-8 distinct regions on the target gene; the reaction process is completed within 60 min with high efficiency, in a close-tube format [(80)].

These features can be useful to prevent contamination, which can occur in PCR during the transfer of samples containing amplicons from tubes to gels for electrophoretic confirmation and preclude the need for expensive

thermo-cycler instruments, as required for PCR. Furthermore, the high efficiency of the LAMP method ensures a sensitivity level adequate for mutation analysis, as requested for detection of JAK V617F in clinical laboratories.

MATERIALS AND METHODS

Target DNA

In order to preliminary test reliability and performance of LAMP assay we established specificity, sensitivity and selectivity by experiments on mixtures of plasmids with mutated JAK2 and plasmids with wt JAK2. Plasmids were synthetically generated by Geneart. A synthetic double strand DNA sequence is cloned in a plasmid then amplified by *E. coli* transformation. Highly purified extracted plasmids are sent to the customer.

Genomic DNA from cell line

Genomic DNA was extracted by GENTRA KIT (Qiagen, Hilden, Germany) from the following in vitro growing human cell lines: HEL (Acute Myeloid Leukemia, AML M6), UKE-1 (ET → AML), and SET-2 (ET → leukemic transformation), positive for JAK2V617F but with different allele burden, and control wild type cell lines including HL60 (AML M2), TF1 (AML), GFD8 (AML), KASUMI (AML, M2), K562 (a t(9;22) Chronic Myeloid Leukemia in erythroid blast crisis), NB4 (a t(15;17) positive Acute Promyelocytic Leukemia, APL), BJAB (a t(8;14) Burkitt, non Hodgkin's Lymphoma), U266B1 (multiple myeloma), RS411 (positive for MLL-AF4), MV4-11 (human, leukemia, acute monocytic) and 697 (childhood acute lymphoblastic leukemia). The continuous cell lines were either taken from the stock of the cell bank (DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), exception to UKE-1 generously provided by Walter Fiedler, Eppendorf Hospital, Hamburg, Germany.

Clinical samples

Experiments were also performed on genomic DNA obtained from patients with PV (n= 63), ET (n= 25), MF (n=4), IF(n=2) and unclassified CMN (n= 2). The genomic DNA was extracted by Gentra kit- (Qiagen Hilden Germany) from purified granulocytes or whole blood. Granulocytes were purified using density centrifugation with Ficoll-Paque Plus (GE Healthcare Biosciences, Upsala, Sweden). As control, DNA was also extracted from both whole blood and purified granulocytes of healthy donors (n=83) and patients with Acute Lymphoblastic Leukemia (ALL, n= 4), Follicular non Hodgkin's Lymphoma (NHL, n= 3), B cell Chronic Lymphocytic Leukemia (B-CLL, n= 2). All these subjects are routinely

followed for clinical reasons at Ospedali Riuniti di Bergamo and gave written informed consent.

LAMP assay

The AS-LAMP assay design

The Loop mediated isothermal AMPlification (LAMP) of DNA was initially described by Notomi as an innovative technology to amplify DNA with high specificity, efficiency and rapidity under isothermal conditions [(80)].

We have designed an innovative assay, we termed Allele Specific LAMP (AS-LAMP) based on the LAMP principle, presenting two innovative reagents.

The AS-LAMP reaction (Figure 11) is based on the use of 4 primers specifically designed to recognize 6 distinct regions on the target gene: a pair of outer primers (F3 and B3) and a pair of inner primers (FIP and BIP). The reaction is conducted at constant temperature in presence of a DNA polymerase with strand displacement activity. The FIP and BIP primers anneal and are extended on the target DNA and the newly synthesized DNA chains are then displaced by extension of F3 and B3. The displaced product generates a "stem-loop structure" which represents the starting structure for a classical LAMP reaction (Figure 12).

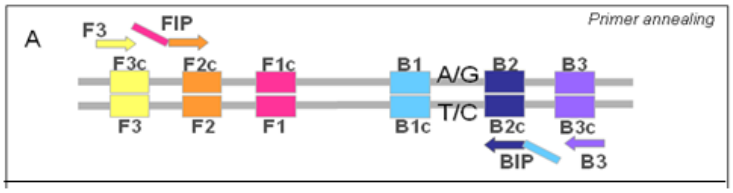

Fig. 11 Outline of LAMP primers placement. FIP and BIP anneal respectively on F2c and B2c regions; their tails anneal on the F1 and B1 regions sited on the extension products of FIP and BIP on their target. These DNA strands are displaced by F3 and B3 extension on the target, leading to the following step.

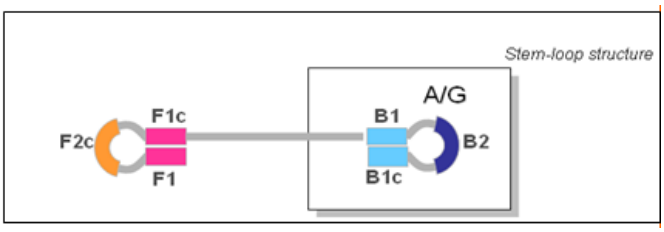

Fig. 12 Stem loop structures. "Dumbbell" structures are generated by FIP, BIP, F3 and B3 activity and they promote the following exponential-like amplification. The mutation is present in the region between B2 and B1 sequence.

Primers were purposely designed so that the mutation V617F is in the region between B2 and B1 on the target. This region corresponds to a loop single strand structure in the starting structure of LAMP.

The design includes an additional primer with a particular stem loop structure, called **self annealed loop primer** (sLB), complementary to the loop region in which the JAK2 T mutated base is located. The sLB extensible primer consists in a central loop sequence able to selectively recognize and hybridize to the region where is present the point mutation and in a 5' end sequence and a 3' end sequence, complementary to each other to form a stem (Figure 13).

Fig 13. Self annealed Loop primer. The region complementary to the target DNA (green line) has a melting temperature (Tm) with the specific mutant sequence 6°C higher than with the wild type sequence.

If the target in solution presents the JAK2 T mutated base, the sLB breaks its internal interaction since primer-target hybridization Tm is higher than the intra-molecular hairpin structure Tm. The sLB binds its target allowing the amplification. If the target in solution presents the JAK2 G wild type base, the intra-molecular hairpin structure Tm is higher than the primer-target hybridization Tm. Any aspecific annealing of the sLB are prevented

thanks to its structure that causes an auto-sequestration of the primer in absence of the specific sequence which cannot be extended by the polymerase.

The second innovative element introduced in reaction is a **Peptide Nucleic Acid** (PNA) probe specific for the JAK2 G wild type nucleotide. PNA's backbone is composed of repeating N-(2-aminoethyl)-glycine units linked by peptide bonds. PNA oligomers show great specificity in binding to complementary DNA strands, with a PNA/DNA base mismatch being more destabilizing than a similar mismatch in a DNA/DNA duplex. The melting temperature of the PNA on wt JAK2 sequence is 9°C higher than on the mutant sequence. If the target in reaction is WT, the PNA forms a stable duplex with its target, preventing the aspecific annealing and extension of the sLB and therefore suppressing the amplification. If the target in solution is mutated, the PNA does not anneal due to the single-base mismatch (Figure 14).

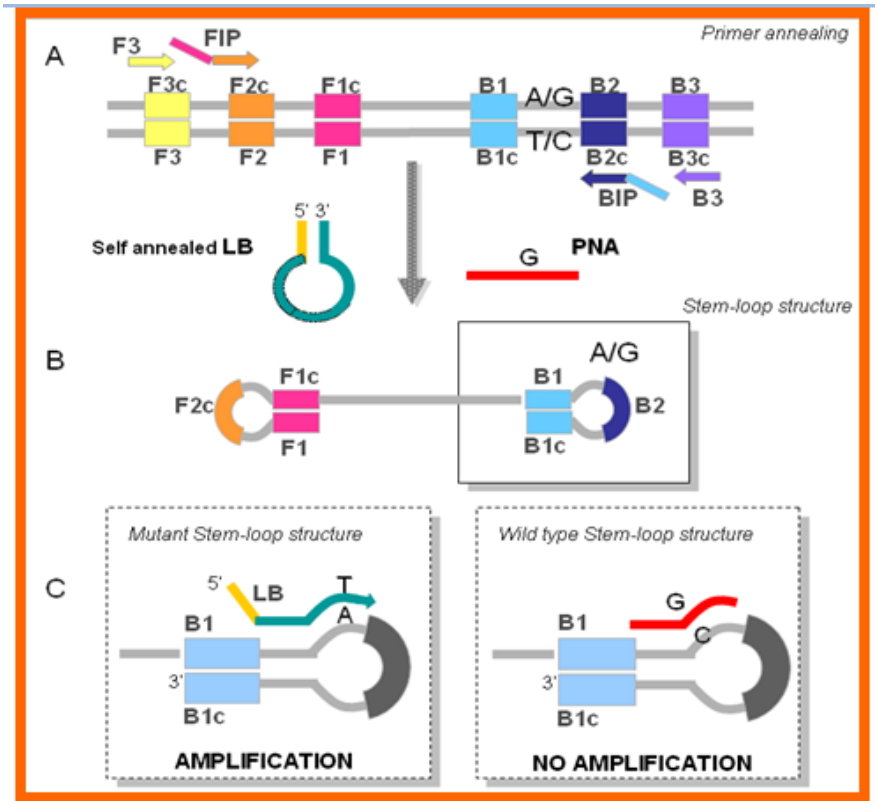

Fig.14: AS-LAMP principle

AS-LAMP primers (F3, B3, FIP, BIP, sLB), specifically designed to amplify the point mutation V617F in JAK2 gene were designed manually with the support of VisualOmp software (DNA Software, Inc.), while the PNA was designed using the formula for thermal stability (Tm) prediction of PNA/DNA duplexes [(99)].

Primers were synthesized by SGS and PNA by (Eurogentec, Liege, Belgium). Their sequences are listed in Table 4.

name	sequence
F3	5' GCATCTTTATTATGGCAGAGAG 3'
B3	5' TGCTCTGAGAAAGGCATTA 3'
BIP	5'GCTTTCTCACAAGCATTTGGTTTTAAATTAGCCTGTAGTTTTACTTACTCTC 3'
FIP	5' GCTGCTTCAAAGAAAGACTAAGGAAATGGACAACAGTCAAACAAC 3'
sLB	5' GTCTCCACTGGAGTATGTTTCTGTGGAGAC 3'
PNA	^{NH2}gagtatgtgtctgtgga^{COOH}

Table 4. AS-LAMP primers for point mutation *V617F JAK2*.

AS-LAMP - Turbidimetry

The assay in turbidimetry was performed at 65°C constant temperature for 1 hour on the LA200 turbidimeter (Teramecs Co, Kyoto, Japan) instrument for real-time monitoring. The variation of absorbance in terms of arbitrary units (a.u.) can be analyzed to find the threshold time for each sample tested. The threshold time is the minute at which the sample absorbance, after baseline subtraction, reaches the a.u. value representing the threshold (in this case 0.1). The threshold time (T(min)t) reached by each sample is correlated with its logarithm of DNA copies/µl.

A LAMP control reaction to validate negative results is also performed in parallel by a mutant-insensitive amplification of the JAK2 gene using the same basic primer set described above and avoiding the PNA and self-annealed mutant loop primer in the reaction mixture.

The amplification resulting by the LAMP control assay is indicative of a correct annealing and extension of the LAMP primers as well as of the absence of inhibitors within the reaction solution.

Reaction mixtures (25 µL) contained 160 nmol/L of F3 and B3 primers, 640 nmol/L of sLB primers, 640 nmol/L of PNA, 1.28 mmol/L of FIP and BIP primers, 1.4 mmol/L of each dNTP, 20 mmol/L Tris-HCl, 10 mmol/L KCl, 8 mmol/L MgSO4, 10 mmol/L (NH4)2SO4, 0.1% Tween20, 0.32 U/mL Bst polymerase (New England Biolabs), 5 µL of DNA samples (20 ng/µL) and distilled water (made up to the final volume of 25 µL/tube).

In experiments performed to assess the sensitivity and selectivity of the AS-LAMP a DNA denaturation preceded the LAMP reaction: plasmid aliquots were heated at 95°C for 10 minutes, rapidly cooled in ice for 10 minutes and finally added to reaction mix immediately before the incubation at 65°C.

AS-LAMP (duplex) - Fluorescence

AS-LAMP was also performed using a fluorescence detection. The fluorescence is generated by adding an intercalating dyes (Yo-Pro-1 iodite, Molecular Probes, Invitrogen) in the reaction mixture. LAMP reactions in fluorescence were performed at 65°C for 30 minutes using the reagents at the same concentrations as in turbidimetry but adding an additional primers set specifically designed to amplify the endogenus ABL gene as a internal control.

The amplification resulting by the LAMP internal control assay is informative to exclude false-negative results due to low quality or absence of the DNA due to a failure in the extraction procedure. The sequences of ABL primers set are listed in Table 5.

name	sequence
F3	5' ACCAAAAATGGCCAAGGCT 3'
B3	5' TCAGCGAGATGGACCTCTG 3'
FIP	5' GGCCCATGGTACCAGGAGTGT**TCCCAAGCAACTACATCACG** 3'
BIP	5' CCGCAATGCCGCTGAGTATCT**TCTCACTCTCACGCACCA** 3'
LF	5' TTCTCCAGACTGTTGACTGG 3'
LB	5' AGCGGGATCAATGGCAGC 3'

Table 5. LAMP primers for ABL internal control.

AS-LAMP reaction were run in a real time Optical thermoblock (GenieI, Optigene) under isothermal conditions and fluorescence emission was monitored in green channel corresponding to the peaks of emission of the intercalating dyes (Yo-Pro-1 iodite, Molecular Probes, Invitrogen).

The optical instrument provides fluorescent excitation and quantification of fluorescent emission. The fluorescence increases significantly when the intercalating dye binds the double-stranded DNA during the hybridization (annealing) and extension reaction. The fluorescence signal increases proportionally to the amount of amplification product. Because intercalating dyes bind to all double-stranded DNA, fluorescence signal can represent signal from a specific product as well as non-specific products like primer-dimers.

Furthermore it is not possible to discriminate the amplification of the target from the amplification of the internal control. To overcome this limitation, after the completion of the amplification reaction, an annealing analysis has been performed allowing to discriminate the specific product(s) of amplification.

Annealing curves provide information about the purity of the amplification product and the annealing temperature of the amplified product(s). Annealing curves are generated, after the denaturation at 100°C of the LAMP product(s) followed by slowly cooling the amplified DNA from 95°C to 70°C. As the temperature decreases, amplified complementary DNA strands anneal and the intercalating dye is incorporated, increasing the fluorescence signal. LAMP JAK2V617F and LAMP ABL products were identified by different annealing temperatures respectively of 83°C and 91°C.

Reaction mixtures (25 μL) contained JAK2 primers set at 160 nmol/L of F3 and B3 primers, 640 nmol/L of sLB primers, 640 nmol/L of PNA, 1.28 mmol/L of FIP and BIP primers and ABL primers set at 10 nmol/L of F3 and B3primers, 40 nmol/L of LF and LB primers, 80 nmol/L of FIP and BIP primers, 1.4 mmol/L of each dNTP, 20 mmol/L Tris-HCl, 10 mmol/L KCl, 8 mmol/L MgSO4, 10 mmol/L (NH4)2SO4, 0.1% Tween20, 1uM Yo-Pro-1 iodite (Invitrogen), 0.32 U/mL Bst polymerase (New England Biolabs), 5 μL of DNA samples (5 ng/μL) and distilled water (made up to the final volume of 25 μL/tube).

Detection of JAK2V617F by ASO-PCR amplification

The Allele Specific Oligonucleotide (ASO) PCR for the JAK2V617F mutation was determined as described [64].

Confirmation of positivity was performed by amplification of exon 14 and subsequent digestion (PCR-RFLP) with the restriction endonuclease BsaXI (New England Biolabs, Ipswitch, MA, USA) [100] . The G-T mutation destroys a BsaXI site in the wild type JAK2 sequence. Digested amplified products were separated by electrophoresis on an ethidium bromide-impregnated 2% agarose gel.

This approach allows both normal and mutant alleles to be visualized and can distinguish between homozygous and heterozygous mutations.

RESULTS

AS-LAMP (Allele Specific-Loop Mediated Isothermal Amplification) is a novel isothermal DNA amplification method for the detection of the somatic point mutation V617F in JAK2 gene presents with high frequency in Myeloproliferative Neoplasms. The mutated JAK2 allele differs from the wild type by just one nucleotidic exchange (from Guanine toTymine) leading to the Valine to Phenylalanine transition at codon 617 (V617F).

Amplification is performed using a set of primers specifically designed for the detection of V617F JAK2 mutation and a thermostable DNA polymerase, Bst polymerase, which shows a high strand displacement activity that allows a continuous amplification of DNA by the formation of characteristic loop structures; no denaturation steps are needed and reaction is carried out at isothermal conditions.

As previously mentioned (see Introduction) the primer set for a basic LAMP reaction consists of 4 primers (F3, B3, FIP, BIP) annealing to the target region in a peculiar way that allows the formation of an initial stem-loop structure and of subsequent elongated DNA strands consisting in concatamers of inverted repeats of a basic module. The addition of two innovative elements such as PNA and sLB (self-annealed Loop primers) plays an important role in the specific discrimination between mutant and wild type JAK2 alleles. AS-LAMP, generates, within one hour, a large amount of amplified DNA, visible to the naked-eye, monitorable by Real-Time turbidimetry and fluorescence and not requiring gel separation.

AS-LAMP in turbidimetry

Validation of AS-LAMP principle on plasmid controls

To initially obtain proof of principle and to test specificity and sensitivity of AS-LAMP for JAK2V617F detection, we first synthesized two synthetic plasmids containing the sequence of JAK2 mutant and JAK2 wild type genes.

Sensitivity on plasmids

The AS-LAMP assay was optimized on plasmid controls carrying, or not, the JAK2V617F mutation. The sensitivity of the assay was also evaluated considering the selectivity of the AS-LAMP. Selectivity is defined as the ability of the method to detect the specific target in presence of a high

background constituted by the non-specific target. Thus the level of sensitivity was tested on mutated plasmid (35,000 cps), wild type plasmid (35,000 cps) and on mutated plasmid serially diluted into wt plasmid at 1%, 0.5%, 0.1%, 0.05% and 0.01%. 35,000 copies represent approximately the number of copies of JAK2 allele contained in 100 ng of genomic DNA per reaction (based on a typical amount of 6 pg of DNA per diploid cell). Amplification was reproducibly obtained down to 17 copies/reaction. Semilogarithmic graph, (inset of figure 1) where the threshold time, i.e. the reaction time corresponding to the threshold value of turbidity of 100 AU, is plotted against the base-10 logarithm of the DNA amount. LAMP amplification curves obtained by real-time turbidimetry on synthetic plasmid are shown in Figure 15.

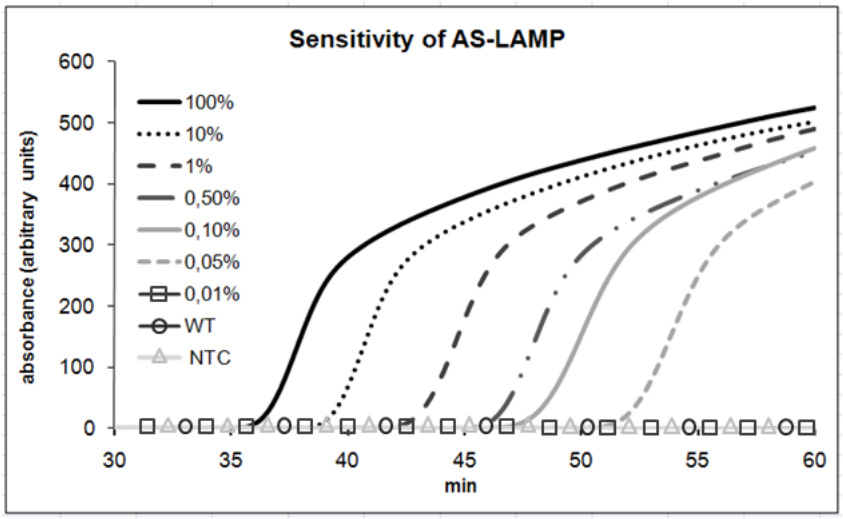

Fig. 15 Validation of AS-LAMP on synthetic plasmids: sensitivity and selectivity. The AS-LAMP assay was performed on samples containing decreasing amounts of JAK2 mutant plasmid in a background of JAK2 wild type plasmid. Amplification was reproducibly detected down to 17 copies per reaction (0.05%). NTC, not target control.

Specificity on plasmids

The specificity was tested using 35,000 copies per reaction of wild type JAK2 plasmid, including in the experiments a positive control carrying the mutated sequence. Amplification occurred exclusively using the plasmid carrying V617FJAK2 mutation while the plasmid containing the wild type

sequence and no-target-controls (NTC, water) produced no amplification within one hour reaction (Figure 16). These results indicate that AS-LAMP is able to discriminate sequences which differ only for a single base nucleotide with a high level of reliability.

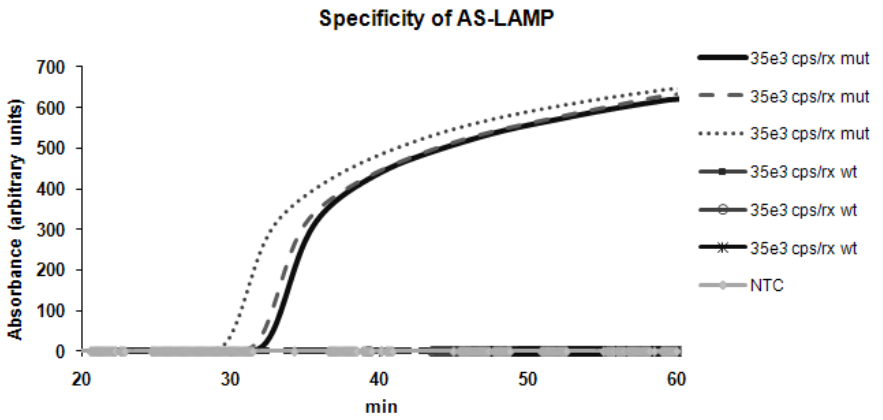

Fig. 16 Validation of AS-LAMP on synthetic plasmids: specificity. Real time amplification curves of triplicate experiments on V617FJAK2 plasmid (Mut) or wild type JAK2 plasmid (Wt). Amplification is obtained only in the samples carring V617FJAK2 mutation.

Validation of AS-LAMP on Genomic DNA from cell line

After the preliminary proof of principle of the performance of AS-LAMP on synthetic plasmids, we set up a series of experiments to evaluate our technology in conditions closer to those found in biological systems. We conducted experiments on more complex and realistic models represented by human genomic DNA extracted from human cell lines presenting, or not, the mutation of interest.

Sensitivity on cell lines

To evaluate the ability of the AS-LAMP assay to detect low amounts of JAK2V617F mutated DNA, 100 ng per reaction of genomic DNA from the UKE-1 cell line was serially diluted (100, 10, 1, 0.1, 0.05, 0.01 and 0%) into wild-type DNA from BJAB cells. The reproducible sensitivity of AS-LAMP is 0.1% mutant-to-wild type DNA ratio, being detected in 100% of cases (100 replicates). The 0,05% dilution is detected in 50% of cases, while the 0.01% in 37.8% of cases, being the level of maximum sensitivity of the assay (Figure 17). A linear relationship (R2= 0.99) between the threshold

time (T(t)min) and the logarithm-dilution factor of the mutant DNA can be observed between the 100% and 1% dose.

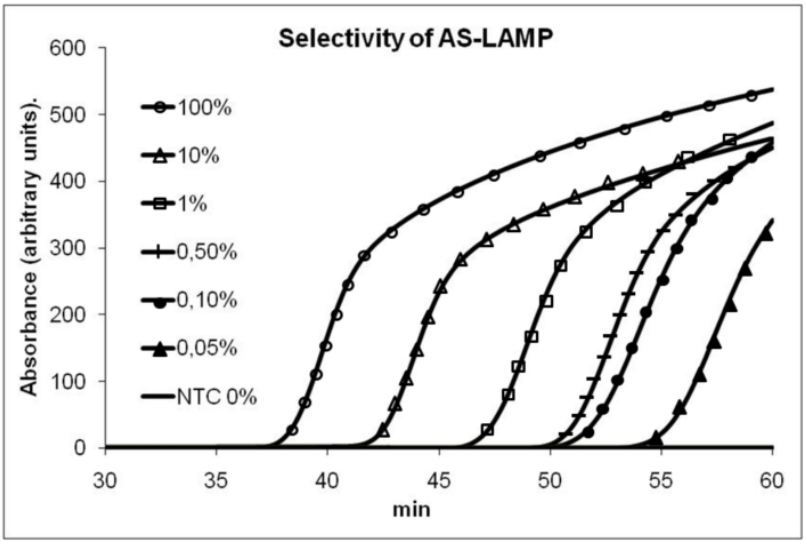

Fig. 17. Validation of AS-LAMP on DNA from cell lines: sensitivity. Serial dilutions of mutant UKE-1 DNA in wild type BJAB DNA at concentrations of 100, 10, 1, 0.5, 0.1, 0.05, and 0% were analyzed by AS-LAMP. Graph shows representative curves for each Mutant/wild type ratio always detectable on a 3 repetitions basis. Mutant alleles could be detected down to 0.05% mutant-to-wild type ratio within one hour. Negative samples did not generate amplification signals.

Specificity on cell lines

To evaluated the specificity of the AS-LAMP, we performed the assay using 100 ng per reaction of genomic DNA from three JAK2V617F mutant (UKE-1, SET-2 and HEL) and eight JAK2 wild type cell lines (HL60, TF1, GFD8, KASUMI, K562, NB4, BJAB, U266B1). Results of amplification reactions are shown in (Figure 18); After one-hour reaction no amplification was observed in JAK2 wild-type cell lines, while all the JAK2V617F mutants proved positive. These results confirm the complete blocking of amplification of the wild-type thanks to the use of PNA and sLB. All the negative results were validated by a control reaction (JAK2 wild type LAMP assay) performed in parallel to exclude inhibitors or incorrect reaction conditions.

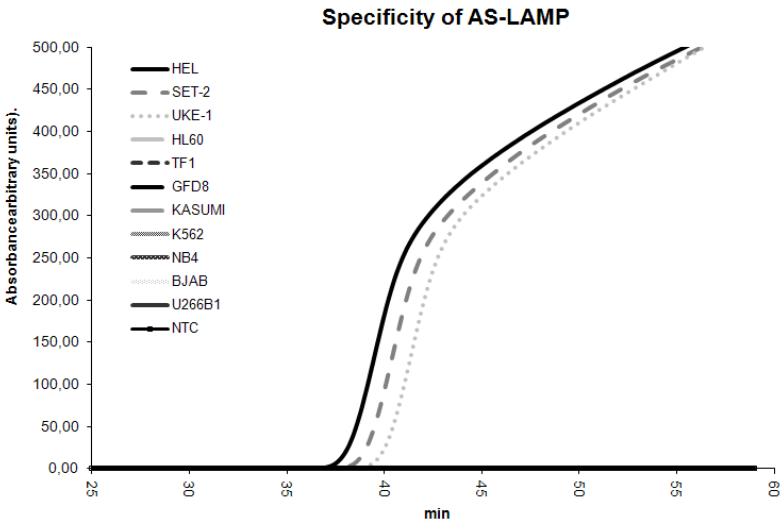

Fig. 18. Validation of AS-LAMP on DNA from cell lines: specificity. Seven wild type (HL60, TF1, GFD8, KASUMI, K562, NB4, BJAB, U266B1) and three JAK2V617F mutant cell lines (UKE-1, SET-2 and HEL) were analyzed by AS-LAMP. Within 60 minutes, only DNA samples obtained from mutant cell lines showed an amplified product, while no amplification was detected in all the wild type samples.

Comparison between AS-LAMP and ASO-PCR assay on clinical samples

The AS-LAMP assay was further validated on **66** samples obtained from PV (n=36), ET (n=24), MF (n=4) and unclassified CMN (n= 2) patients previously analyzed and found JAK2V617F positive by conventional ASO-PCR. Clinical sample DNA was extracted from peripheral blood purified granulocyte and subjected to the ASO-PCR assay by Ospedali Riuniti di Bergamo (in compliance with the institute's ethics and human research guidelines). A 100% concordance between the two assays was demonstrated for all samples. None of the seven negative controls, (2 Acute Lymphoblastic Leukemia, 2 Follicular Non Hodgkin's Lymphoma, 2 Chronic Lymphocytic Leukemia, and 1 healthy donor) gave false positive results (**Table 6**).

Sample type	Total samples	Positive by ASO-PCR	Positive by AS-LAMP	Concordance
Polycitemia Vera	36	36	36	100%
Essential Thrombocytemia	24	24	24	100%
Myelofibrosis	4	4	4	100%
Idiopatic Erytrocytosis	2	2	2	100%
Negative Controls*	7	0	0	100%

* negative controls are: 2 ALL, 2 Follicular NHL, 2 B-CLL and 1 healthy donor

Table 6: Sample types analyzed by ASO-PCR and AS-LAMP

In addition, **80** DNA samples obtained from peripheral blood granulocytes, of healthy donors (n=73) and patients with Acute Lymphoblastic Leukemia (n=2), Follicular non-Hodgkin's Lymphoma (NHL, n=2) and B cell Chronic Lymphocytic Leukemia (B-CLL, n=2) proved consistently negative.

AS-LAMP in fluorescence

The AS-LAMP method was further improved by the implementation of an internal control reaction in a duplex format, using fluorescence-based signal detection. While turbidimetry cannot discriminate two simultaneous amplifications in the same tube, the fluorescent AS-LAMP format allows to amplify simultaneously different genes recognizing the respective amplification product(s) by **annealing** analysis at the end of the reaction. In fact an annealing curve profile characteristic for each type of amplicon present in the reaction solution can be generated, allowing discrimination among different fluorescent amplification products.

The instrument (GenieII, Optigene) records the total fluorescence generated by YO-PRO 1 binding to double-stranded DNA as temperature changes, and plots the fluorescence in real time as a function of temperature. The first derivative of this plot, dF/dT, is the rate of change of fluorescence in the reaction, and a significant change in fluorescence accompanies the annealing of the double-stranded LAMP products. A plot of -dF/dT vs. temperature will display these changes in fluorescence as distinct peaks. The annealing temperature of each product is defined as the temperature at which the corresponding peak occurs. This analysis confirms the specificity of the system as well as reveals the presence of primer-dimers. Because of their small size, primer-dimers usually anneal at lower temperatures than the desired product. Additionally, non-specific amplification may result in LAMP products that melt at temperatures above or below that of the desired product.

Furthermore the AS-LAMP signal detection based on fluorescence improved the sensitivity of the method. The highest level of sensitivity allows to avoid the step of purification of granulocytes from whole blood, today necessary to increase the detection sensitivity of JAK2 mutation analysis, especially in cases where the frequency of cells carrying the mutant JAK2 allele is low (e.g. in the early stages of the disease or after treatment).

The reaction consists in the same set of primers used to detect the mutation JAK2V617F in turbidimetry assay but adding the intercalating dyes (YO-PRO 1 iodite, Invitrogen) in the mixture of reaction. Furthermore, as internal control, another set of primers specifically designed to amplify the

endogenous housekeeping ABL gene has been added. The duplex reaction was then run in isothermal conditions in a real-time thermoblock (GenieI, Optigene) and monitored in the green channel. Due to the simultaneous occurrence of two independent reactions, the fluorescence assay required a new optimization process to maintain the ability to discriminate the JAK2V617F mutation. This request is due because of different efficiencies of the two single amplifications (ABL and JAK2V61F respectively) that coexist in the same reaction tube. The direct consequence is that the two reactions must be well balanced to ensure a correct use of common reagents by the two amplification reactions avoiding one system takes over on the other. Since the ABL internal control assay was faster and more efficient in respect to the V617FJAK2 one, its primers set concentration was decreased in order to leave sufficient common reagents to the other system ensuring a rapid and sensitive detection of the JAK2 mutation. Furthermore the ABL assay, optimized to amplify weakly the control gene, offers an adequate control even for recognition of inhibitors that may cause delay in amplification times, easily confusable with low positive results.

Validation of duplex AS-LAMP on Genomic DNA from cell line

Sensitivity on cell lines

The sensitivity of the duplex AS-LAMP assay was assessed using 25 ng per reaction of genomic DNA from UKE-1 cell line (which carries the V617F mutation) serially diluted in BJAB wild type DNA (negative for V617F mutation). At the end of the amplification reaction, an annealing curve from 99°C to 80°C at 0.05°C, has been performed to confirm the product of amplification. This eliminates the need for gel electrophoresis allowing a closed-tube system. Annealing curve raw data is generally represented by plotting fluorescence over temperature, but to make analysis more convenient, the negative first derivatives (-dF/dT) are used, revealing annealing temperatures at peaks. The negative first derivatives of the annealing curves in the duplex AS-LAMP assay showed two annealing peak referring to two simultaneous amplifications. (Figure 19). The amplification of JAK2V617F mutation exhibits an annealing curve with a peak at about 83°C while the amplification of the ABL internal control shows a peak at the higher temperature of about 91°C. The duplex assay sensitivity was detected down to 0.5% of mutated DNA in wild type, confirmed by an high number of replicates. The presence of the internal control does not interfere with the sensitivity of the assay. The time-to-results is up to 30 minutes for detection of low doses of mutation.

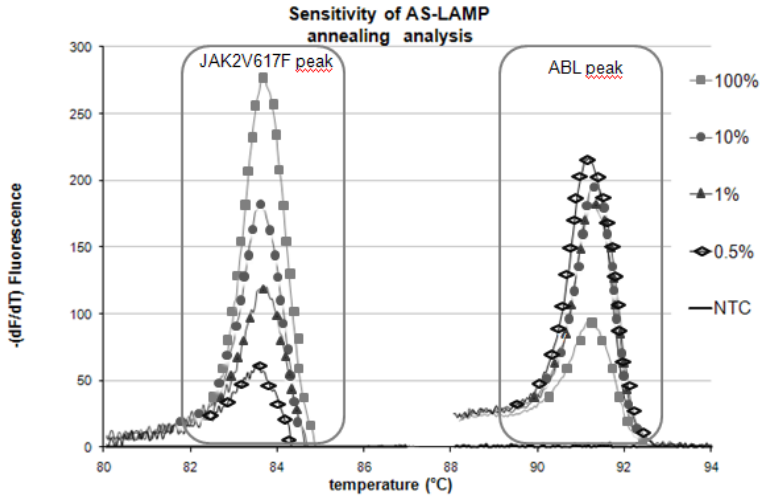

Fig. 19. Validation of AS-LAMP on DNA from cell lines: sensitivity. Anneal derivative results. DNA from the UKE-1 cell line (homozygous for the V617F mutation) was serially diluted with DNA from BJAB cell lines and amplified in the AS-LAMP assay. UKE-1, undiluted (100%); 10%, UKE-1 diluted 1/10; 1%, UKE-1 diluted 1/100; and 0.5%, UKE-1 diluted 1/200. NTC, no template control. AS-LAMP generates annealing peaks distinct to the JAK2V617F mutation (about 83°C) and ABL internal control amplification (about 91°C). Derivative annealing peaks characteristic of JAK2V617F and ABL (internal control) are shown. Mutant alleles could be detected down to 0.5% mutant-to-wild type ratio within 30 minutes. Amplicons were renatured and annealed at the rate of 0.1°C/s.

Specificity on cell lines

To establish the specificity, we performed AS-LAMP duplex assay using 25 ng per reaction of several genomic DNA extracted from cell lines negative for JAK2V617F mutation (REH, RS411, MV4, 697, HL60), as shown in Figure 20.

The ABL gene of all negative cell lines has been correctly amplified, generating the expected annealing curve . No amplification occurred in NTC while the positive UKE-1 cell line produced an amplification with an annealing peak specific for JAK2V617F mutation. Therefore AS-LAMP was able to rapidly detect and amplify the specific target DNA. The assay was highly reproducible in at least three independent experiments on the same samples, consistently producing identical results.

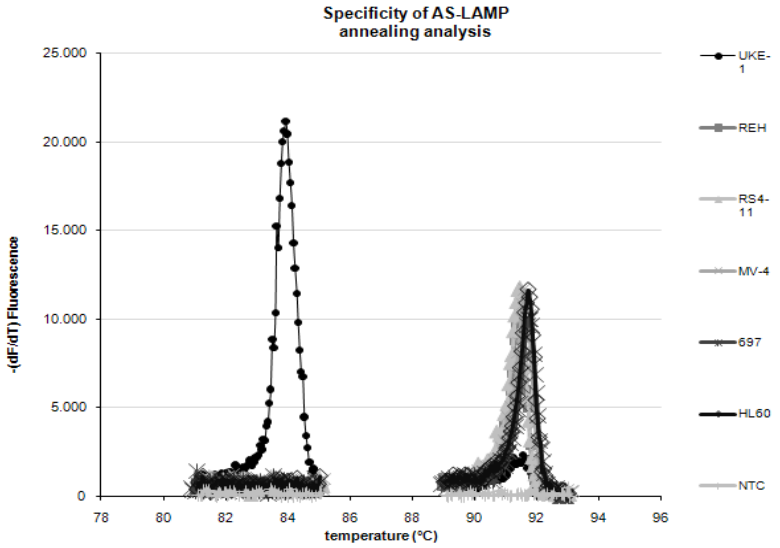

Fig. 20. Validation of AS-LAMP on DNA from cell lines: specificity. Five wild type (REH, RS4-11, MV4, 697, HL60) and one JAK2V617F mutant cell lines (UKE-1) were analyzed by AS-LAMP (duplex). Within 30 minutes, only DNA samples obtained from the mutant cell line showed an JAK2V617F peak at 83°C, while the only ABL peak at 91°C was detected in all the wild type samples. Amplicons were renatured and annealed at the rate of 0.1°C/s.

Comparison between duplex AS-LAMP and ASO-PCR assays on clinical samples

After the first phase in which we evaluated the sensitivity, the specificity and the performance of AS-LAMP duplex assay on genomic DNA from cell lines, we applied our assay for detecting the JAK2V617F mutation in real clinical samples in comparison with an established method, allele specific oligonucleotide PCR (ASO-PCR) performed by Ospedali Riuniti di Bergamo (Italy). Given the great sensitivity of the AS-LAMP (duplex), we tested both DNA extracted from peripheral blood granulocytes and whole blood. We examined **27** clinical specimens carrying the JAK2V617F (PV). 10 out of 27 were purified by granulocytes. All this samples were positive with both LAMP and ASO-PCR. 17 out of 27 samples were tested whit LAMP on whole blood and with ASO-PCR on corresponding granulocytes purified. All ASO-PCR positive samples (assessed on granulocytes purified) were positive with LAMP (assessed on whole blood). In addition we tested **19** ASO-PCR JAK2 negative clinical specimens (10 healthy donors whole blood and 9 patients with various myeloproliferative disorders and leukemia

granulocytes purified). All 19 samples, but one were negative with both ASO-PCR and AS-LAMP. One patient (affected by ET) was initially negative for JAK2 mutation assessed by ASO-PCR, whereas it was clearly positive with LAMP. A further investigation demonstrated that the sample was indeed a real low positive inasmuch as, when tested again later on in the clinical evolution of the patient, it was eventually detectable by ASO-PCR but at the limit of detection. Presumably, this sample was negative by ASO-PCR when tested initially because of the limited sensitivity of this technology.

DISCUSSION

Myeloproliferative Neoplasms (MPNs), comprising Polycythaemia Vera (PV), Essential Thrombocythaemia (ET) and Idiopathic Myelofibrosis (IMF), include an heterogeneous group of diseases characterized by excessive production of various blood myeloid lineages[53]. The identification of an acquired somatic point mutation in the Janus Kinase 2 (JAK2) gene in the majority of patients with Polycythemia Vera (PV) and approximately half of patients with Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF) has lead to a breakthrough in the understanding of the molecular pathogenesis of these disorders[28–32]. The JAK2 gene encodes a receptor-associated cytoplasmatic tyrosine kinase, which plays a key role in regulating normal hematopoiesis, transducing the activating signal of several cytokines regulating myeloid cell proliferation and differentiation. A point mutation at position 1849 in exon 14 (guanine to thymine) results in an amino acid substitution of valine to phenylalanine in codon 617 (V617F) which causes the constitutive, cytokine-independent activation of JAK-2 kinase activity leading to uncontrolled proliferation of myeloid precursor cells.

The molecular analysis of V617FJAK2 mutation in patients suspected of MPNs is mandatory to achieve a correct diagnosis, improve clinical classification, decide therapy and predict prognosis[66].

Several molecular diagnostic techniques have been developed to detect the V617F mutation but each of them presents important limitations[111]. For the routine diagnosis in hospital laboratories, some of these methods, eg, polymerase chain reaction (PCR) followed by enzymatic digestion (BsaXI) [112;113], or sequencing [29;28;30;31] are considered labor-intensive and poorly sensitive. Other methods are relatively technically complex, expensive and time consuming such as the pyrosequencer, [76,114] fluorescence probes [79;115;116] or denaturing high-performance liquid chromatography [117] for being applied widely on a routine basis.

Loop-mediated isothermal AMPlification (LAMP) is a powerful, innovative nucleic acid amplification technique which is emerging as an easy to perform and rapid tool for molecular diagnostics applications in clinical routine [80].

LAMP amplifies DNA or RNA target with high specificity, efficiency and rapidity under isothermal conditions. Its specificity is conferred by the peculiar amplification mechanism, which allows to virtually eliminate primer dimers and aspecific amplifications. It is fast, inasmuch as reactions

are completed within 60 minutes; it is relatively inexpensive thanks to the use of a strand-displacement polymerase, avoiding the use of Taq DNA polymerase and expensive thermocycler instruments. All these peculiarities make LAMP intrinsically different from PCR and allow to achieve superior performance in those applications in which the unique characteristics of this method are magnified.

Based on LAMP, this work presented a novel molecular assay, the Allele-Specific Loop mediated AMPlification (AS-LAMP) for the identification of the JAK2V617F mutation.

Key elements of the AS-LAMP assay are a self-annealed Loop primer (sLB) specific for the mutated JAK2 sequence and a Peptide Nucleic Acid (PNA) blocker designed to bind exclusively the wild type gene. The discrimination between wild type and mutated base in guaranteed by a the cooperation between the PNA, that suppresses the aspecific amplification of wild type alleles, and the sLB primer that efficiently and selectively amplifies the mutated ones. These elements make the nucleic acid detection by AS-LAMP also highly sensitive, allowing the detection of the mutation in patients with few mutated cells.

AS-LAMP can be performed in two different detection methods. The first is based on turbidimetry and has been tested with success on plasmids, DNA from cell lines and clinical specimens; the second, successfully tested on DNA from cell line and validated on clinical samples, presents the same primers to detect the mutation of the turbidimetric assay, with the further addition of another primers set specifically designed to amplify the internal control gene ABL. The reaction is performed with an intercalating dye (Yo-Pro 1) in a single reaction solution for **fluorescent** detection. In this last version, the intercalating dye is incorporated during LAMP reaction and allows a real-time detection of the DNA amplification. To discriminate the amplification of the target gene (mutated JAK-2) from the internal control gene, an annealing analysis was performed at the end of amplification for accurate discrimination of the specific product(s) of amplification.

The turbidimetric version benefits from ease and low cost, while the fluorescence detection improves the assay introducing the internal control, necessary to validate the negative samples, and improves the sensitivity and the time to results.

The AS-LAMP, both turbidimetric and fluorescent versions, allows a rapid and robust identification of DNA samples harboring the JAK2V617F mutation. No false positive or false negative results were registered on clinical samples previously tested by the reference assay ASO-PCR. The superior specificity obtained with AS-LAMP in a high number of replicates (n=203) is due to the intrinsic features of the LAMP in which at least six different target genomic regions are recognized by primers instead of only two regions recognized in a PCR assay. In addition, specificity is also gained by the suppression of the amplification of the wild type target, thanks to the PNA blocker and by the specific V617F mutation recognition of sLB.

In particular the fluorescent AS-LAMP proved to represent a highly sensitive nucleic acid amplification technology. Sensitivity of AS-LAMP in detecting JAK2V617F DNA was as high as 0.5% using a low amount of DNA (25 ng per reaction) of mutant cell line (UKE-1) in wild-type cells. The high sensitivity of the assay allows the detection of the mutation in DNA obtained directly from peripheral whole blood rather than in purified blood PMN (in which the somatic mutation is found), a very time consuming procedure required for PCR detection.

The fluorescent AS-LAMP assay was successfully tested on clinical specimens. Remarkably, when compared to other molecular methods currently employed to detect JAK2V617F mutation, AS-LAMP proved more sensitive than ASO-PCR, more accurate than enzymatic digestion of the PCR products and does not require the use of Real-Time PCR instrumentation. A patient with low levels of JAK-2 mutated cells who was negative with PCR was successfully detected by AS-LAMP.

An additional key characteristic of the fluorescent AS-LAMP is the unique and remarkable opportunity to run the internal control in the same tube of JAK2 amplification, thus ruling out false negative results due to poor or absent amplification.

In conclusion fluorescent AS-LAMP is a highly sensitive and specific assay for the rapid (less than 30 minutes) detection of JAK2 mutation in whole blood of patients with Myeloproliferative Neoplasms. All these characteristics make this method feasible and convenient in routine diagnostic laboratories even in the absence of major technical infrastructures or highly qualified personnel. Considering the widespread request for molecular detection of JAK2V617F mutation in patients with suspected chronic myeloproliferative disorders, this highly performing assay may represent a substantial improvement over existing nucleic acid amplification technologies.

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Papers and Patent

Part of the work presented in this thesis is submitted as a paper titled "A novel, highly sensitive and rapid Allele Specific-Loop mediated Amplification assay (AS-LAMP) for the detection of the JAK2V617F mutation in Chronic Myeloproliferative Neoplasms" on *Haematologica*.

An European patent application (no. 09 165 252.9) concerning a method for detecting single point mutations by an improvement of the LAMP (Loop mediated isothermal AMPlification) method, was also obtained.

Posters:

A Novel, Highly Sensitive, Rapid, Non-PCR Based Method for the Detection of the JAK2V617F Mutation in Chronic Myeloproliferative Neoplasms. Presented at ASH congress, New Orleans 2009.

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A Novel, Highly Sensitive, Rapid, Quantitative, Non-PCR Based Method for the Detection of the JAK2V617F Mutation in Chronic Myeloproliferative Neoplasms. Presented at EHA, 15th congress, june 10-13, Barcellona 2010.

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