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Review Article

Clonal Hematopoiesis: Role in Hematologic and Non-Hematologic Malignancies

Ugo Testa, Germana Castelli and Elvira Pelosi.

Department of Oncology, Istituto Superiore di Sanità, Rome, Italy.

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Abstract. Hematopoietic stem cells (HSCs) ensure the coordinated and balanced production of all hematopoietic cell types throughout life. Aging is associated with a gradual decline of the self-renewal and regenerative potential of HSCs and with the development of clonal hematopoiesis. Clonal hematopoiesis of indeterminate potential (CHIP) defines the clonal expansion of genetically variant hematopoietic cells bearing one or more gene mutations and/or structural variants (such as copy number alterations). CHIP increases exponentially with age and is associated with cancers, including hematologic neoplasia, cardiovascular and other diseases. The presence of CHIP consistently increases the risk of hematologic malignancy, particularly in individuals who have CHIP in association with peripheral blood cytopenia.

Keywords: Hematopoiesis; Hematopoietic stem cells; Clonal hematopoiesis; Gene mutations; Next generation sequencing.

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Correspondence to: Ugo Testa, Department of Oncology, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161. Rome, Italy. E-mail: ugo.testa@iss.it

Hematopoiesis, Hematopoietic Stem Cells and Aging. Hematopoietic stem cells (HSCs) are the blood-forming stem cells that possess the property of both self-renewing and of differentiation generating all hematopoietic elements; the biological activity of HSCs is essential during all life to maintain hematopoiesis and to promote hematopoietic cell regeneration in stress conditions or after transplantation. HSCs are phenotypically and functionally heterogeneous.

Fetal Hematopoiesis. The hematopoietic system is generated through a complex developmental process starting from the early stages of embryonic development. In mammals, the first transient waves of blood cell generation occur at the level of the extra-embryonic yolk sac; two waves of yolk-sac hematopoiesis have been described: a primitive wave of hematopoiesis leads to the production of primitive nucleated erythrocytes and primitive macrophages; the second wave of hematopoiesis is characterized by the generation of erythron-myeloid progenitors (EMPs) and lymphoid progenitors that transiently migrate and seed into the fetal liver.[1]

Generation of HSC. HSCs are originated from a peculiar population of endothelial cells (hemogenic endothelium) located at the level of the dorsal aorta or the aorta-gonad-mesonephros (AGM) region around 30-42 days post-conception.[1] Following their budding into blood vessels, HSCs undergo a migration process to the fetal liver, where the stem cell pool considerably expands.[1] The final step of the developmental generation of the hematopoietic system is represented by the migration of HSCs to the bone marrow, where these cells further expand and differentiate, orchestrating the whole hematopoiesis.[1]

In human embryonic tissues, the hematogenous potential is present inside the embryo, while hematopoietic cells originating in the yolk sac do not contribute to the generation of HSCs sustaining definitive hematopoiesis.[2]

However, the yolk sac contributes to the generation of macrophagic cells that are maintained in adult life. Studies in murine hematopoiesis have shown three

waves of macrophage cell development: (i) A primitive wave of macrophage generation related to primitive hematopoiesis occurring at the level of the yolk sac (in this phase, in addition to macrophages, are also generated primitive erythroid and megakaryocytic cells); these primitive macrophages are responsible for the generation of microglia in adult mouse brain. (ii) The second wave of macrophage production is related to erythron-myeloid (EMP) progenitors responsible through their differentiation for the generation of tissue-resident macrophages. (iii) The third wave of macrophage generation is mediated by the differentiation of HSCs formed in the AGM region of the embryo and colonizing the fetal liver; these macrophages represent a population of long-lived macrophagic cells persisting throughout life. In mouse, most adult tissue macrophages resident in the liver, brain, epidermis, and lung originates from yolk sac EMLs distinct from HSCs.[3] Studies carried out in human embryonic yolk sac cells support the yolk sac origin of a part of macrophages on day 23-25 post-conception, a population of early yolk sac-derived primitive macrophages was observed; on day 39-42 post-conception, erythron-myeloid progenitors are present in the yolk sac, generating Mac1 macrophages: these macrophages have a distinct identity compared to HSC-derived macrophages, as evidenced by their expression of CDH5 and HBE1.[4] Analysis of human embryonic heads showed that most microglia cells originated from primitive yolk sac-derived macrophages.[4] Importantly, HSC-independent macrophages maintain a peculiar transcriptomic and epigenetic identity in adult life compared to their HSC-derived counterparts.[4] Single-cell RNA sequencing studies have led to the identification of three macrophage subsets: one of these subsets detected both in mouse and in humans is characterized by TIMD4, LYVE1 and/or FOLR2 (TFL^+) expression: these macrophages are maintained through self-renewal with minimal monocyte input.[5] Atkins et al. reported a human pluripotent stem cell (PSC) *in vitro* differentiation model for yolk sac hematopoiesis: activin A, BMP4 and FGF2 signaling drives PSC differentiation to KDR^+-$CD235a/b^+$ mesoderm cells that generate yolk sac-type primitive erythroid cells and macrophages via hemogenic endothelial cells and these cells, in turn, generate erythroid-myeloid progenitors with multipotent differentiation capacities.[6] This study supports the hypothesis that the development of hematopoiesis is mediated through the formation of hemogenic endothelium that in the yolk sac primes a primitive wave of hematopoiesis associated with transient multipotency, and in the AGM region, a definitive wave of hematopoiesis, associated with sustained multipotency and long-term self-renewal capacity (HSC wave).

Recent studies carried out in zebrafish embryos suggest that both hemogenic endothelium and aortic endothelium originated from a common angioblast precursor; this angioblast precursor generates arterial ECs and HECs: these cells display a spatial separation in the dorsal aorta where most ECs are in the floor and most HECs in the roof.[7] The specification of ECs or HECs from the precursor angioblasts is modulated by ETV2 levels through differential regulation of Fli1a, Notch, and Sclβ.[7] In addition, enforced RUNX1 expression in ECs promotes the transition to HECs.[7]

The process through which HE generates hematopoietic stem/progenitor cells is an endothelial to hematopoietic transition. Intra-aortic hematopoietic clusters (IAHCs) contain immature HSCs, HSCs, and committed progenitors.[8] The single-cell transcriptomic analysis of HE cells, of cells undergoing EHT transition, and whole IAHCs isolated from mouse embryo aortas allowed identifying transcription factor networks activated during the HE transitions to IAHCs.[8]

Studies in mouse and human embryos have led to a partial characterization of HE and IAHCs.[9-11] One of the studies in mice showed that the transition from ECs to pre-HECs is characterized at molecular level by increased accessibility of chromatin regions enriched for SOX, FOX, GATA, and SMAD binding motifs.[9] The transition from pre-HE to HE is associated with reduced RUNX1 enhancer accessibility.[9] Two populations of IAHCs are generated: an initial wave of lympho-myeloid biased progenitors, followed by precursors of HSCs.[9] The second study in mice was based on a single-cell transcriptomic analysis of the dorsal aorta from embryonic day 8 to E11.[10] At E10, two types of endothelial cells were observed (EC and HEC) and HSC-competent HECs; the sequential analysis showed that primitive vascular endothelial progenitors at E8 first undergo an EC arterial fate choice, followed by a hemogenic (HEC) fate conversion.[10] The study on human embryos showed that arterial endothelial cells with hemogenic potential in wk4 human embryos were characterized by upregulation of RUNX1, MYB and ANGPT1 expression; endothelial cells expressing CD44 were particularly enriched in hemogenic potential.[11] HE cells primed to hematopoietic transition were characterized by transient overexpression of EMCN, PROCR, and RUNXT1.[11]

The placenta represents, in addition to the yolk sac, another site of extra-embryonic hematopoiesis. In humans, at weeks 5-6 of development, the human placenta contains $CD34^+$ cells and erythron-myeloid progenitors as assayed by the common *in vitro* clonogenic assays.[12] At the stage of 5-7 weeks of gestation, the human placenta is a site for terminal maturation (enucleation) of primitive erythrocytes, characterized by the synthesis of embryonic hemoglobin.[13] Despite the early presence of $CD34^+$ cells in the human placenta, HSCs are detected in this organ only after week 9 of gestation.[14] Repopulating HSCs were detected in the human placenta from 15 to 24 week

gestation but were absent at term.[15]

Calvanese et al. have recently performed a single-cell transcriptome analysis of human hematopoietic tissues from the first trimester to birth, and through this analysis, they provide an overview of the whole development process occurring during embryonic and fetal life.[16] HSC origin in human embryos was tracked to hemogenic endothelial cells characterized by the positivity of ALDH1A1 and KCNK17 markers; these cells were regulated from a subset of endothelial cells identified as pre-hemogenic IL33+ALDH1A1+ endothelial cells; this process occurred at the level of intra-aortic hematopoietic clusters.[16] The single-cell RNA sequence of $CD34^+$ and/or $CD31^+$ cells isolated from the AGM region of 4.5-5 weeks old human embryos allowed to define of a molecular signature that characterized HSCs and included the co-expression of RUNX1, HOXA9, MLLT3, MECOM, HLF and SPINK2 genes.[16] AGM HSCs also possessed a distinct endothelial signature, characterized by the expression of genes such as PROCR and EMCN. Clustered cells expressing this RNA expression signature were observed in AGM, placenta, yolk-sac, umbilical and vitelline vessels, head, and heart.[16]

Interestingly, AGM HSCs also possessed a distinct endothelial signature, characterized by the expression of genes such as PROCR and EMCN.[16] The analysis of this RNA signature in fetal livers of different ages showed that the HSC transition to the liver occurs around six weeks of gestation; furthermore, an extended analysis of gene expression profile suggested that fetal liver HSCs undergo a maturational process after eight weeks, progressively exhibiting features like those observed in adult HSCs with the acquisition of the HSC maturity markers CD133 and HLA-DR.[16] The comparative analysis of HSCs populating different sites suggested that extra-embryonic HSCs are positioned one step downstream from the most immature AGM HSCs and could colonize the liver to initiate multilineage hematopoiesis.[16]

The migration of HSCs from the AGM region to the fetal liver is a fundamental event in the development of hematopoietic tissue because, in the hepatic environment, the migrated HSCs undergo an intensive expansion process to generate the HSC pool required to sustain hematopoiesis for the lifetime of an individual. The fetal liver remains the main site of hematopoiesis until it is replaced before birth by the bone marrow. Fetal liver HSCs exhibit several properties different from adult bone-marrow stem cells, related to higher cycling, self-renewing activity, and a higher engraftment/repopulating activity of fetal liver compared to cord blood and bone marrow.[17] Fetal HSCs concomitantly display two functional properties, such as high proliferating and repopulating activity, while in adult HSCs, proliferation and repopulating activity are two inversely related functional properties.[18,19] Studies in murine fetal liver HSCs suggest that these cells possess the unique property to tolerate high proliferation without reducing their multipotential differentiation capacities through a mechanism seemingly related to increased DNA damage response observed in fetal HSCs compared to postnatal HSCs.[20] Gene expression studies have shown that a purified cell population enriched in long-term repopulating HSCs ($GPI\text{-}80^+$-enriched fraction) purified from fetal liver exhibited an enhanced expression of some genes related to aryl hydrocarbon receptor (AHR) family and of *RGCC, LMNA* and *ID* genes.[21] The most expressed gene of the AHR family, *TIPARP,* is a negative regulator of AHR activity and AHR inhibitors favor HSC expansion.[21] *LMNA* gene encodes the nuclear lamina protein lamin A/C and its expression is preferentially observed in repopulating HSCs and declines with aging.[21] *ID* and *RGCC* genes are related to cell-cycle control and their expression of fetal HSCs may contribute to the increased proliferation and engraftment capacities of fetal HSCs.[21]

Fetal liver HSCs migrate and seed the bone marrow, where these cells, in the presence of a different microenvironment, generate a program of definitive hematopoiesis, promoting an extensive myeloid diversification with the production of neutrophils, eosinophils, and basophils and dendritic cell subsets; furthermore, in fetal bone marrow, B lymphoid elements undergo a consistent expansion, starting a process of generation of the adaptive immune repertoire, subsequently completed in post-natal and adult bone marrow.[22]

Fetal and adult hematopoietic stem cells display different proliferation rates (higher in fetal compared to adult HSCs), gene expression profiles, and lineage differentiation biases. The molecular mechanisms responsible for these developmental-related changes in HSCs are largely unknown. However, a recent study provided evidence that the transition from proliferating fetal HSCs to quiescent adult HSCs is mainly related to intrinsic mechanisms, gradual events independent of the microenvironment, occurring in a stochastic manner and, at least in part, mediated by the type I interferon.[23]

HSC self-renewal. HSCs have the unique property of replicating to create two daughter identical cells and to differentiate to generate a progeny of hematopoietic cells, and through these essential biological functions, they are able to both maintain and restore blood cell production. However, adult HSCs are characterized by a very low replication rate. In humans, the analysis of the changing ratio with the age of maternal/paternal X-chromosome phenotypes in blood cells from females allowed to define the replication rate of human HSCs, estimated on average of one replication every 40 weeks, with a range comprised between 25 to 50 weeks.[24]

Both intrinsic mechanisms mediated by molecular regulators and extrinsic mechanisms mediated by environmental signals regulate the quiescence of HSCs; these mechanisms include transcription factors, cell-cycle regulators, microenvironmental mediators, and epigenetic factors. All these mechanisms imply a peculiar regulation of cell metabolism of HSCs, and the transition from quiescence to activation is accompanied by marked changes in cell metabolism (protein synthesis, oxidative phosphorylation, glycolysis, autophagy). Recent studies have identified some important regulators of HSC quiescence/proliferation.

Cell-cycle components are important regulators of quiescence/proliferation of HSCs. The HSC pool is heterogeneous concerning the repopulating capacities, and HSCs can be subdivided into long-, intermediate- and short-term (LT, IT, and ST) HSCs. Both LT-HSCs and ST-HSCs are quiescent but differ in the timing of exit from quiescence that is longer for LT-HSCs compared to ST-HSCs, and this difference seems to be related to the level of cyclin-dependent kinase 6 (CDK6) that is higher in ST-HSCs than in LT-HSCs.[25]

The study of transcriptional signatures of human quiescent LF-HSCs showed some remarkable differences compared to those observed in activated ST-HSCs: in fact, the Act/HSPC (activated/hematopoietic stem progenitor cells) signature is observed in activated ST-HSCs and is characterized by the activation of CCCTC-binding factor (CTCF) binding sites; silencing of CTCF expression derepressed expression of stemness genes and maintained the long-term repopulating activity of quiescent HSCs.[26] These observations suggest that chromatin interactions mediated by CTCF control the transition of HSC from quiescence to an activated condition.[26]

Other studies have supported a major role of lysosomes in the control of HSC quiescence.[27,28] These organelles are not simply catabolic degradation structures but are also major signaling centers for complex molecular assembly. A first study showed a connection between lysosomal activity and metabolic activity of HSCs.[27] Quiescent, highly-repopulating HSCs are characterized by low mitochondrial membrane potential (MMP), this potential being higher in primed, activated HSCs.[27] Cycling, primed HSCs are characterized by high glycolysis, and inhibition of glycolytic activity in these cells induces an enhancement of their repopulating capacity.[27] Quiescent HSCs are characterized by the presence of large, scarcely active lysosomes. Inhibition of lysosomal activation in HSCs suppresses glucose uptake and further stimulates their repopulating activity.[27] In the second study, Gracia-Prat et al. explored the peculiar mechanisms of lysosomal activity observed in HSCs; particularly, they examined how transcription factor EB (TFEB) and MYC regulate the catabolic and anabolic processes required for HSC quiescence or activation.[18] Particularly, TFEB-mediated induction of the endolysosomal pathway triggers membrane receptor degradation, limiting HSC metabolic activation and mitogenic activation, promoting stem cell quiescence and self-renewal; in contrast, MYC promotes biosynthetic processes and inhibits lysosomal catabolic functions, thus driving HSC activation.[28]

Some transcription factors are selectively expressed at the level of HSCs and play a key role in the maintenance of the self-renewal capacity of these cells. Thus, Hepatic Leukemia Factor (HLF) is highly expressed in normal HSCs and multipotent progenitors (MPP) and is rapidly lost during the differentiation of these cells.[29] HLF-deficient mice are viable with normal hematopoietic parameters, including a normal HSC pool. However, when these mice were challenged through transplantation showed an impaired capacity to reconstitute hematopoiesis and were gradually exhausted after transplantation.[30] In mouse embryos, HLF expression is limited to intraembryonic HSCs (intra-aortic and fetal liver) but not to extra-embryonic HSCs (yolk sac).[31] HLF expression marks human HSCs at all stages of hematopoietic development, from intra-aortic embryonic hematopoiesis to cord blood and bone marrow.[32]

MLLT3 is a transcription factor whose expression is highly enriched in fetal, neonatal, and adult human HSCs and is gradually decreased in culture, determining a loss of HSC activity.[33] Enforced expression of MLLT3 in human HSCs reduces this decrease in stem activity during *in vitro* culture and potentiates about tenfold the expansion of repopulating HSCs.[33] Importantly, fusion proteins involving MLLT3 have the property to transform normal HPCs into leukemic stem cells. Another factor exerting an essential role in the control of HSC self-renewal is the RNA-binding protein Musashi 2 (MSI2); this factor plays a key role in the expression of master regulators of HSCs through a post-transcriptional mechanism: particularly, MSI2 expression promotes HSCs and HPCs proliferation through downregulation of aryl hydrocarbon receptor (AHR) signaling.[34] The expression of MSI2 in the HSCs is promoted by two transcription factors, PLAG1 and USF2,which are able to bind to the MSI2 gene promoter resulting in increased gene transcription.[35] In line with these findings, an AHR small molecule antagonist, StemReginin (SR1) promotes ex vivo expansion of transplantable human HSCs/HPCs: a phase I/II clinical trial using SR1-expanded cord blood stem/progenitor cells for transplantation showed faster neutrophil engraftment compared to unmanipulated stem/progenitor cells, but not an improvement in hematopoietic recovery.[36] A small molecule screen allowed to identify pyrimidoindole derivatives (UM171) as stimulators of cord blood HSC expansion *in vitro*. Short-term expansion of cord blood HSCs/HPCs using UM171 is a safe and feasible strategy and is under active

clinical evaluation in the treatment of patients with hematological malignancies lacking a suitable HLA-matched bone marrow donor.[37] Importantly, the UM171 expansion markedly improved the usability of CB units stocked in CB banks, allowing the use of smaller CB units for transplantation purposes.[38]

HSC differentiation. Hematopoiesis is a finely regulated process of cell differentiation through which HSCs generate blood elements of all hematopoietic lineages belonging to myeloid and lymphoid lineages. Historically, two theories have been proposed to explain the process of HSC commitment: an instructive model and a stochastic model.[39-41] Following the instructive model, the HSC differentiation choices are driven by external signals mediated by cytokines. According to the stochastic model, the commitment of HSCs is promoted by spontaneous, stochastic variations of cell phenotypes that are selected through selective signals/mechanisms mediated by cytokines.[39-41] A remarkable difference between the two models is related to the level of cell heterogeneity that is expected to be high in the stochastic model and low in the instructive model; the stochastic model suggests the existence of cell-to-cell variability, particularly at the early steps of hematopoietic differentiation before the occurrence of selective processes.

The hematopoietic system represents a highly complex biological system of cell differentiation, leading to the controlled generation of different hematopoietic blood cell types through the differentiation of small populations of HSCs and Hemopoietic Progenitor Cells.[39-41] Historically, the study of hematopoietic cell differentiation was promoted by the isolation of single stem/progenitor cells grown *in vitro* using colony assays or transplantation into myeloablated mice. These studies allowed to define HSCs and HPCs with various differentiation potentials into a cell differentiation hierarchy with HSCs at the apex and mature cell types at the bottom; between these two extremes, there are many defined intermediate stages, the first one being represented by the bifurcation of HSCs into myeloid and lymphoid branches, through the generation of a common myeloid progenitor (CMP) and a common lymphoid progenitor (CLP); the subsequent steps are represented by the generation of unilineage hematopoietic progenitors, generating the first undifferentiated precursors of the various blood lineages entering into the maturation compartment.[39-41] During the years 2005 to 2015,this model was integrated through new acquisitions that have led to considering the HSC compartment more heterogeneous both in terms of self-renewal and differentiation capacities, the presence of a multipotent progenitor LMPP (Lymphoid Myeloid Pluripotent Progenitor) linking the myeloid and lymphoid lineages below the HSC stage. From 2016 onwards, a continuum model of hematopoietic differentiation was proposed, suggesting that hematopoietic lineage commitment is more reliably represented by a continuous process of differentiation trajectories rather than by stepwise differentiation series of distinct hematopoietic progenitor cell populations.[39-41] The development of new technologies of single-cell transcriptomics and proteomics, as well as lineage tracing and functional studies, have led to the important conclusion that there is a continuum of the lineage commitment from HSCs up to mature blood elements, with most of the lineage choices being promoted at the level of HSCs and MPPs.[39-41]

The development of fluorescently labeled HSCs from transgenic donor mice allowed analyzing their differentiation capacities along five different hematopoietic lineages (erythroid, megakaryocytic, neutrophilic, monocytic, B-lymphoid, and T-lymphoid cells). This assay identified a class of myeloid-repopulating progenitor cells able to generate a cell progeny composed of platelet (MkRP), platelet-erythrocyte (MERPs), or platelet-erythrocyte-neutrophil-monocyte lineages (CMRPs). These repopulating progenitors may be originated through direct differentiation of HSCs through asymmetric cell divisions generating one multipotent stem daughter cell and one lineage committed repopulating daughter progenitor.[42] These repopulating progenitors display the capacity of repopulating *in vivo* part of the hematopoietic system but cannot be serially transplanted, thus indicating that they do not possess self-renewal capacity.[43,44]

Single-cell and HSC transplantation cell tracking experiments supported a consistent differentiative heterogeneity of hSCs with the evidence about the existence of some myeloid-biased HSCs and some HSCs adopting a fate towards effective and stable replenishment of a megakaryocyte/platelet lineage tree but no other cell lineages.[44] These findings were confirmed by Rodriguez-Fraticelli and co-workers, who used transposon tagging to clonally trace the fates of progenitors and stem cells in native hematopoiesis; this analysis showed the existence of some long-term HSCs are a source of megakaryocyte-restricted progenitors, suggesting that in mice the megakaryocyte lineage id the predominant fate of long-term HSCs.[45] Finally, Upadhaya et al. have used a system for *in vivo* genetic labeling of HSCs, combined with high-dimensional single-cell analysis to characterize the kinetics of HSC differentiation under native hematopoiesis; this study showed early emergence of megakaryopoiesis, the subsequent divergence of erythroid and myeloid development from lymphopoiesis.[46]

Two studies have combined lineage tracing and single-cell RNA sequencing to obtain simultaneous evaluations of clonal history and cell identity in murine

hematopoiesis.[47,48] This method elucidates how single HSCs and their corresponding progeny develop through the continuous differentiation steps shown by single cell transcriptomics. The results of the study of Weinreb et al. showed that in the hematopoietic differentiation process, different sequences of molecular events might lead to the same differentiation terminal event; a notable example is given by monocytes. Furthermore, sister cell experiments provided evidence that cells with very similar gene expression profiles can be committed to different cell fates, thus suggesting that transcriptional networks alone are insufficient to determine the potential of the hematopoietic cell towards different fates.[47] The second study, performed by Pei et al. using a similar methodological approach, showed that HSCs are heterogeneous, with differentiation-inactive, multilineage, and lineage-restricted HSC clones corresponding to different regions of the transcriptional landscape of hematopoiesis.[48]

Adult hematopoiesis. Studies in human hematopoietic cells support the revised model of hematopoietic differentiation based on the observation that HSC and multipotent progenitors progressively acquire lineage biases along various differentiation fates. Through an integrative analysis of transcriptomic, flow cytometry, and functional data at a single-cell level, Velten et al. explored the early steps of hematopoietic cell differentiation at HSC and HPC stages: this analysis supported the existence of a continuum of low-primed undifferentiated HSCs and HPCs; in this context, the separation of megakaryocytic/erythroid and lympho-myeloid represented the main routes of lineage specification.[49] According to these findings, a model of human hematopoietic differentiation was proposed based on a continuum and transient state of lineage commitment at the stem/progenitor cell compartment level. Buenrostro et al. have used another approach to explore a regulatory landscape of human hematopoietic differentiation through single-cell epigenomic analysis based on an assay for Transpase Accessible Chromatin with high single-cell RNA sequencing on ten phenotypically defined HSC and HPC subsets; this analysis showed the existence of an association of changes in chromatin accessibility with changes in transcription factor expression during differentiation.[50] Particularly, the transcription factor-chromatin accessibility variability in HSCs follows megakaryocytic-erythroid/lymphoid pathways and shows the existence of two granulo-monocytic subsets: the more primitive and least-primed subset of HSCs and HPCs is characterized by low cell cycling activity, low RNA content, low gene expression, low cellular respiration and expression of HOX motif; the stem/progenitor cells primed along cell differentiation display an increased cell cycling activity, increased gene expression and gradient of expression of transcription factor regulators ID3, CEBP and GATA1 toward lymphoid, myeloid and erythroid differentiation, respectively.[50]

Additional studies strongly supported the continuum nature of human hematopoiesis. Thus, Psaila et al. have performed a single-cell analysis of human megakaryocyte-erythroid progenitors isolated from cord blood, showing a consistent differentiation capacity of these cells: pre-MEPs predominantly display erythroid-myeloid differentiation but with residual myeloid potential; MEPs were strongly biased to erythroid differentiation; Mk-MEPs primarily showed megakaryocytic differentiation capacities.[51] In addition, Karamitros et al. performed a single-cell analysis of lympho-myeloid progenitors present in human cord blood, indicating that lymphoid-primed multipotential progenitors (LMPPs), granulo-monocyte progenitors (GMPs) and multi-lymphoid progenitors (MLPs) are functionally unipotent, bipotent and multipotent and transcriptionally heterogeneous.[52] Single-cell analysis of human hematopoietic progenitors further supported the continuum differentiation model of human hematopoiesis.[53]

These studies have supported the view that: (i) lineage commitment occurs at early stages of hematopoietic differentiation from primed HSCs; (ii) hematopoietic progenitor cells are highly heterogeneous and classical erythroid-megakaryocytic, and granulo-monocytic progenitors englobe oligopotent progenitor cells; (iii) the two main branches of hematopoietic differentiation involve a GATA2-positive branch of erythroid, megakaryocytic and eosinophilic/basophilic/mast cell progenitors and a GAAT2-negative branch of lympho-myeloid progenitors, including progenitors of neutrophils, monocytes, and dendritic cells.

Single-cell transcriptomic and proteomic studies, coupled with flow cytometry analysis, have allowed defining some immunophenotypic profiles associated with differentiation properties of human stem/progenitor cells. Human hematopoiesis is mostly sustained by CD34-positive cells, a cell surface marker identifying the large majority of HSCs and HPCs. Using a combination of cell surface markers, Notta and co-workers distinguished a subset of $CD34^+CD38^-CD90^+CD49f^+$ cells enriched in functional HSCs and a subset of $CD34^+CD38^-CD90^-CD49f^-$ cells enriched in MPPs.[54] The combination of single-cell transcriptomic studies and xenotransplantation assays allowed to identify $CD34^+CD38^-CD45RA^-EPCR^+$ cells as a cell subset highly enriched in multipotent HSCs: 1/3 of these cells display functional properties of repopulating HSCs; furthermore, these cells are slow cycling and exhibit a low metabolic profile.[55] $EPCR^+$ cells are at the apex of a HSC/HPC hierarchy: $CD34^+CD38^-CD90^+$ cells display a

lower stem repopulating capacity, estimated in the order of 1/119 cells; $CD34^+EPCR^+$ cells can generate $CD34^+EPCR^-$ cells but not the contrary; $CD34^+EPCR^-$ cells can generate MPPs and more committed progenitor cells.[55] Unicellular transcriptomic studies showed that $CD34^+EPCR^+$ cells display a multipotent/stem profile with a moderately myeloid biased phenotype.[55] Importantly, $CD34^+EPCR^+$ cells resulted in being relatively homogeneous, as expected for cells that can be located at the apex of hematopoiesis.

The techniques that simultaneously measure mRNA and surface protein expression in single cells allowed to define of cytometry assays carefully reflecting single-cell RNA sequencing-based molecular data at the level of various hematopoietic differentiation stages.[56] Aksoz et al. have explored the individual transcriptome profile of human bone marrow hematopoietic cells highly enriched in HSCs ($CD34^+CD38^-CD90^+CD45RA^-$) and showed that these cells displayed a consistent heterogeneity: HSCs with multilineage signatures correlated with high cellular output signatures, whereas platelet bias and low-cellular-output signatures correlated at the single-cell level.[57]

One of the fundamental biologic properties of HSCs consists in their capacity to undergo symmetrically (with the generation of two HSCs or two HPCs) or asymmetric (with the generation of one HSC and one HPC) divisions; through this unique biologic property, HSCs can adapt to the regenerative need of hematopoietic tissue. Asymmetric cell divisions imply the unequal repartition of cellular components during cell division, a condition that determines a different cell fate of daughter cells. Recent studies have suggested that asymmetric cell divisions are determined by an asymmetric reorganization of the cytoskeleton during cell division, determining a condition of cellular polarization with consequent asymmetric distribution of cell fate determinants. Studies carried out in murine HSCs have shown that the asymmetric distribution of the cellular degradative machinery (including lysosomes, autophagosomes, mitophagosomes, and the NUMB-can protein) is associated with the activation/differentiation in that the daughter cells receiving this machinery maintain a stem cell condition.[58] The asymmetric distribution of lysosomes plays a relevant role in the mechanism of cell fate determination of human HSCs. In fact, Loeffler et al. have shown that human HSCs, undergoing asymmetric divisions, receive more lysosomes at the level of daughter cells, maintaining their stemness condition. In contrast, daughter cells receiving fewer lysosomes are more prone to undergo cell differentiation.[59] Interestingly, in addition to lysosomes, active mitochondria can also be asymmetrically partitioned: daughter cells receiving more lysosomes tend to receive fewer active mitochondria during an asymmetric HSC division.[59]

The model of continuum hematopoietic differentiation implies a possible involvement of HSCs and MPPs in the homeostatic maintenance of hematopoiesis. Initial studies performed in mice have supported the view that unperturbed hematopoiesis is mainly maintained by MPPs and committed progenitor cells but not by HSCs.[60-61] However, recent studies have challenged this view, providing evidence through different *in vivo* labeling systems that murine adult HSCs considerably contribute to steady-state, unperturbed hematopoiesis.[62-64] Particularly, Chapple et al. showed that adult murine HSCs contribute robustly to steady-state hematopoiesis, with a major efflux toward the myeloid lineages compared to lymphoid lineages;[63] Sawai et al. showed that murine HSCs give a major contribution to all blood lineages, including myeloid cells and lymphocytes, except tissue macrophages and B1a lymphoid cells;[62] Sawen et al. provided evidence that HSCs contribute to native hematopoiesis, but the HSC contribution to multilineage hematopoiesis declines with increasing age.[64]

Role of HSCs in hematopoietic reconstitution after bone marrow transplantation. Recent studies have explored the contribution of HSCs to hematopoietic reconstitution after bone marrow transplantation. These studies took advantage of the clonal tracking studies carried out in gene therapy-treated patients exploiting vector integration sites (ISs) as molecular markers for monitoring and assessing the dynamics of hematopoietic reconstitution induced by infusion of bone marrow cells genetically manipulated.[65-66] The analysis of ISs at the level of different blood elements after transplantation allowed to define the kinetics of blood cell generation from individual HSCs. Studies carried out in murine systems have shown that individual ISs are, in the majority of instances, present in either myeloid or lymphoid blood cells and only in a few cases are shared in both these cell types.[67] Lu et al. have used a high-sensitivity quantitative cloning tracking technology to explore HSC commitment after transplantation in the absence of conditioning and after conditioning with irradiation or with anti-c-kit antibody treatment.[68] Under conditions of unperturbed hematopoiesis, donor HSCs homogeneously contribute to the various stages of hematopoiesis, thus suggesting that HSC lineage commitment develops with an equal contribution from each clone.[68] At variance of unconditioned mice, in irradiated mice, a small fraction of engrafted HSC clones constantly expanded faster than other clones during differentiation and generated most neutrophils and B-lymphocytes; it was estimated that in both irradiated and c-kit conditioned mice, about 50% of total neutrophil and B-lymphoid production is generated from the differentiation these few HSC dominant clones.[68] The conditioning regimens (such as the irradiation dose) and

the transplantation conditions (such as the number of helper cells used in the transplantation procedure) consistently induce HSC lineage bias; lineage bias is originated from dominant differentiation events occurring at distinct lineage commitment steps.[68]

Studies in non-human primates have confirmed the results observed in mice. A quantitative method for the assessment of the self-renewal and differentiation patterns of lentivirally-labeled macaque HSCs allowed to show that: (i) individual HSC clones may display stable myeloid or lymphoid bias for many years; (ii) output of individual HSCs and HPCs was stable for many years, with very limited evidence of clonal succession.[69]

In humans, the study of patients treated with genetically manipulated HSCs/HPCs allowed the unique opportunity to explore the dynamics of human hematopoietic reconstitution at the clonal level, exploiting the capacity to identify the differentiated progeny at clonal level through the analysis of the insertion of the therapeutic vector in a unique genomic site.[65] Furthermore, the longitudinal study of individual clones of hematopoietic cells for many years after transplantation of genetically modified HSCs/HPCs allowed to define peculiar patterns of clonal dynamics during early and steady-state reconstitution phases: in the initial phase of engraftment, the generation of myeloid cells is ensured by committed myeloid progenitors, such as CMPs and GMPs; after this phase of engraftment there is the early phase of reconstitution that is ensured during the first 18 months post-transplantation by short term-repopulating HSCs and MPPs and from 18 to 24 months post-transplantation by long-term repopulating HSCs; the phase of steady-state hematopoiesis, occurring after 24 months post-transplantation, is ensured by long-term repopulating HSCs.[65-66] These studies also showed that lymphoid-biased stem/progenitor cells may be capable of long-term survival and can be maintained independently of their generation from HSCs.[66] Another study based on the analysis of patients undergoing HSC/HPC gene therapy for Wiscott-Aldrich Syndrome or beta-hemoglobinopathies provided evidence that HSCs/HPCs can be classified into three groups according to their clonal lineage outputs, reflecting stable, distinct differentiation programs: myeloid-dominant, lymphoid-dominant and balanced.[70]

Bone Marrow Transplantation Derived Observations. The observations made during the last 20 years on bone marrow transplantation also support the existence of a decrease in HSC function associated with aging. In 2001, a retrospective analysis of a large cohort of patients who have undergone bone marrow transplantation (BMT) and analyzed various donor-related parameters that could affect overall survival (OS), disease-free survival (DFS), acute and chronic graft-versus-host disease (GVHD), engraftment and relapse. Among the various donor parameters evaluated, age was the only donor trait significantly associated with OS and DFS for both HLA-matched and HLA-mismatched transplants: the use of younger donors lowers the incidence of GVHD and improves survival after BMT.[71] The same authors confirmed these findings through the analysis of 11,039 BMTs from unrelated donors; after adjusting for patient disease and transplantation characteristics, survival was better for transplantations of grafts from young donors: for every 10-year increment in donor age, there was a 5.5% in the hazard ratio for overall mortality.[72] The study of a cohort of 889 patients who have undergone haploidentical BMT showed that increasing donor age by decade was associated with poorer OS. In addition, worse progression-free survival and a higher frequency of GVHD; these less-favorable results with older donors were related to worse non-relapse mortality.[73]

When bone marrow cells are infused in patients for transplantation, only 5-30% of HSCs get home to the bone marrow, while the remainder is lost and distributed in the lung, spleen, and liver. The relatively low homing/engraftment of infused HSCs/HPCs is more pronounced in clinical studies carried out using gene-edited stem/progenitor cells, a phenomenon related to the gene editing procedure or the use in these studies of purified populations of HSCs/HPCs deprived of T helper cells.[74] To bypass this limitation, some clinical studies have used the intra-bone administration of genetically-corrected autologous HSCs/HPCs, a procedure that accelerates the kinetics of hematopoietic recovery post-transplantation.[75] Transplantation studies carried out in immunodeficient mice showed that intra-bone marrow transplantation of HSCs and HPCs, but not of LT-HSCs: the higher engraftment of HPCs compared to HSCs seems to be due to a higher expression of the CXCR4 receptor on HPCs compared to HSCs.[76] The removal of HPC and the transplantation of an HSC-enriched cell population intra-bone improved the engraftment HSCs.[76] Induction of a higher expression of CXCR4 on HSCs improves homing and engraftment of these cells.[76]

Aging of Hematopoietic Stem Cells. Aging is associated with a decline and alterations of mature blood cells and there is evidence that a combination of intrinsic and extrinsic mechanisms is responsible for these changes. Growing evidence suggests that changes occurring at the level of HSCs are, in large part, responsible for the aging of the hematopoietic system.

At the level of intrinsic mechanisms, several studies have characterized the changes occurring at the level of the HSC compartment in mice and in humans.

The studies carried in murine HSCs have shown that: (i) the number of HSCs increases with age, but the competitive repopulating activity of these cells declines, thus suggesting a decrease of HSC biologic function associated with age; (ii) aging mouse displays a decrease

in lymphopoiesis, associated with an increase in myelopoiesis, changes that are at least in part related to a prevalence of myeloid biased HSCs in elderly mice.[77-79] A fundamental study by Ganuza et al. used a non-invasive *in vivo* color-labeling system to evaluate the changing clonal complexity of steady-state hematopoiesis during murine lifespan.[80] Steady-state hematopoiesis is characterized by a mechanism of clonal instability in which pools of HSCs increase and decrease their contribution to hematopoiesis during life.[80] The clonal complexity of hematopoiesis consistently decreases with age, particularly at the HSC and MPP cell compartments.[80] Aging was associated with a consistent increase in the functional heterogeneity of HSCs and with a reduction in their repopulating activity.[80] Serial transplantations exerted marked effects on hematopoiesis, as evidenced by: (i) a marked reduction of clonal diversity; (ii) an increase of clonal instability; (iii) an increase of mutational burden, a phenomenon much more evident in aged bone marrow than in young bone marrow.[80]

Studies carried out in human bone marrow showed an increase of cells with an HSC immunophenotype ($CD34^+CD38^-CD90^+CD45RA^-$) among $CD34^+$ cells associated with aging.[81] Immunophenotypic evaluation of HPC subsets showed a significant decrease of CLPs in elderly bone marrow.[81] Elderly HSCs displayed a reduced capacity to generate a lymphoid progeny and an increased myeloid-differentiation capacity.[81] Thus, aged human HSCs are less quiescent and exhibit myeloid-biased differentiation potential compared to young HSCs.[81] At the gene expression level, elderly HSCs transcriptionally up-regulate genes associated with cell cycle, myeloid lineage specification, and myeloid malignancies.[81] Other studies confirmed that aged human BM displays a reduced content of CLPs, associated with increased frequencies of MEPs.[82] Kuranda et al. confirmed these results showing an increased frequency of $CD34^+CD38^-$ cells in the elderly bone marrow; however, xenotransplantation experiments in NOD/SCID mice showed that the number of repopulating HSCs does not change with aging.[83] Elderly bone marrow HSCs showed a reduced myeloid reconstitution.[83]

Studies in murine HSCs have explored the cycling activity of HSCs. In this context, a study by Kowalzyk et al. provided evidence of a consistent decrease of the G1/S phase cells among old HSCs compared to young HSCs (7% vs. 22%, respectively); this finding was interpreted as evidence that aged HSCs traverse through G1 faster.[84]

Ethinyl deoxyuridine (EdU) is a chemical compound used for fast and sensitive detection of DNA synthesis.[85] Kovtonyuk et al., using this technique, measured the cycling status and the compartment sizes of HSCs, HPCs, and granulocytes in mice of four different ages: 3-week, 2-month, 1-year, and 2-year-old mice.[86] The compartment size gradually increased with age from 3 wk old mice to 2-year-old mice; in contrast, the cycling activity of HSCs decreased progressively and significantly with age.[86] Thus, an increase of HSC in dormancy is responsible for the increased size of the HSC pool in aging.[86]

Cdc42 is a small Rho GTPase present in two activation states: an active guanosine-triphosphate (GTP)-bound state and an inactive guanosine diphosphate (GDP)-bound state. Cdc42 expression is altered in aging HSCs, and this event causes a loss of polarity of these cells.[87] In fact, Florian et al. showed that Cdc42 expression is high in aging HSCs and correlates with a loss of polarity in aged HSCs.[87] Experiments with pharmacologic inhibitors of Cdc42 restored the polarization of aged HSCs and the levels and spatial distribution of histone H4 lysine 16 acetylation.[87] The loss of polarization induces several effects on aged HSCs, such as preferential self-renewing symmetric divisions, resulting in the degeneration of daughter HSCs with reduced regenerative potential and lymphoid differentiation capacities.[88] A recent study showed that also the aging of human HSCs is associated with changes in Cdc42 activity; Amoah and coworkers showed that: (i) the number of aged HSCs increased, and these cells display a delayed response *in vitro* to cytokine stimulation; (ii) Cdc42 activity in aged human HSCs is increased and correlates with an increased number of HSCs; (iii) the frequency of HSCs exhibiting a polarization for Cdc42 and tubulin decreases with aging; (iv) treatment of aged human HSCs with casin, a Cdc42 inhibitor, restores cell repolarization and rapid response to cytokines.[89]

DNA damage is universally considered one of the fundamental mechanisms driving tissue aging: DNA damage affects most aspects of the aging phenotype.[90] Proliferating progenitor cells are dependent on reliable homologous recombination (HR) pathway for DNA reparation, while quiescent HSCs use a different mechanism of DNA reparation called the error-prone nonhomologous end joining (NHEJ) repair pathway.[91] This mechanism exposes quiescent stem cells to the risk of genomic rearrangements that can persist and contribute to developing hematopoietic abnormalities.[91] The condition of HSC quiescence and the concomitant decrease of DNA repair and response pathways are conditions that favor DNA damage accumulation in HSCs during aging.[92] Furthermore, cycling old HSCs display increased levels of replication stress activity, associated with cell cycle defects and chromosome gaps and breaks: this condition determines a functional decline of aged HSCs.[93]

In murine HSCs, the induction of the quiescence state in response to conditions that model physiological stress, such as chronic blood loss or infection, provoked the

gradual decrease of normal HSC activity.[94] This observation suggests a link between physiological stress and DNA damage in normal HSCs.[94]

5-hydroxymethylcytosine binding, cell-specific (HMCES), is a gene enabling single-stranded DNA binding activity, involved in cellular response to DNA damage stimulus and protein-DNA covalent cross-linking. HMCES represents a guardian of genome integrity and long-term self-renewal capacity of HSCs during the stress response, such as response to myeloablation and transplantation.[95]

The presence and the level of expression of DNA damage repair mechanisms is a major determinant of radiosensitivity of hematopoietic cells. In addition, marked differences in radiation sensitivity exist between the lymphoid and myeloid cells, with lymphoid cells being significantly more sensitive than cells of the myeloid lineage; in the myeloid lineage, monocytes/macrophages are the most radio-resistant cell types.[96]

Irradiated human HSCs/HPCs, but not committed HPCs rapidly undergo apoptosis through an ATM-dependent process. This apoptotic process is inhibited by interaction with bone marrow stromal cells.[97] HSCs/HPCs showed reduced NHEJ processes compared to committed HPCs.[97] The interaction with stroma does not affect the level of NHEJ activity. 10% of HSCs/HPCs surviving to irradiation display clonal chromosomal aberrations.[97]

The Discovery of Clonal Hematopoiesis of Indeterminate Potential (CHIP). During their lifespan, cells divide and may accumulate somatic mutations: most of these mutations are neutral; however, a minority of mutations may increase cellular fitness and confer a growth advantage, resulting in clone expansion. This phenomenon was observed in many normal tissues and increases with age.[98] A median somatic mutation frequency of 2.8×10^{-7} per bp was estimated using human dermal fibroblasts; this frequency of mutational events is higher than that observed in germline tissues, calculated in the order of 10^{-8} per bp.[99]

Whole exome sequencing studies were used to quantify and track somatic mutations in normal hematopoietic cells.[100,101] These studies were based on isolating single stem/progenitor cells by fluorescence-activated cell sorting and expanding these cells *in vitro* to generate clonal populations of hematopoietic cells sufficient to permit extensive whole genome sequencing.[100,101] According to the results obtained, it was estimated in one study carried out on a single subject a mutational accumulation rate of 11.7 mutations per year[100] and in the other study, carried out on seven healthy donors ranging in age from 0 (umbilical cord blood) to 63 years, a constant accumulation rate of 14 mutations per year.[101] Importantly, blood mutations occurred in a characteristic trinucleotide context.[101] The analysis of 11 healthy subjects of different ages showed a positive correlation between the number of base substitutions in hematopoietic stem/progenitor cells and the age of the donors, with an accumulation of 14.6 base substitutions per year of life.[102] The analysis of the mutational spectra of normal stem/progenitor cells showed two predominant mutational signatures: hematopoietic stem/progenitor signature (previously identified as a signature predominantly observed in normal stem/progenitor cells) and single base substitution signature 5.[103] Most point mutations consist of 1 bp deletion of a C or a T and 1 bp insertion of a T, a process commonly ascribed to polymerase slippage during replication of the replicated DNA strand.[104] In addition to mutational events, chromosomal studies have shown some recurring aging-related alterations.

A notable example is loss of the Y chromosome, detected in 43% of men older than 70, while sub chromosomal rearrangements have been observed in 2-3% of older individuals.[105] The complex cellular and molecular mechanisms orchestrating and regulating cell division represent a major source of mutational events and chromosomal abnormalities generation.[106] Furthermore, genome integrity is also compromised by other molecular mechanisms, such as defective DNA-repair mechanisms.[106] Endogenous and exogenous mutagens favor the generation of the genetic alterations ineluctably associated with aging.[106]

Among the various forms of tissutal clonal expansion, clonal hematopoiesis was intensively investigated. The term clonal hematopoiesis of indeterminate significance (CHIP) was introduced to describe individuals with a hematologic malignancy-associated somatic mutation in peripheral blood or bone marrow cells but without any other diagnostic criteria for a hematologic malignancy.[107] CHIP must be distinguished from myelodysplastic syndromes (MDS) by the absence of cytopenias and the diagnostic morphologic criteria for dysplasia that define MDS and can be considered analogous to monoclonal gammopathy of undetermined significance and monoclonal B-lymphocytosis.[107]

Initial studies aiming to measure the ratios of X-inactivation if females led to the identification of age-associated skewing (AAS) in blood cells, particularly at the level of the myeloid compartment; a potential cause of AAS is the acquisition of somatic mutations inducing a growth advantage, with consequent clonal hematopoiesis: in line with this hypothesis, Busque et al. reported the occurrence of *TET2* and *DNMT3A* mutations in one of three individuals with AAS; the subsequent exploration of 179 older women with AAS showed the presence of *TET2* mutations (nonsense, missense and frameshift mutations) in 5.6% of these subjects.[108]

Three pivotal studies reported in 2014 the main

biological features associated with CHIP: CHIP-associated mutations are rare in individuals younger than 40 years of age but rose significantly with age, particularly after 60 years; the risk of leukemic progression is influenced by VAF, the number and the type of mutant genes; the majority of mutations were observed at the level of three genes, *DNMT3A, TET2* and *ASXL1*; the presence of CHIP was associated with an increased risk of hematologic malignancies, an increase in all-cause mortality and in the risk of coronary heart disease.[109-111]

As it will be discussed, CHIP is associated with aging and is over-represented in some diseases irrespective of age, where it may contribute to disease outcomes and all-cause mortality.

Age-associated CHIP, gene mutations. In 2014, three studies reported the age-related accumulation of mutations in leukemia-related genes (*DNMT3A, TET2, ASXL1*) using NGS with variant allele frequency >0.02 (2%), due to the error rate. Benign mutant clones were rarely detected in individuals younger than 60 years but in 10-20% of individuals older than 60-70 years.[109-111] Based on these studies, CHIP was operatively defined as the presence of somatic mutations otherwise detected in hematologic malignancies in subjects' blood without any evidence of a morphologically defined hematologic malignancy, with a VAF of 2% or greater.[107] The most frequent mutations occurred at the level of three genes, *DNMT3A, TET2* and *ASXL1* followed by less frequent mutations of *TP53, JAK2, SF3B1, SRSF2, GNB1* and *CBL*.[109-111] McKerrell et al. confirmed these findings by performing an ultra-deep targeted analysis of 4,219 individuals and reporting clonal hematopoiesis in 0.8% of individuals under 60 years, rising to 19.5 in those ≥90 years.[112] *DNMT3A-R882* mutations were the most frequent; the mutations involving spliceosome genes *SF3B1* and *SRSF2* were detected only in individuals aged >70 years.[112] Another study based on ultra-deep targeted sequencing involved the analysis of 2530 hematologically normal individuals with an age range from 55 to 101 years. 13.7 % of subjects displayed CHIP, ranging from <10% in the 55-59 year group to >40% in the 90-101 year group.[113] Some remarkable differences were observed between *DNMT3A* and *TET2* mutations: *TET2* mutations were more age-dependent, associated with a modest neutropenic effect, familial aggregation and chronic obstructive pulmonary disease; *DNMT3A* mutations do not have an impact on hematologic parameters.[113]

Young et al. explored the occurrence of CHIP by targeted error-corrected sequencing, which enables the detection of clonal mutations as rare as 0.0003 VAF; they observed that CHIP could be detected using this very sensitive technique in nearly 95% of 50-70 years old individuals.[114] Furthermore, the exploration of clonal variants in purified T lymphocytes, B lymphocytes and myeloid cells purified from the blood of 13 individuals showed the presence in 10/13 cases of the same clonal single nucleotide variant in both lymphoid and myeloid elements, thus supporting the cellular origin of CHIP at the level of hematopoietic stem/progenitor cells.[114]

Arends et al. performed a detailed analysis of clonal hematopoiesis in 437 elderly individuals in different blood cell populations (**Table 1**). VAFs of the main CHIP-mutated genes were significantly higher in monocytes, granulocytes, and NK-cells compared to B and T-cells.[115] Importantly, in all cases analyzed, CHIP mutations were detected at the level of the $CD34^{+}CD38^{-}$ HSCs and at the level of myeloid-committed progenitors.[115]

Zink et al. explored the occurrence of CHIP in a population of 11,262 Icelanders by whole-genome sequencing. They observed a frequency of 12.5% in the whole population, variable with age and reaching a frequency of 23%, 32%, and 52% in the age groups of 65-75,75-85, and 85-110 years, respectively (**Table 1**).[116] In this population, the presence of CHIP was associated with higher death rates and an increased risk of hematological malignancy.[116]

Another recent study of targeted error-corrected NGS confirmed the presence of CHIP in the large majority of individuals ≥80 years: 57% in the 80-82 yr group and 72% in the ≥86 yr group.[20] In this old population of individuals, only the presence of multiple mutations was associated with an increased risk of death and of developing hematological malignancy.[117]

Rossi et al. reported the analysis of CHIP in 1794 individuals aged ≥80 years: somatic mutations were observed in about one-third of these subjects[118] (**Table 1**). Somatic mutations were observed in about one-third of individuals aged >80 and were associated with reduced survival.[118] Most variants occurred in 3 genes: *DNMT3A, TET2 and ASXL1;* a significant prevalence of *TET2* and *ASXL1* mutations after the age of 90 years was observed; mutations in *JAK2* and splicing genes, multiple mutations, and variant allele frequency ≥0.096 had predictive value for increased probability of developing myeloid neoplasms.[118]

Kar et al. reported the data of CH in a large cohort of 200,453 UK Biobank participants aged between 38-72 years analyzed by whole-exome sequencing.[119] *DNMT3A, TET2* and *ASXL1* genes were the most commonly mutated genes in CHIP, followed by DNA damage response genes *PPM1D, TP53, ATM* and splicing factor genes *SRSF2* and *SF3B1*; and *JAK2* and *GNB1* genes.[119] However, the age-related rise in prevalence differed for the different driver genes: compared to *DNMT3A* mutations, mutations in *ATM* were detected three years before, while *ASXL1, PPM1D, SRSF2, SF3B1* mutations were observed 1-3 years later.[119]

Table 1. CHIP studies involving aged individuals.

Subjects	Frequency of CHIP	Mutations in CHIP	Main features of individuals with CHIP
Normal healthy subjects (Jaswal et al. 2014) 17,182 healthy persons Whole exome sequencing	Variable following age (yr) 20-29 0%; 30-39 0,.1% 40-49 1.7%; 50-59 2.5% 60-69 5.6%; 70-79 9.5% 80-89 11.6%; 90-99 16% 100-108 29%	DNMT33A (59%), TET2 (10.6%), ASXL1 (9.1%), TP53 (4.9%), JAK2 (4.5%) SF3B1 (3.9%), GNB1 (3.2%), CBL (1.8%), SRSF2 (1.6%) 93% with 1 mutation, 7% with two mutations	Individuals with CHIP with 1 or 2 mutations he hematologic parameters comparable to those of individuals without CHIP. Among individuals with a VAF of ≥0.1 the risk of developing a hematologic cancer is increased by a factor nearly 50. Individuals with CHIP who were 70 years of age or older have an increased risk of death related to hematologic neoplasms and to an increased incidence of cardiovascular diseases.
Normal healthy subjects (Zink et al. 2017) 11,262 Icelanders Whole genome sequencing	12.5 in the whole population, variable with age, reaching a frequency of 23%, 32% and 52% in the age groups of 65-75, 75-85 and 85-110 years, respectively.	The most frequent mutations occurred at the level of DNMT3a; TET2, ASXL1, PPM1D, JAK2, TP53, SRSF2 and SF3B1.	In this population the presence of CHIP was associated with higher death rates and increased risk of hematological malignancy.
Individuals aged ≥80 years (van Zeventer et al. 2021) Targeted error-corrected NGS at a VAF ≥1%	62% Variable following age, from 57% in the 80-82 years to 72% in the ≥86 years group.	DNMT3A 35% TET2 27% ASXL1 6% Spliceosome 4% TP53 3% Multiple variants of DNMT3A and TET2 are frequently observed.	No effect of CHIP on hematologic parameters. No differences in the incidence of CHIP related to the sex. An elevated risk of exposure to DNA damaging agents was not significantly associated with differences in the prevalence of CHIP; however, ASXL1 and spliceosome gene variants are more frequent in these individuals. No association with cardiovascular diseases, but with COPD was observed. Overall, CHIP did not confer an increased risk of death; however, individuals with multiple genetic variants have an increased risk of death and of developing hematologic malignancies.
299 twin pairs ≥70 years (Hansen et al. 2020) Targeted NGS covering CHIP mutations	36% Variable following age, from 29% at 75 yrs to >64% in the group >85 years.	DNMT3A 48.5% TET2 38% ASXL1 10% TP53 5% PPM1D 4% JAK2 3.5%	20 twin pairs had mutations within the same genes but the exact same mutation was observed in only 2 twin pairs. No differences in casewise concordance between monozygotic and dizygotic twins was observed for any gene, subgroup, or CHIP mutations overall, and no significant heritability was detected. 127 twin pairs were discordant for at least mutation, and in 48% of these cases, the affected twin died first.
1794 individuals aged >80 years. (Rossi et al. 2021) Targeted NGS-covering CHIP mutations	32.6%	DNMT3A, TET2, ASXL1, PPM1D, SF3B1, BCOR, JAK2, TP53 were the most recurrently mutated.	TET2, DNMT3A and ASXL1 comutations, splicing gene mutations and VAF> 0.096 are independent predititctor for developing myeloid neoplasms. ASXL1, TET2, DNMT3A and JAK2 mutations are high-risk for vascular events. The individuals with cytopenias and with CHIP bearing splicing genes, ASXL1, DNMT3A or TET2 comutations and/or VAF >0.096 have a high-risk of leukemic transformation.

Fabre et al. reported the longitudinal dynamics of CHIP clones over a median of 13 years; 92.4% of these clones expanded at a stable exponential rate over observation: different mutations exhibited different growth rates.[120] Thus, *DNMT3A* and *TP53* grow with an average annual growth rate of about 5%; clones bearing mutations in *TET2, ASXL1, PPM1D* and *SF3B1* displayed a growth rate of about 10%; fast-growing clones with *SRSF2, PTPN11* and *U2AF1* mutations expand with a growth rate of 15-20%/yr.[120] The individual growth variability of fast-growing clones, such as those associated with *SRSF2-P95H* mutant or *U2AF1,* was low compared to the consistent individual variability of slow-growing clones, such as those related to *DNMT3A* mutations.[120] The reconstruction of the dynamics of development of the different mutant clones showed that: *DNMT3A*-mutant clones preferentially expand early in life and exhibit a slower growth in old age, whereas splicing gene mutations expand later in life, and *TET2*-mutant clones grow and expand at all ages.[120]

The compendium of all genes driving clonal hematopoiesis is far from complete and recent studies have identified new drivers. Pich et al. have adopted a reverse somatic calling approach, exploring the analysis of whole-genome and whole-exome blood/tumor paired samples of two large cohorts of cancer patients.[121] Using this approach, they identified more than 60 genes showing signs of positive selection in clonal hematopoiesis and thus with the properties of clonal hematopoiesis drivers.[121] Beauchamp and coworkers reported analysis of sequencing data from 84,683 individuals and identified novel drivers of clonal hematopoiesis in the 5-methylcytosine reader *ZBTB33* (0.18%) and in *YLPM1* (0.07%), *SRCAP* (0.06%) and *ZNF318* (0.12%).[122] Functional studies in mouse models suggested that mutated *ZBTB33* induces the expansion of hematopoietic stem cells.[122]

Growing evidence indicates that elderly individuals have evidence of CHIPs even in the absence of known driver mutations. Thus, Zink and coworkers, in their screening of 11262 Icelanders, provided proof of clonal hematopoiesis with and without driver mutations: using a comprehensive genome sequencing approach, they detected a much higher proportion of individuals with

CHIP compared to the frequency observed using a detection approach based on the analysis restricted to the 18 genes including all high-impact mutations observed in hematopoietic tissue.[123] Poon et al. have developed a strategy to decipher the genome-wide rate of positive selection based on the analysis of the VAF distribution of synonymous mutations: most synonymous mutations reach high VAF due to genetic hitchhiking, a phenomenon implying the co-occurrence of synonymous mutations in association with positively selected driver mutations which might be undetected.[123] Thus, the high number of VAF synonymous variants provides information about the genome-wide rate of driver mutations. The application of this framework to data from the physiological blood of normal individuals showed that a large part of mutations driving clonal expansions is located outside of canonical cancer driver genes.[123] Mitchell et al. have evaluated the clonal dynamics of hematopoiesis during the human lifespan;[124] this study was based on the analysis by whole-exome sequencing of colonies grown from single stem/progenitor cells, enabling comprehensive identification of somatic mutations and reconstruction of lineage relationships between cells, similarly to the study previously performed by Lee Six et al..[100] HSC/Progenitors accumulated 17 mutations/year after birth and lost 30bp/year of telomere length. Hematopoiesis in adults aged <65 years was largely polyclonal, with consistent clonal diversity and with a population of 20,000-200,000 stem/progenitor cells contributing to blood cell production; in individuals aged >75 was oligoclonal, with 12-18 independent clones globally contributing to 30-60% of total hematopoiesis and each contributing to 1-34% of blood production.[124] Most clones start their expansion before the subjects reach 40 years of age. However, only 22% of these clones had known driver mutations; genome-wide selection analysis estimated that 3% to 8% of mutations were drivers.[124] Thus, this study raised several fundamental observations on age-related clonal hematopoiesis, showing that: the prevalence of clones with more than 1% of VAF is virtually universal over the age of 70 years; (ii) the number of expanded clones per individual is 10-20; (iii) the fraction of overall hematopoiesis sustained by mutant clones in 30-60%; (iv) clonal expansions are generated from mutations occurring decades earlier.[124]

Abascal and co-workers explored the somatic mutation landscape at single-molecule resolution in individuals of different ages observing that differentiated blood granulocytes displayed remarkably similar mutation loads and signatures compared to their corresponding stem/progenitor cells, although mature granulocytes had undergone considerably more cell divisions.[125] This observation suggests that mutational events occurring in hematopoietic stem cells may be independent of cell division.[125] Similar comments have been made for the colon and other tissues.[125]

Although the development of clonal hematopoiesis is an age-related event, the generation of somatic mutations in hematopoietic cells occurs even in fetal hematopoietic stem/progenitor cells. Using an error-corrected sequencing approach enabling the detection of variants with a VAF as low as 0.01%, Wong et al. reported the presence of clonal hematopoiesis in 18.2% of cord blood samples, with a VAF ranging from 0.2% to 0.6%.[126] Hasaart et al. explored the occurrence of mutations in normal and Down syndrome human fetal hematopoiesis and observed that: in fetal liver hematopoietic stem/progenitor cells, there is an accumulation of about 100 base substitutions which is about two times and 5.8 times higher than in cord and in post-infant hematopoietic stem/progenitor cells, respectively.[127] Most of these mutations are located in introns, a minority in exons, and none are classified as drivers.[127] Interestingly, fetal stem/progenitor cells displayed a higher relative contribution of single base substitution signature 1 compared to post-infant stem/progenitor cells.[127] Campbell et al. have explored the accumulation of somatic mutations in fetal hematopoietic stem/progenitor cells to investigate the dynamics of human prenatal development and the origins of primitive and definitive hematopoiesis, showing that fetal progenitors acquire tens of somatic mutations by 18 weeks after conception.[128]

The heritability of CHIP was explored in a large group of 299 twin pairs ≥70 years: 20 twin pairs had CHIP mutations within the same genes, but the same mutation was observed in only two twin pairs; furthermore, no difference in case wise concordance between monozygotic and dizygotic twins was observed for any gene, subgroup, or CHIP mutations overall, and no significant heritability was detected.[129] One hundred twenty-seven twin pairs were discordant for at least one mutation, and in 48% of these cases, the affected twin died first.[129]

The CHIP study was largely confined to the analysis of somatic variants involving a subset of genes recurrently mutated in myeloid malignancies. However, recently, Niroula et al. hypothesized that clonal hematopoiesis can also be detected in the lymphoid lineage and could represent a condition of increased risk for developing lymphoid malignancies. Thus, they defined a list of 235 genes recurrently mutated in lymphoid malignancies and examined somatic variants in these lymphoid driver genes using wide-exome sequencing data from 46,706 individuals aged 40-70 years with no previous diagnosis of hematologic malignancy in the UK Biobank resource: 1.3% individuals carried variants in one of these lymphoid driver genes and were referred as lymphoid CHIP (L-CHIP).[130] L-CHIP increased with age as well as myeloid-

CHIP (M-CHIP); at variance with M-CHIP, L-CHIP variants were distributed along a wide number of genes whose frequency of occurrence was similar, such as *DUSP22, FAT1, KMT2D, SYNE1, ATM, KMT2C, PCLO, PEN, ARID1A, NEB, MGA*.[33] Importantly, L-CHIP was associated in individuals screened in the UK Biobank with an increased incidence of lymphoid malignancies, particularly evident among individuals with larger clones; importantly, the incidence of lymphoid malignancies was much lower among individuals without CHIP or with M-CHIP; finally, only one individual with L-CHIP developed a myeloid malignancy.[130] In addition, some rare individuals displayed both L-CHIP and M-CHIP: these individuals had a higher frequency of myeloid than lymphoid malignancies.[130]

Clonal hematopoiesis, when measured with a VAF sensitivity of >0.02, is clearly increasing with age; this age-related effect is due to a cell-autonomous mechanism linked to an increase in HSC self-renewal and positive selection.[131] In fact, the mutations in epigenetic regulators, such as *DNMT3A* and *TET2* ,provide an advantage by increasing self-renewal of stem/progenitor cells and thus favoring their expansion, while the mutations in genes involved in DNA damage response may increase cell survival.[131]

CHIP detection in mice could provide an important animal model to explore the mechanisms and physio-pathological consequences of clonal hematopoiesis. To this end, Chin et al. screened for the most common CHIP mutations in 4-month-old wild-type C57BL/6j mice, the most extensively used mouse strain for hematologic studies.[132] Hematopoietic clones with non-synonymous mutations in CHIP genes were only detected in 2% of mice at 24 months. However, in transplanted mice, the CHIP clones expanded: the detection of the same mutations in multiple recipients of the same donor supported the view that CHIP mutations could be acquired early in life.[132] In conclusion, the aged mice cannot provide a suitable model to study human clonal hematopoiesis.

Interestingly, a recent study showed an experimental approach based on CRISPR/Cas9 technology to develop a simple model of clonal hematopoiesis.[133] Site-specific mutations were introduced in specific sites of *ASXL1, DNMT3A,* and *TET2* in $CD34^+$ progenitors derived from umbilical cord blood. The biological effects induced by these genetic modifications were assayed in short-term and long-term cultures, evaluating changes in self-renewal and cell differentiation; *TET2,* but not *DNMT3A* and *ASXL1* mutations induced enhanced self-renewal in short-term cultures; all the three mutants and particularly the combined three mutants elicited a clear increase of self-renewal, as evidenced by long-term culture experiments.[133] In addition, the analysis of clonal expansion after long-term culture showed a mutation-specific impact on stem/progenitor cells.[133]

The study of long-term survivors of allogeneic stem cell transplantation grafted with CHIP-positive donors offers the unique opportunity to explore the expansion of CHIP clones during hematopoietic reconstitution. Boettcher et al. have studied 5 of these patients exhibiting donor-engrafted CHIP: 4/5 cases displayed increased CHIP clones' size in recipients compared with donors, as measured by VAF; CHIP mutations were constantly found in the myeloid lineage, but with variable penetrance in the B and T lymphoid lineages; telomere shortening was observed in granulocytes, supporting a proliferative activity of hematopoietic stem cells.[134] Wong et al. have made similar observations in a group of patients transplanted with younger unrelated donors: some rare clonal mutations engrafted in recipients and persisted over time.[135] Other studies have evaluated whether the presence of CHIP-related mutations after either autologous[136] or allogeneic matched sibling transplantation[137] influences transplant outcomes. Both studies concluded that the presence of CHIP did not affect transplant outcomes, including the time to hematopoiesis recovery, relapse incidence, transplant-related mortality, and progression-free and overall survival.[136,137] However, the risk of acute graft versus host disease was higher in allogeneic CHIP-positive donors compared to CHIP-negative donors.[136,137]

In conclusion, these studies showed that engraftment, repopulation, and long-term survival with donor CHIP are possible; after transplantation, survival of hematopoietic cells with donor CHIP is only modest and associated with a low clonal expansion in recipients. However, it cannot be excluded that some CHIP-related variants could be associated with increased clonal fitness. It is important to note that one of the five patients reported in the study of Boettcher et al.[134] developed a myelodysplastic syndrome. Particularly, one donor-recipient pair developed MDS, diagnosed 18 and 21 years posttransplant, respectively, and in both cases, derived from a shared founding clone.[134] Nevejan et al. reported four subjects of CHIP-positive donor cell-derived hematologic neoplasms (DCHN) in 263 HLA identical sibling transplantation: the recipient patients, but not the donors, developed myeloid neoplasia 17-20 years posttransplant; a higher VAF of the pathogenic variant was observed in recipients compared to the donors; a variable presence of driver variants in CHIP was observed.[138] These observations show that malignant progression of donor-engrafted clonal hematopoiesis in sibling recipients may occur many years after stem cell transplantation.[138]

Chromosomal alterations in clonal hematopoiesis. In addition to variants related to point mutations, other studies have defined the presence of chromosomal

alterations observed in clonal hematopoietic cells. This condition, called age-related mosaic chromosomal alterations (mCAs), and observed in DNA derived from blood elements and related to clonal structural somatic alterations (deletions, duplications, or copy number neutral loss of heterozygosity), present in a more or less small fraction of peripheral leukocytes, can indicate the existence of a condition of clonal hematopoiesis. Two pivotal studies in 2012, based on the analysis of DNA purified from peripheral blood of normal individuals, reported a frequency of chromosomal mosaic abnormalities (duplications, deletions, copy-neutral loss of heterozygosity) increasing with age: <0.5% before 50 years and rising to 2-3% at >70 years.[139,140]

Recent studies have characterized mCAs in British and Japanese normal populations. The characterization of the British population was carried out on the basis of DNA analysis of 151,202 UK Biobank participants; 4.94% of individuals displayed mCAs: most detected mCAs (70%) were present at inferred fractions <5%.[141] The detected mCAs were classified as loss or gain or copy-number neutral loss of heterozygosity. The most common events were the loss of chromosome X in females and the loss of chromosome Y in males; commonly deleted regions <1 MB involve tumor suppressor genes; focal deletions most frequently involved *DNMT3A, TET2* and 13q14; gains on chromosome 15 were much more frequent in elderly males, while 16p11.2 deletions and 10q terminal deletions were more frequent in females.[141] mCAs displayed associations with germline variants on the same chromosomes. CNN-LOH events on chromosome 1p are strongly associated with rare risk haplotypes at the level of the *MPL* proto-oncogene at 1p34.1; CNN-LOH events on chromosome 11q are associated with a rare risk haplotype surrounding the *ATM* gene at 11q22.3; CNN-LOH and loss events at chromosome 15q are associated with a rare inherited 70-kb deletion spanning the *TM2D3* and *TARSL2* genes at 15q26.3.[141] Terao et al. have reported the results of a study based on the analysis of mosaic chromosomal alterations in a population of 179,417 participants in the Japan BioBank.[142] This analysis detected a frequency of 15.5% of individuals with mCAs; detectable mosaicism reached 40.7% in men and 31.5% in women over 90-y, thus suggesting that mCAs are almost inevitable in elderly individuals.[142] This study highlighted the existence of several remarkable differences between Japanese and European individual in the frequency of some mCAs: mosaic deletion of the *TRA* locus on chromosome 14q, indicating clonal expansion at the level of the T cell lineage, were common in the Japanese individuals but rare in the European individuals (82% vs. 11%) and deletions at the *IGH* and *IGL* immunoglobulin loci, indicating clonal expansion at the level of the B cell lineage, were common in the European but rare in Japanese individuals (39% vs. 5% on chromosome 14 and 58% vs. 2% on chromosome 22); the three mCAs, chromosome 12 gain, 13q loss, and 13q CN-LOH, events commonly observed in CLL and in individuals who later develop CLL, are less frequent in Japanese individuals than in European individuals, in line with the lower incidence of CLL in the former ones compared to the latter ones.[142]

Zekavat et al. reported the results of a very large study based on the analysis of mCAs from 768,762 individuals from 5 biobanks without hematological cancer at the time of DNA acquisition.[143] These authors explored the association of mCAs with hematological traits providing evidence that autosomal mCAs and loss of chromosomes X and Y are significantly associated with altered hematologic features.[143] Particularly, autosomal mCAs are related to elevated lymphocyte and white blood cell leukocyte counts; loss of chromosome Y with increased white blood cell leukocyte counts, neutrophil, monocyte, and platelet counts; loss of chromosome X with increased white blood cell leukocyte counts, lymphocyte, monocyte, and neutrophil counts.[143] Autosomal mCAs were more associated with an increased risk of hematological cancer (particularly lymphoid leukemia) than the loss of chromosome Y.[143] Loss of chromosome X was associated with an increased risk of CLL, lymphoid leukemia, and AML development.[143]

As mentioned above, the loss of the Y chromosome is the most common chromosome abnormality in the hematopoietic lineage of aging men. Peripheral leukocytes often exhibit mosaic loss of chromosome Y in aging men, with an estimated frequency of >40% in the UK BioBank.[144] The origin of the loss of the Y chromosome may be explained by chromosome mis-segregation events during mitosis.[144] A recent study provided evidence that 75% of men with loss of chromosome Y also carried mutations in genes typically associated with CHIP.[145] The study of the mutational profile of the monocytes of 26 individuals with loss of chromosome Y showed frequent CHIP pathogenic variants in *TET2, DNMT3A, SF3B1, ASXL1* and *TP53* genes; furthermore, *BCOR, ZRSF2, BCORL1, FBWW7, FLT3* and *GATA2* gene resulted also to be frequently mutated.[145] Another recent study showed that patients with chromosome Y loss in their bone marrow cells often have mutations of *DNMT3A, TET2* and *ASXL1* genes.[146] The analysis of bone marrow cells of elderly subjects with loss of Y chromosome showed that individuals with ≥75% of metaphases with LOY have a greater likelihood of having myeloid-associated mutations and a higher risk of developing myeloid neoplasia.[146] Interestingly, a recent study reported the results of the study of the association of mLOY with alterations in blood cell counts in a large cohort (206,353 UK males) of UK BioBank men: associations between mLOY and reduced erythrocyte count, elevated platelet count, elevated

leukocyte count (particularly for neutrophils and monocytes and less for lymphocytes); these associations were independent of the effects of aging and smoking.[147]

Some recent studies have provided a fundamental contribution to the study and to the understanding of clonal hematopoiesis through the combined study of single-nucleotide variants and copy number alterations.[130,148] Saiki et have investigated the occurrence of CHIP-related gene mutations by targeted NGS and CNAs by gene array in 11,234 Japanese individuals (in large part aged ≥60 years) without hematological malignancies from the BioBank Japan cohort, including 672 individuals with subsequent development of hematological malignancy and have studied the effects of these genetic alterations on hematological phenotypes, including the development of hematological malignancies.[148] 27.3% of these individuals exhibited CHIP-related mutations: 20.6% with one mutation, 5.2% with two mutations and 1.5% with at least three mutations; the most frequent alterations were *DNMT3A* (13.5%), *TET2 (9.5%), ASXL1* (2.2%) and *PPM1D* (1.4%).[28] CNAs, including CNN-LOH or uniparental disomy (UPD), were detected in 20.1% of cases, of which 3.7% exhibited multiple CNAs; 14qUPD, +21q, del(20q), and +15q were frequent CNAs, whereas del(20q), 16pUPD and 17pUPD were associated with the largest mean clone size.[148] The evaluation of the combined occurrence of CNAs and CHIP mutations showed a frequency of 40% in individuals ≥60 years and 56% of cases who developed hematological malignancies.[28] 16% of all individuals displaying clonal hematopoiesis have both types of genetic lesions: mutations in *TP53, TET2, JAK2, SF3B1,* and *U2AF1* were more frequently accompanied by concomitant CNAs; the maximum clone size in clonal hematopoiesis-positive cases correlated with the total number of CHIP mutations and CNAs.[148] Interestingly, in some cases, multiple co-occurring lesions were estimated to be present in the same large clone. In other cases, the mutations and CNAs affect the same genetic loci.[148] In addition to age, other factors affected CHIP mutations and CNAs: *ASXL1, PPM1D, TP53,* splicing factors and CNAs, including +15,del(20q), +21,and 14qUPD correlated with male gender and smoking.[148] Furthermore, individuals with high platelet counts have a higher frequency of *JAK2* mutations and 9pUPD, while individuals with cytopenia of any type display frequent *U2AF1* mutations and del(20q).[148] Both CHIP mutations and CNAs were significantly associated with higher mortality deriving from hematological malignancies; hematological-related mortality in clonal hematopoiesis-positive individuals was more attributable to myeloid than to lymphoid neoplasms; the highest risk of hematological mortality was related to *U2AF1, EZH2, RUNX1, SRSF2* and *TP53* mutations and +1q CNA.[148] Importantly, the presence of both CHIP mutations and CNAs was associated with increased hematological-related mortality compared with that of CHIP mutations or CNAs alone; this combined effect seems to be largely related to the total number of alterations rather than by the type of alterations.[148] The results of this study underscore the importance of measuring both lesions for an accurate evaluation of the risk of developing a hematological malignancy in individuals with clonal hematopoiesis.

Niroula et al. have analyzed CHIP mutations in 55,383 individuals and autosomal mCAs in 420,969 individuals with no history of hematologic malignancies in the UK BioBank and Mass General Brigham BioBank.[130] As discussed above, this study distinguished myeloid and lymphoid somatic gene mutations at the CHIP level; furthermore, this study used a similar strategy to differentiate myeloid and lymphoid mCAs. Thus, to categorize mCAs, in a group of individuals with a hematologic malignancy, mCAs have been defined as M-mCA (0.38% in the whole population) or L-mCA (0.83%) according to their differential prevalence in myeloid or in lymphoid malignancies; mCAs common to both malignancies were defined as ambiguous (A-mCA, 0.32%); a significant number of mCAs (2%) cannot be classified.[130] The presence of M-mCA increased the risk of myeloid malignancies, L-mCA increased the risk of lymphoid malignancies, and A-mCA increased the risk of both lymphoid and myeloid malignancies.[130] Frequencies of myeloid malignancies, such as acute myeloid leukemia, myelodysplastic syndrome, and myeloproliferative neoplasms, are higher among individuals with M-CHIP and M-mCA; L-CHIP and L-mCA are associated with increased risk of chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL) and also with follicular lymphoma and diffuse large B-cell lymphoma (DLBCL); the annual rate of myeloid malignancies increased from 0.02%-0.03% among individuals with no clonal hematopoiesis to 0.17% and 0.82% among individuals with M-CHIP and M-mCA, respectively and the annual rate of lymphoid malignancies increased from 0.01% among individuals without clonal hematopoiesis to 0.22% and 0.6% among individuals with L-CHIP and L-mCA, respectively.[130] Some individuals exhibited concomitant M-CHIP and M-mCA and others concomitant L-CHIP and L-mCA; these individuals showed an increased tendency to develop myeloid and lymphoid malignancies, respectively, compared to those with a single type of genetic alteration.[130] Interestingly, in a significant proportion of cases M-CHIP and L-CHIP, mCAs (mostly CNN-LOH) overlapping the mutated genes were present, resulting in biallelic variants in specific driver genes.[130] Analysis of standard hematological parameters showed that patients with both abnormal myeloid cell parameters and M-CHIP/M-mCA alterations and with both abnormal lymphoid cell parameters and L-CHIP/L-mCA

alterations exhibit a particularly increased risk of developing myeloid and lymphoid malignancies, respectively.[130]

482,378 individuals from the UK biobank were investigated for the coexistence of different types of clonal hematopoiesis: LOX, LOY, autosomal mCAs (gains, losses, and copy neutral LOH), CHIP, and MPN (myeloproliferative neoplasia).[149] Positive phenotypic associations between autosomal mCAs with LOY, LOX, CHIP, and MPN were observed; CHIP was positively associated with MPN but negatively associated with LOY; individuals with higher cellular fractions with autosomal mCA events have a greater probability of also having LOX, CHIP and MPN; higher autosomal mCA cellular fractions were inversely associated with LOY; individuals with higher VAF of CHIP have more chances to have detectable autosomal mCAs.[149] Furthermore, 6.4% of individuals with CHIP and 5.3% of individuals with autosomal mCAs displayed both CH types: these individuals display a peculiar pattern of co-occurrence of either CHIP mutations or mCAs; some of these individuals show co-localization of mutational and CA events at the level of *TET2, DNMT3A* and *JAK2:* the analysis of the VAF of CHIP mutations and the cellular distribution of mCAs suggests that the acquisition of the CHIP mutation preceded the acquisition of autosomal mCAs.[149] Genome-wide studies suggested genetic correlations between LOY and LOX and between LOY and MPN, suggesting the existence of shared biologic mechanisms promoting or predisposing clonal expansion development.[149]

The presence of an unexplained alteration in a hematological trait must be explored at the molecular level for its possible association with clonal hematopoiesis. Van Zeventer et al. examined a cohort of 144,676 adults for the presence of monocytosis (defined as monocytes $\geq 1x10^9$/L and ≥10%), not associated with known pathological conditions.[150] Among individuals ≥60 years, the prevalence of monocytosis was 0.8% and increased with age; these older individuals with monocytosis displayed more frequently CHIP than age-matched individuals without monocytosis (50.9% vs. 35.5%, respectively).[150] Monocytosis has been associated with more frequent multiple gene mutations and mutations in spliceosome genes but not of isolated mutated *DNMT3A, TET2* and *ASXL1*.[150] Myeloid malignancies developed in 4 individuals with monocytosis, all displaying CHIP.[150]

Germline Risk of Clonal Hematopoiesis and Rare Inherited and Not-Inherited Diseases Associated with Clonal Hematopoiesis Expansion. Studies carried out in the last years have provided evidence that inherited factors shape the incidence of clonal hematopoiesis.[151] The simultaneous analysis of germline and somatic genetic variation on a population scale has shown some heritable germline susceptibility to CHIP, mCA and mLOY.[151] The most remarkable finding emerging from these studies is that the DNA damage response and telomere maintenance pathway genes are commonly implicated in genetic association with clonal hematopoiesis subtypes.[151] Bick et al. performed the widest population screening of the inherited causes of clonal hematopoiesis in a population of 97,691 individuals analyzed by whole-genome sequencing; they identified 4,229 individuals with CHIP.[152] Three main germline genetic determinants of CHIP were identified: germline genetic variants at the *TERT* locus were identified as predisposing to clonal hematopoiesis; one locus on chromosome 3 in an intergenic region spanning *KPNA4* and *TRIM59;* one locus on chromosome 4 near *TET2*.[152]

Recent studies have evidenced a consistent contribution of clonal hematopoiesis in the genesis of several rare inherited or not-inherited conditions associated with frequent myeloid neoplasms.

Erdheim Chester Disease (ECD) is a rare histiocytic neoplasm classified as a macrophage dendritic neoplasm in the 2016 hematopoietic and lymphoid tumors classification. About 50% of ECD patients display a $BRAF^{V600E}$ mutation, and the rest usually have other activating mutations in the MAPK pathway. As a result, about 10% of ECD patients develop myeloid neoplasms. A recent study by Cohen Aubart et al. reported the targeted NGS of bone marrow cells of 120 ECD patients: mutations associated with CHIP were identified in 42.5% of patients; the most frequent mutations were *TET2* (22%), *ASXL1* (9%) and *DNMT3A* (8%).[153] In addition, 15% of these patients developed myeloid neoplasms: 31% of ECD patients with CHIP developed a myeloid neoplasia, compared to 3% among those without CHIP; 89% of patients who developed a myeloid neoplasia have CHIP, compared to 34% among those who did not develop myeloid neoplasia.[153]

DNMT3A overgrowth syndrome (DOS), also known as Tatton-Brown Rahman Syndrome (DOS), is one of the overgrowth syndromes caused by constitutional mutations in genes encoding epigenetic regulators and characterized by complex phenotypes. DOS patients had *de novo* heterozygous germline mutations in *DNMT3A*, missense, frameshift, and nonsense mutations, all involving the functional domain of DNMT3A protein. The clinical features of DOS include overgrowth and intellectual disability. The analysis of the 200 known DOS patients worldwide showed that 8 of these patients developed hematological malignancies.[154] The study of DNA methylation in peripheral blood cells of DOS patients showed that heterozygous *DNMT3A* mutations induce a focal hypomethylation phenotype, most severe with the dominant negative $DNMT3A^{R882H}$.[155] A very recent study reported a detailed analysis of the hematological phenotype of individuals with DNMT3A

overgrowth syndrome, characterized by the presence of nonanemic RBC macrocytosis, a relative decrease in peripheral blood lymphocytes (with an increase of the CD4/CD8 ratio), and monocytes and an increase in neutrophils; this hematological phenotype was recapitulated in murine models of DNMT3A overgrowth syndrome.[156]

Shwachman-Diamond Syndrome (SDS) is a genetic disorder (ribosomopathy) associated with a high risk of developing myeloid neoplasia early in life; this disorder is due to a biallelic mutation in the *SBDS* gene encoding the SBDS protein required for the formation of the translationally active 80S ribosome. Recent studies have clarified the mechanisms responsible for the frequent generation of myeloid neoplasms in these patients. Thus, Xia et al. have explored clonal hematopoiesis's occurrence in different types of congenital neutropenia, including SDS.[157] CHIPs were observed in 59% of SDS cases; mutations of *TP53* were observed in 48% of SDS CHIPs, while mutations of this gene were undetectable in the CHIPs of controls or of cyclic neutropenia or severe congenital neutropenia patients.[157] *TP53* mutations were present in a low fraction of bone marrow cells, and their presence correlates with the age of patients.[157] Kennedy et al. performed a detailed molecular characterization study of a large cohort of 110 SDS patients. The analysis of bone marrow cells of 86 patients without diagnosis of myeloid neoplasia provided evidence of CHIP in 72% of cases: the majority of these CHIP-positive patients displayed more than one mutation; recurrent mutations in *EIF6* (59%), *TP53* (40%), *PRPF8* (10.8%), *CSNK1A1* (7.2%) were observed.[158] It was proposed that germline *SBDS* mutations induce a fitness constraint that determines the selection of somatic clones through two distinct mechanisms: *EIF6* mutations induce EIF6 inactivation with consequent amelioration of the ribosome defect of SDS with no effects on leukemic transformation; *TP53* mutations drive a maladaptive pathway with leukemic potential through inactivation of tumor suppressor checkpoints without correcting the ribosome defect.[158] Leukemia development in these patients was associated with acquiring biallelic *TP53* alterations.[158] Furutani and co-workers reported the study of 153 subjects with SDS and explored the hematologic phenotype of these patients, particularly for that concerns the hematologic complications: (i) absolute neutrophil counts were positively associated with age; (ii) platelet counts and bone marrow cellularity were negatively associated with age; (iii) severe marrow failure necessitating transplantation was observed in 8 patients; (iv) 17% of patients developed a malignancy (16 MDS and 10 AML) at a median age of 12.3 years; (v) 1 patient developed a lymphoid malignancy.[159]

Sterile Alpha Motif Domain 9 (*SMAD9*) and its paralogue *SAMD9*-like (*SAMD9L)* are two genes located on chromosome 7q21, encoding two cytoplasmic proteins. The function of these two genes seemingly originated from a common ancestral gene duplication, remains enigmatic. Germline *SAMD9* and *SAMD9L* cause a variety of multisystem syndromes with a propensity for cytopenia, bone marrow failure, and an elevated risk of early-onset myeloid neoplasms, particularly myelodysplastic syndromes.[160-162] Germline *SAMD9* and *SAMD9L* mutations predispose to myelodysplastic syndromes. Schwartz, in an extensive study of evaluation of the genomic landscape of pediatric MDSs, showed that germline variants in *SAMD9* or *SAMD9L* were present in 17% of primary MDS patients; these variants were lost in the tumor mechanism by a rescue mechanism involving either chromosomal deletions though monosomy 7 or copy number neutral loss of heterozygosity (CN-LOH).[163]

Another recent study further supported the existence of rescue mechanisms essential for the leukemic transformation of *SAMD9* or *SAMD9L* germline mutated cells; in fact, it was shown that 61% of these patients undergo somatic genetic rescue, resulting in clonal hematopoiesis involving both maladaptive (monosomy 7) and adaptive (isodisomy 7q) mechanisms.[164] Bone marrow single-cell DNA sequencing showed multiple competing somatic genetic rescue events in individual patients.[164]

A recent study showed that SMAD9 and SMAD9L are multifunctional proteins that determine alterations in cell cycle, cell proliferation, and protein translation in hematopoietic stem/progenitor cells; mutant *SAMD9* or *SAMD9L* induce the generation of a cellular environment that causes DNA damage repair defects.[165]

Clonal Hematopoiesis in Cancer. Clonal hematopoiesis increases with age and is a condition that predisposes to hematologic malignancies. In addition, many studies have shown that clonal hematopoiesis occurs with higher frequency in individuals with lymphoid and solid tumors and increases following exposure to genotoxic stress. Furthermore, clonal hematopoiesis may represent a major determinant for the risk of therapy-related neoplasms (t-MN), comprising therapy-related acute myeloid leukemia and myelodysplastic syndrome, as late complications of cytotoxic therapy, chemotherapy, and/or radiotherapy, used in the treatment of both malignant and non-malignant diseases.

The terrorist attack on the World Trade Center (WTC) in 1993 generated a unique environmental exposure to aerosolized dust, gases, and potential carcinogens in a small population of individuals; it represented a peculiar opportunity to explore the effect of potential environmental carcinogens in clonal hematopoiesis development.[166] Deep targeted sequencing of blood samples showed a significantly

higher proportion of individuals with CHIP among WTC-exposed first responders compared to non-WTC-exposed firefighters after controlling for age, sex, and race/ethnicity (10% vs. 6.7%, respectively).[166] CHIP mutations predominantly affected *TET2* and *DNMT3A*.[166]

In 2017, Coombs et al. reported the analysis of paired tumor and blood samples derived from 8,810 individuals with non-hematological malignancies by deep-coverage, targeted NGS; in these patients, clonal hematopoiesis was identified in 25% of cases and was associated with increased age, tobacco smoking and prior radiotherapy.[167] In these patients, clonal hematopoiesis was associated with increased white blood cell counts, increased absolute monocyte and neutrophil counts, increased mean corpuscular volume, and decreased platelet count.[167] The most frequently mutated genes at the level of clonal hematopoiesis were *DNMT3A, TET2, PPM1D, ASXL1, ATM,* and *TP53*; *TP53* and *PPM1D* mutations were significantly associated with prior exposure to cancer chemotherapy.[167] Clonal hematopoiesis in these patients was associated with an increased incidence of subsequent hematologic cancers.[167] These findings were confirmed in a larger analysis involving 17,469 cancer patients: clonal hematopoiesis-associated mutations were observed in 26.5% of these patients, mostly involving *DNMT3A, TET2,* and *PPM1D*; clonal hematopoiesis was most frequent among skin cancer and non-small cell lung cancer patients.[168]

Clonal hematopoiesis mutations can be tentatively identified in unpaired NGS assays of tumor samples of solid tumors, but these mutations putatively related to clonal hematopoiesis must be confirmed by paired blood sequencing.[169,170]

Gao and coworkers reported the results of a combined analysis of mCAa and CHIP mutations in a cohort of 32,442 cancer patients: the incidence of mCAs was low, increasing with age from under 1% in individuals under 50 years to more than 3% among individuals more than 80 years old; mCAs were most frequently observed among cancer patients with soft tissue sarcoma, thyroid and lung cancer; mCA was positively associated with external beam radiation therapy but not with cytotoxic treatment.[171] 63% of clonal hematopoiesis cases with mCAs co-occurred with at least one gene mutation, particularly with high mutation number and VAF.[47] CHIP mutations were observed in 30% of these cancer patients. mCAs displayed a peculiar pattern of co-occurrence with gene mutations, showing mechanisms of cooperation between chromosomal alterations and gene mutations through *cis* (biallelic inactivation) and *trans* (functional cooperation) mechanisms.[171] These patients exhibited clonal hematopoiesis with composite genotypes, a subgroup associated with a high risk of developing a hematologic malignancy.[171]

Other studies have evaluated the effects of anticancer treatments (chemotherapy, radiation therapy, targeted therapy, and immunotherapy) on clonal hematopoiesis. Furthermore, it was investigated a possible contribution of clonal hematopoiesis to the generation of therapy-related myeloid neoplasms (t-MN), a condition that occurs after treating primary malignancies using chemotherapy and/or radiation therapy or after immunosuppression for solid organ transplant or autoimmune disease.[172,173] Two recent studies have explored the evolution of hematopoietic cells under cancer therapy, exploring the mutational footprints induced by cancer therapies.[174] Cells of AMLs secondary to treatment with platinum-based drugs show the mutational footprint typical of these drugs. These mutations also appeared in non-malignant cells at the drug exposure time. Furthermore, platinum drugs produce a mutagenic effect of the same magnitude in both normal and tumoral cells.[175] In contrast, no trace of the 5-fluorouracil mutational signature is found in AMLs secondary to exposure to 5-FU, suggesting that cells establishing the leukemic process could be quiescent during treatment (in fact, 5-FU-induced mutations appear only in cells that are nor quiescent during drug exposure).[175] In a second study, using wide genome sequencing therapy-related myeloid neoplasias (tMN) samples from patients exposed to various anti-cancer drugs and pre-therapy samples, have characterized the mutational impact of chemotherapy and used chemotherapy-induced signatures (CIS) as a temporal barcode to reconstitute tumor evolution.[176] tMNs evolving without CISs display features similar to those observed in *de novo* AMLs. In contrast, tMNs with CIS, such as those occurring in platinum- or melphalan-treated patients, are hypermutated and more frequently associated with complex genomes, including chromothripsis and copy number alterations.[176] Interestingly the procedure of treatment involving high-dose melphalan and ASCT allows clonal hematopoiesis to escape chemotherapy exposure and to be reinfused to expand to malignancy; these patients can develop tMN bearing no evidence of melphalan-induced mutations.[176]

TMNs are molecularly heterogeneous and are represented by tMDSs and tAMLs; the majority of tMNs display chromosomal abnormalities associated with MDS or *KMT2A* rearrangements.[177] At molecular level, the majority (about 80%) of these tMNs display an abnormal karyotype, while a minority (about 20%) is associated with a normal karyotype; TMNs with normal karyotype compared to those with abnormal karyotype are enriched for mutations in *TET2, NPM1, ASXL1, SRSF2, RUNX1* and *STAG2,* many genes frequently mutated in CHIP.[177]

Clonal hematopoiesis in lymphoma. A consistent number of studies explored the occurrence of CHIP in patients

with various types of lymphoma or myeloma. Wong et al. investigated 119 patients with a history of malignancy (lymphoma and myeloma): 81 received previous chemotherapy with or without radiation, and they were explored by error-corrected NGS for 46 genes associated with clonal hematopoiesis.[178] 28.4% of patients exposed to cytotoxic therapy displayed clonal hematopoiesis possessing at least one mutation with VAF ≥2% and 82.7% with VAF ≥0.1%; the incidence of clonal hematopoiesis, as well as the number of genetic variants, is higher in patients receiving cytotoxic therapy compared to those who not received it and to the healthy controls.[178] The analysis of the CHIP mutational spectrum showed several remarkable differences between patients who received cytotoxic therapy and those not receiving these treatments. While the frequency of *DNMT3A* and *TET2* mutations was similar in these two groups, *TP53* and *PPM1D* mutations displayed a markedly higher frequency in patients who received cytotoxic therapy compared to those who did not receive it.[178] In addition, 73% of patients with clonal hematopoiesis after cytotoxic therapy displayed multiple variants; 59% had multiple variants in the same gene, 31% had multiple variants in DNA damage response genes, and 26% had three or more variants.[178] In these patients, *DNMT3A, PPM1D* and *TP53* mutations are present in both myeloid and lymphoid elements, suggesting their origin from hematopoietic stem cells.[178] In a set of 40 of these patients undergoing autologous stem cell transplantation, it was evaluated the effect of transplantation on clonal hematopoiesis expansion; two gene variants, *DNMT3A* and *PPM1D*, were mostly modulated by transplantation: of 51 *DNMT3A* variants, 33% significantly increased ≥ 2-fold in VAF after transplantation, while 6% decreased; of 23 *PPM1D* variants, only about 9% increase in VAF after transplantation, while 30% decreased.[178] Finally, the leukemogenic potential of expanded *TP53* mutant hematopoietic clones was higher than that of clones bearing mutations at the level of other DNA damage response genes, such as *PPM1D*.[178]

Husby et al. explored 565 lymphoma patients undergoing autologous stem cell transplantation in the context of transplant centers in Denmark: 25% of these patients displayed at least one CHIP mutation; overall, patients with CHIP did not reveal an inferior survival; however, those with mutations in DNA repair genes, such as *PPM1D, TP53, RAD21* and *BRCC3* showed a reduced overall survival.[179]

Gibson et al.[180] explored the occurrence of clonal hematopoiesis mutations in the blood of 401 lymphoma patients (Hodgkin and non-Hodgkin lymphoma) undergoing autologous stem cell transplantation (ASCT): about 30% of these patients displayed in peripheral blood clonal hematopoiesis-associated mutations; 69% of these patients showed 1 mutation and 31% ≥ 2 mutations.[180] In these patients, mutations in *PPM1D* and *TP53* are much more frequent compared to the values reported for aged individuals: *PPM1D* and *TP53* mutations accounted for 32% and 11% of all the clonal hematopoiesis-related mutations observed in these patients.[180] Importantly, patients with CHIP, particularly those with CHIP bearing PPM1D exhibited a shorter overall survival than those without CHIP.[180] In another study, Eskelund et al. evaluated a homogeneous cohort of 149 mantle cell lymphoma (MCL) patients undergoing ASCT and achieving a condition of minimal residual disease negativity (MRD^-): CHIP clones consistently expanded during chemotherapy and ASCT and stabilized after the end of therapy; no clinical impact of CHIP in this cohort of good-prognosis patients was observed; 98% of CHIP mutations were already detectable before exposure to any chemotherapy.[181]

Few studies have explored CHIP in Hodgkin lymphoma (HL) patients. Husby et al. reported the occurrence of CHIP in 14% of HL patients undergoing ASCT.[179] Venanzi explored the presence of CHIP in 40 HL patients and observed positivity in 12.5% of cases.[182] Massive genome sequencing of tumor tissue showed that only one of the five CHIP-positive patients, mutant clonal hematopoiesis seeded the neoplastic clone.[182]

Saini et al. explored the occurrence of clonal hematopoiesis among 114 patients with large B-cell lymphoma treated with anti-CD19 CART cells; 36.8% of these patients displayed CHIP most frequently mutated in *PPM1D* and *TP53* genes.[183] CHIP-positive patients had a more frequent incidence of immune-effector cell-associated neurotoxicity syndrome (ICANS) than CHIP-negative patients (45% vs. 25%) Higher incidence of ICANS and cytokine release syndrome were observed among patients with CHIP associated with *DNMT3A, TET2* and *ASXL1* gene mutations.[183] In addition, the cumulative 24-h incidence of tMN was significantly higher in CHIP-positive than in CHIP-negative patients (19% vs. 4.2%, respectively).

The presence of CHIP in lymphoma patients may also be associated with drug-associated toxicity. Thus, TET2-clonal hematopoiesis was observed in 8.2% of patients with lymphoma; importantly. In addition, TET2-CH was associated with anthracycline-induced cardiotoxicity.[184]

The exploration of CHIP in patients with angioimmunoblastic T-cell lymphoma (AITL) is particularly interesting in that these tumors harbor frequent mutations in the epigenetic modifiers *TET2, DNMT3A,* and *IDH2* and in the small GTPase *RHOA*.[185] In a cohort of 25 patients with AITL, it was found that 15/22 displayed CHIP: in these patients, identical mutations were detected in the neoplastic T cells and in myeloid compartments, including *DNMT3A* and *TET2* mutations; four of these patients developed myeloid neoplasia, all of which shared CHIP-type mutations with their AITL tumor counterpart.[185] These observations

indicate that CHIP is prevalent in AITL patients and can evolve to AITL and MNs.[185]

Blastic plasmacytoid dendritic cell neoplasm (BPDCN) is an example of another lymphoma condition associated with prevalent CHIP incidence. BPDCN is an aggressive tumor with features of cutaneous lymphoma and/or leukemia; 20% of these patients displayed MDS or chronic myelomonocytic leukemia with a shared clonal origin.[186] NGS on bone marrow cells, skin, or sorted cells provided evidence of mutations in BPDCN (100% of cases) and in bone marrow hematopoietic cells (65% of cases), with similar high frequencies of *TET2* (58% vs. 60%) and *ASXL1* (40% vs. 33%) mutations; karyotypic abnormalities were frequently observed in BPDCN (66%) but only very rarely in BM hematopoietic cells (2%).[186] These observations support a high prevalence of bone marrow clonal hematopoiesis in BPDCN.[186]

Clonal hematopoiesis in multiple myeloma. Several studies have evaluated CHIP occurrence in multiple myeloma (MM) patients. However, only two studies have characterized large sets of MM patients. One of these two studies was based on the analysis of the hematopoietic stem cell products of 629 MM patients treated with autologous stem cell transplantation at Dana Farber Cancer Institute: using deep targeted sequencing (VAF≥1%) observed a CHIP prevalence corresponding to 21.6%.[187] Recurrently mutated genes in these patients were *DNMT3a, TET2, TP53, ASXL1* and *PPM1D*.[187] In patients not receiving immunomodulatory drug maintenance (IMDM), the presence of CHIP is associated with decreased overall survival due to an increase in MM progression; in patients receiving IMDM, drug treatment improved overall survival and progression-free survival, regardless of CHIP status.[187] The second study was based on the analysis of MM patients enrolled in PETHEMA and GEM2012MENOS65 trials. At diagnosis, about 10% of MM displayed MDS-associated phenotypic alterations (MDS-PA) and 11.6% monocytic MDS-PA.[188] Bulk and single-cell sequencing studies of CHIP-associated genes at the level of $CD34^+$ progenitors showed the occurrence of CHIP in 50% of cases with MDS-PA and 22% in those without MDS-PA; *TET2* and *NRAS* were the most recurrently mutated genes.[188] MDS-PA at diagnosis predicted an increased risk of hematopoietic toxicity and was independently associated with shorter PFS and OS.[188]

Waldenstrom macroglobulinemia (WM) is an indolent NHL characterized by immunoglobulin M-secreting lymphoplasmacytic cells and mutations at the level of genes involved in B-cell signaling. A recent study showed that CHIP was identified in 14% of patients with IgM monoclonal gammopathy of undetermined significance (MGUS), smoldering WM (SWM), or WM; the most recurrent mutations involved *DNMT3A, TET2* and *ASXL1* genes.[189] In addition, patients with CHIP had an increased risk of progression from MGUS to SWM to WM but not worse overall survival.[189]

Clonal hematopoiesis in solid tumors. Several studies have characterized in patients with solid tumors: (i) the association between the presence of some mutant genes in CHIP and the response to chemotherapy; (ii) the influence of various chemotherapy treatments on the mutations observed at the level of CHIP and of t-MNs.

PPM1D mutations were observed in pMNCs of patients with solid cancers, such as breast, colon, ovarian, and lung cancer. Thus, Zajkowicz et al. reported frameshift *PPM1D* mutations in 0.92% of non-small cell lung cancer patients; all the positive patients had squamous lung cancer (1.5% of positive patients).[190] Pharoah et al. reported *PPM1D* mutations in 0.37% of ovarian cancer patients at the level of lymphocyte DNA: all positive cases corresponded to patients analyzed after chemotherapy treatment.[191] Studies carried out in ovarian cancer patients showed that *PPM1D* mutations detected in peripheral blood mononuclear cells were significantly associated with prior chemotherapy and, in patients treated with chemotherapy, with older age.[192] Interestingly, in these patients, *TP53* mutations were not related to previous chemotherapy and age.[192]

Truncating mutations in the terminal exon of *PPM1D* induce a chemoresistance phenotype that results in the selective expansion of *PPM1D*-mutant hematopoietic cells in the presence of chemotherapy; this finding explains the clonal expansion of *PPM1D*-mutant cells.[193]

A recent study explored a very large set of cancer patients (24,439 individuals) and observed CHIP in 30% of these patients: 68% of these patients had one mutation in CHIP, and 32% had two or more mutations; the most frequently mutated genes were the epigenetic regulators *DNMT3A* and *TET2* and the genes involved in DNA Damage Response (DDR) pathway, including *PPM1D, TP53,* and *CHEK2*; 90% of the mutations observed in CHIP were classified as driver myeloid mutations.[194] The spectrum of gene mutations observed in CHIP was similar in different cancer types, with the exception of DDR gene mutations, particularly of the *PPM1D* gene, which were enriched in ovarian and endometrial cancers.[194] The presence of specific gene mutations was associated with some pathogenic events: (i) mutations of the spliceosome genes *SRSF2* and *SF3B1* were less frequent than other CH mutations and are associated with age; (ii) CHIP mutations in the DDR genes *TP53, PPM1D* and *CHEK2* were strongly associated with prior oncologic therapy (mutations in *PPM1D* were mainly associated with previous exposure to platinum and radionuclide therapy, but also with topoisomerase II inhibitors and taxanes; mutations in *TP53* were

associated with previous platinum, radiation therapy, and taxanes; mutations in *CHEK2* were associated with platinum and topoisomerase II inhibitors); (iii) CHIP mutations in *ASXL1* gene were strongly associated with smoking.[194] Furthermore, the fitness associated with mutations in epigenetic regulators or splicing regulators was not markedly modulated by oncologic therapy.[194] The environmental factors most strongly associated with the development of CHIP myeloid driver mutations are represented by radiation therapy, platinum (mostly carboplatin) chemotherapy and exposure to topoisomerase II inhibitors.[194] The characterization of the clonal dynamics of evolution of CHIP mutations in 525 cancer patients in a median lapse time of 23 months provided evidence that 62% remained stable, 28% increased, and 10% decreased in clonal size; the growth rate was most pronounced for CHIP mutations in DDR genes.[194] The incidence of CHIP far exceeds that of t-AML, and the main determinants of the risk of a CHIP transforming into therapy-related myeloid neoplasia are related to the type of CHIP mutations (mostly *TP53* and spliceosome genes *SRSF2, U2AF1* and *SF3B1* mutations), the number of CHIP mutations and clonal size.[56] As discussed above, *TP53* is one of the mutated genes frequently involved in t-AML: the analysis of 34 t-MN seemingly evolving from CHIP displayed *TP53* mutations in 44% of cases; 73% of these *TP53*-mutant t-MNs displayed pre-tMN *TP53* mutations; 73% of *TP53*-mutated t-MNs showed complex karyotype alterations, an event acquired at the level of neoplastic transformation, but absent in pre-neoplastic CHIPs.[194]

The incidence of t-MN is variable in different cancer types and treatment regimens; t-MN usually develops 3-10 years after exposure to chemotherapy and/or radiation treatment; t-MN occurs more frequently in patients who received alkylating agents or topoisomerase II inhibitors and less often in those who received taxanes or antimetabolites; high-dose chemotherapy followed by autologous stem cell transplantation increases the risk of developing t-MN.[195-197]

Several recent studies support the view that CHIPs may have a relationship with t-MN development, at least in a significant proportion of patients. Thus. Wong et al. explored 22 cancer patients who developed t-MN by error-corrected NGS whole genome sequencing; for 7 of these patients displaying clonal *TP53* mutations, peripheral blood or bone marrow samples were available 3-8 years before the development of t-MN: four of these seven patients showed biallelic *TP53* mutations in peripheral blood or bone marrow years before chemotherapy treatment.[60] In addition, studies in chimeric mice supported a competitive advantage of *TRP53*-mutated HSCs over *TRP53*-WT HSCs.[198]

Takahashi and coworkers have explored the possible association between clonal hematopoiesis and the risk of t-MNs; they initially explored a group of 14 cancer patients developing t-MN and found that 71% displayed preleukemic mutations in their peripheral blood samples.[199] In a control group of cancer patients, CHIP was found in 31 cases: these CHIP-positive patients exhibited a higher frequency of t-MN development after a five-year follow-up than the rest of the CHIP-negative patients (30% vs. 7%).[199] Finally, in another cohort of lymphoma patients treated with CHOP chemotherapy, 7% developed t-MN and 80% of them displayed CHIP.[199] In line with these observations, Gillis et al. reported that cancer patients (that were 70 years or older at diagnosis and were treated with chemotherapy) with t-MN were more likely to have CHIP than those who do not develop t-MN (62% vs. 27%, respectively); the mutational spectrum of patients with t-MN compared to those without t-MN differed for a higher prevalence of *TP53* mutations.[200]

Other recent studies explored the possible role of clonal hematopoiesis in t-MN observed in gynecological cancer patients. For example, Kwan et al. showed that 2.1% of ovarian cancer patients enrolled in the ARIEL2 and RIEL3 studies based on the administration of rucaparib, a PARP1 inhibitor, developed t-MN.[201] Furthermore, the frequency of homologous recombination repair gene mutations in the tumor was associated with an increased prevalence of t-MN; the frequency of pre-existing CHIP containing *TP53* gene mutations with a VAF of 1% or greater was significantly higher in PBMNs from cases with t-MN compared to those without t-MN (45% vs. 13.6%).[201] The longitudinal analysis showed that pre-existing *TP53*-mutated CHIPs underwent an expansion in patients who developed t-MN.[201]

Khalife-Hachem performed a molecular analysis of 77 cancer patients with gynecologic and breast cancer both developing t-MN involving the molecular profiling of myeloid neoplasia at diagnosis of t-MN and CHIP in the peripheral blood or bone marrow at the time of diagnosis of primary cancer.[202] The molecular characterization of t-MNs provided evidence that these leukemias can be classified into three different subgroups, according to Lindsley et al.:[203] TP53/PPM1D subgroup, MDS-like subgroup (defined by the presence of mutations such as *SRSF2, SF3B1, U2AF1, ASXL1, EXH2, BCOR, STAG1*) and de-novo/pan AML subgroup. CHIP was detected in 66% of these patients.[202] The patients with *TP53* or *PPM1D* mutations had more treatment lines and more complex karyotypes (7.5 months median OS); the patients with MDS-like features were older and with more gene mutations (14.5 months OS); the patients with de-novo/pan-AML mutations were younger with more balanced chromosomal translocations (25.2 months OS).[203]

Targeted sequencing of known cancer genes showed that clonal hematopoiesis occurred in about 4% of children after cytotoxic treatments.[194] t-MNs represent a

major cause of premature death in childhood cancer survivors and affect about 7-11% of children undergoing treatment for neuroblastoma or sarcoma. Coorens et al. reported the analysis of two children affected by neuroblastoma and developing t-MN after cytotoxic treatment of their tumors, including autologous stem cell transplantation; in both these patients, there was evidence of CHIP mutations present in peripheral blood/bone marrow at the time of diagnosis of primary cancer.[204]

The molecular mechanisms through which mutant *TP53* mutations promote hematopoietic stem and progenitor cell expansion are largely unknown. However, a recent study provided evidence that mutant *TP53* induces a growth, and competitive advantage for HSC/HPCs, as shown by transplantation and post-radiation recovery studies.[205] These effects are mediated, at least in part, through interaction between mutant *TP53* and EZH2, thus increasing the expression of genes involved in the self-renewal of HSCs.[205] These observations suggest that EZH2 may represent a therapeutic target for preventing CHIP progression.[205]

Clonal Hematopoiesis and Acute Myeloid Leukemia Development. Initial studies have shown that aged subjects with clonal hematopoiesis mutations have an increased risk of developing hematologic malignancies, including AML.[4-6] Two studies have explored the possible association between CHIP and AML development.

Abelson et al. have used an experimental strategy to distinguish individuals at high risk of developing AML from those with a low risk of developing AML based on the deep sequencing of genes recurrently mutated in AML in the peripheral blood of 95 individuals who later developed AML (pre-AML group), compared with 414 individuals who do not develop AML (control group). Several remarkable differences were observed between these two groups of individuals. For example, (i) CHIP with driver mutations was observed in 73.4% of pre-AML individuals, compared to 36.7% of controls; (ii) 39% of pre-AML individuals harbored a driver mutation with VAF >10%, compared to 4% of controls; (iii) the median number of CHIP driver mutations per individual increased with age and was higher in the pre-AML group compared to the control group; (iv) analysis of the distribution of VAF supported the existence of larger size clones among pre-AML individuals compared to control individuals; (v) the proportion of pre-AML harboring driver mutations such as *DNMT3A, TET2, SRSF2, ASXL1, TP53, U2AF1, JAK2, RUNX1, IDH2* was higher in pre-AML cases compared to controls.[206] Importantly, mutations in certain genes, such as mutations of *TP53* and the splicing factor *U2AF1,*were associated with the highest risk of AML development, while mutations of *DNMT3A* and *TET2* were associated with a lower risk of leukemia development.[206] Interestingly, the comparative, longitudinal analysis of the hematological parameters of the two groups of individuals showed that those who developed AML had significantly higher red cell distribution width (RDW) than the control group; high RDW preceded AML development by several years.[206] Furthermore, the presence of two or more CHIP mutations per individual and CHIP mutations with VAF ≥ 9% were also associated with a high risk of AML development.

A similar approach was adopted by Desai et al.; they explored 188 women developing AML and 212 controls not developing AML during a follow-up of about ten years for the presence of CHIP mutations in their peripheral blood.[207] The group developing AML was explored for CHIP mutations when healthy, years before AML development.[207] Individuals of the pre-AML group displayed a higher number of CHIP mutations than the control group.[207] The most common mutations observed in CHIP with a VAF above 1% were *DNMT3A* (36.7% in pre-leukemia vs 18.8% in controls), *TET2* (25% vs 5.5%), *TP53* (11.2% vs 0%), *SRSF2* (6.9% vs 0%), *IDH2* (6.4% vs 0%), *SF3B1* (5.9% vs 1.1%), *JAK2* (5.3% vs 0.6%) and *ASXL1* (3.% vs 3.3%); collectively, spliceosome genes (*SF3B1, SRSF2, U2AF1*) were detected in 13.8% of pre-leukemia individuals compared to 1.1% of controls.[207] The mutational complexity was higher in pre-leukemia (46.8% of cases with co-mutations) compared to controls (5.5% of cases with co-mutations). Some of these gene mutations showed greater specificity and penetrance for leukemia development, such as *TP53, IDH1/IDH2, and RUNX1/PHF6*, in that 100% of individuals with these mutations developed leukemia.[207] Multivariate analysis showed that some mutations conferred a high risk of AML development: *TP53, IDH1, IDH2, sf3b1, SRSF2, U2AF1, TET2,* and *DNMT3A*, in decreasing order of penetrance.[207] The presence at baseline of these CHIP mutations shortened the time to AML presentation.[207] The mutations of *TP53* and *IDH* genes at any VAF were associated with an increased AML risk; *DNMT3A, TET2,* and spliceosome genes conferred a higher risk of AML development when present at high VAF (i.e., >10%).[207]

Young et al. have explored with a similar approach a pre-leukemia group of 35 cases and a group of 69 controls using error-corrected NGS to assess somatic mutational profile at VAF ≥ 0.1%.[208] They observed AML-associated mutations at the level of CHIP in 97% of all participants; Individuals with mutations ≥ 1% VAF displayed a significantly increased risk of AML development, as well as individuals with higher-frequency clones and those with *DNMT3A R882H/C* mutations.[208]

Watson et al. have analyzed the literature data on blood sequencing from about 50,000 individuals and have reached the conclusion that positive selection and

not drift, is the major force driving and shaping clonal hematopoiesis; this analysis led the authors to quantify the fitness advantage of specific genetic driver variants and their capacity to confer a higher risk of developing AML: thus, using this approach, 20 high-risk variants were identified.[209] Incorporating specific gene variants and their VAF into predictive algorithms may provide an important contribution to predicting the risk of developing AML and thus identifying individuals with a high-risk condition of leukemic development.[209]

As discussed above, the presence of *TP53* gene alterations in CHIPs is the somatic genetic alteration that induces the highest risk of leukemic transformation of these cells. About 10-15% of AMLs exhibit *TP53* alterations, either like mutations either deletions, or a combination of both alterations.[210] Several observations support the view that *TP53*-mutated AML forms a peculiar group of AMLs with typical cellular and molecular properties.[210] *TP53* aberrations in AML include gene mutations, mostly involving the DNA binding domain of the gene, and deletions of different sizes implying the *TP53* locus at the level of chromosome 17p13. Functional studies on missense *TP53* mutant variants commonly observed in AML indicate loss-of-function effects and induction of effects comparable to those observed with complete *TP53* inactivation; these findings have suggested a dominant-negative effect as the primary force of selection of *TP53* mutations in myeloid malignancies.[211] In addition to somatic *TP53* mutations, *Tp53* germline mutations are observed in a minority of AML patients and are more frequent in t-AML.[212] The prognostic impact of different *TP53* mutations is heterogeneous; in fact, Stengel et al. have explored a large cohort of *TP53*-mutated AMLs: *TP53* mutations were detected in 13% of cases (mutation-only 7%; mutation + deletion 5%; deletion - only 1%); all patients with *TP53* mutations alone or in association with *TP53* deletions, but not cases with *TP53* deletions-only, were associated with a poor prognosis and reduced overall survival.[213] Recent studies have addressed the problem of the heterogeneity of VAF of *TP53* mutations observed in AMLs. Prochazka and co-workers characterized *TP53* mutations in 98 AML patients and reported a VAF >40% in 62.2% of cases, VAF 20-40% in 19.4% of cases, and VAF <20% in 18.4% of cases; AMLs with subclonal *TP53* mutations (VAF <20%) displayed fewer complex karyotypes and chromosomal losses than AMLs with clonal *TP53* mutations.[214] All three mutant *TP53* groups showed similarly reduced PFS and OS compared to *TP53*-WT AMLs.[214] Another study confirmed a worse prognosis of *TP53*-mutated AMLs was observed irrespective of the allele burden, including cases with VAF <20%.[215]

Transplantation studies of AML samples with clonal *TP53* mutations into immunodeficient mice provided evidence that these mutations characterize pre-leukemic stem cells in AML; this conclusion was supported by the observation that *TP53* mutations were detected both in myeloid and lymphoid cell compartments, including T lymphocytes.[216] Interestingly, AML specimens with subclonal TP53 mutations also displayed multilineage engraftment potential in transplantation studies in immunodeficient (NOD/SCID) mice.[217]

P53 pathway is deregulated in AML more frequently than predicted based on *TP53* mutational frequency. In fact, loss of p53 function may also originate through the aberrant expression of a protein that acts as a physiological regulator of p53 stability and function. One of these mechanisms is related to the overexpression of MDM2, a negative regulator of *TP53,* overexpressed in a part of AMLs with *TP53*-WT; these AMLs were characterized by the absence of p21 expression and by a negative prognosis.[218] Other studies have shown that overexpression in AML also of MDMX (also known as MDM4) and the pharmacologic inhibition of both MDM2 and MDM4 resulted in inhibition of cell proliferation and induction of apoptosis of primary AML cells.[219]

Absent or reduced protein levels of p53 are observed in 50% of AMLs. P53 haploinsufficiency or loss plays a key role in the development of AMLs with *FLT3-ITD* mutation.[220]

Stem Cell Transplantation, Clonal Hematopoiesis and the Risk of Leukemic Transformation. The study of the expansion and potential leukemic evolution of CHIPs offered a tool to understand better the biology of clonal hematopoiesis and the risk of leukemic progression. In fact, cell-extrinsic stress associated with the post-HSCT reconstitution of autologous (auto-SCT) or donor-derived (allo-SCT) hematopoiesis accelerates the timing of events underlying CHIP evolution. In this context, particularly informative was the study performed by Ortmann et al. on 81 patients with solid tumors or lymphoid diseases undergoing auto SCT.[221] These patients were studied for CHIP-associated mutations at diagnosis, at stem cell collection for transplantation, and at first follow-up PB analysis post-transplantation.[221] 22% of these patients displayed CHIP; 16 of the 28 CHIP mutations found in the post-transplantation analysis were tracked back to the graft: for 15/16 of these mutations, the clone size increased after transplantation.[221] This finding may be interpreted assuming that CHIP mutations conferred a reconstitution advantage to mutated HSCs in the setting of auto-SCT.[221]

Soerensen et al. explored, in a cohort of 1130 cancer patients undergoing auto-SCT, the CHIP mutational profile of 36 patients with non-myeloid malignant disease developing t-MNs following auto-SCT (case subjects).[222] These patients were compared to an equal number of patients undergoing auto-SCT and not developing t-MNs (controls). Two remarkable

differences distinguished these two groups of patients: (a) case subjects were poorer mobilizers of $CD34^+$ cells at leukapheresis compared to controls, thus suggesting that they have a reduced bone marrow function; (b) while case patients and controls display a comparable CHIP mutational frequency at the level of *DNMT3A* and *TET2* genes, case patients exhibited a markedly higher frequency of low VAFs *TP53, ASXL1, ZRSR2, SRSF2* and *SF3B1* mutations.[222] In these patients, t-MNs could directly originate from these CHIPs through a process of both clonal expansion and acquisition of new mutations driven by stresses induced by HSC reconstituting activity.[222] Similar conclusions were reached by Berger et al. in a group of lymphoma patients developing t-MNs after auto-SCT, suggesting that these leukemias developing after auto-SCT originate from HSCs bearing pre-t-MN mutations that are present years before disease onset; in fact, in 70% of these patients CHIPs were identified.[223] Furthermore, 3/7 patients CHIP-positive developing t-MN possess *TP53* mutations at low VAF, exhibiting a marked increase of their VAF in t-MN.[85] However, sequencing studies of CHIP and corresponding t-MNs do not support, in all cases, a direct origin of leukemias from the evolution of CHIPs.[223]

The study of patients who underwent allo-SCT from donors with CHIP offers a unique opportunity to explore the mechanisms of leukemic evolution of clonal hematopoiesis. Frick et al. explored the role of donor clonal hematopoiesis in allo-SCT; the analysis of 500 stem cell donors' blood samples showed clonal hematopoiesis in 16% of donors.[224] The presence of donor CHIP did not affect thrombocyte engraftment time but induced slightly faster leukocyte engraftment; 2/82 recipients of donors with CHIP developed cell malignancies compared to 0/426 in allo-SCT from donors without CHIP.[224] One of the two patients developing donor cell leukemia had a donor *CBLC* mutation with a VAF of 8%, which rapidly increased after transplantation and remained stable until leukemia diagnosis; sequencing of the DCL sample showed acquisition of *TP53 R175H* mutation.[224] The other patient developing DCL exhibited the presence of an *ASXL1* and a *DNMT3A* mutation with a VAF between 2% and 3%.[224]

Recently, Gibson et al. reported the results of targeted error-corrected sequencing on samples of 1,727 donors for allo-SCT and evaluated the effect of donor clonal hematopoiesis on transplantation outcomes. Clonal hematopoiesis was detected in 22.5% of donors; the presence of *DNMT3A*-mutated CHIP was associated with reduced relapse and increased CGVHD; no recipients of donors with CHIP with sole *DNMT3A* or *TET2* developed donor cell leukemia.[225] Donor cell leukemia was observed with a 10-year cumulative incidence of 0.7%; in 7/8 cases, donor cell leukemia evolved from donor CHIP with rare *TP53* or splicing factor mutations or from donors carrying germline *DDX41* mutations.[225] Other studies have reported cases of donor cell leukemia arising from CHIP marked by somatic mutations in leukemia-related genes in donors usually over age 60.[226,227]

Gibson et al. explored six patients in a group of 552 patients undergoing allo-SCT, characterized by a condition of unexplained cytopenia occurring after transplantation.[228] Five of these six patients displayed evidence of clonal hematopoiesis, all characterized by the presence of *DNMT3A* point mutations.[228] In addition, four of these patients were followed through time: 3 did not show any evidence of clonal expansion during the first three years after transplantation; the fourth patient showed clonal expansion, concomitantly with the acquisition of two additional mutations at the level of *TP53* and *ASXL1* genes.[228]

The studies on allo-SCT with CHIP-positive donors showed a low risk of leukemic transformation, limited to a minority of patients. However, a debate is still open about the opportunity or not to screen stem cell donors for clonal hematopoiesis.[229,230]

Heterogeneity of Clonal Hematopoiesis. The heterogeneity of the mutational spectrum of clonal hematopoiesis is an important driver of its potentiality for leukemic evolution. Thus, it was proposed that according to the type and VAF of genes mutated and the number and/or associations of multiple mutations, clonal hematopoiesis can be divided into two different groups: CHIP and clonal hematopoiesis with oncogenic potential (CHOP).[231,232] Thus, the definition of CHIP implies the existence of clones of hemopoietic cells with a minimal allele burden of 2%, the presence of normal blood cell counts, the absence of persistent cytopenia, and the exclusion of any pathology associated with the somatic alterations.[231,232] CHOP is differentiated from CHIP for the presence of disease-related mutations, cell differentiation, and/or cell proliferation.[231,232]

A notable example of the difference between CHIP and CHOP mutations is given by a recent study by Cappelli et al., who studied 150 *NPM1*-mutated AMLs treated with standard induction and consolidation therapy and achieving a condition of complete molecular remission as assessed by the absence of *NPM1* transcripts at the end of treatment.[233] These patients were explored by targeted NGS; at complete molecular remission, 46% of patients displayed at least one mutation, with a VAF cutoff of $\geq 1\%$, 27% had persistent DTA (*DNMT3A, TET2, ASXL1*) mutations, and 15% persisting non-DTA mutations; patients with persistence or acquisition of non-DTA mutations showed a worse prognosis, while CHIP-like mutations did not affect the overall survival.[233] Based on clonal evolution of longitudinal AML samples, it was concluded that in *NPM1*-mutated AMLs, CHIP-like mutations detectable

from diagnosis to relapse were *DNMT3A, TET2, ASXL1, IDH1, IDH2, SRSF2* and CHOP-like mutations, usually acquired at remission and/or persisting or acquired at relapse, include *FLT3TKD, GATA2, NRAS, PTPN11, WT1, TP53, RUNX1*.[233]

Clonal Hematopoiesis in Cytopenias. In most individuals, clonal hematopoiesis is associated with a normal hematologic phenotype, with normal blood cell counts and morphology. However, in a minority of subjects, clonal hematopoiesis is associated with cytopenia.[96] Cytopenia is defined as a condition in which the patients must have, for at least six months, hemoglobin, platelets and neutrophil counts less than 11g/dL, $100x10^9$/L and $1.5x10^9$/L, respectively.[234] If a patient with cytopenia does not fulfil the criteria for diagnosis of myelodysplastic syndrome, it is most probably affected by the so-called idiopathic cytopenia of undetermined significance (ICUS).[235] When a subject with ICUS displays a somatic mutation in myelodysplasia-associated genes, in the absence of diagnostic criteria for MDS, the condition is considered a clonal cytopenia of undetermined significance (CCUS).[235]

The incidence of peripheral blood cytopenias was explored in the general aging population. Thus, the analysis of the incidence of cytopenias in the aging, making use of the prospective and population-based Lifelines cohort comprising 167,729 community-dwelling individuals living in the northern part of The Netherlands, showed anemia in 4,2% of cases, thrombocytopenia in 1.6% and neutropenia in 4.8%.[236] Anemia and thrombocytopenia increased with older age, while neutropenia showed no increase in prevalence with older age; anemia and thrombocytopenia and particularly the concomitant presence of anemia and thrombocytopenia were associated with reduced overall survival; for individuals aged ≥60 years the incidence of hematological malignancies was higher among individuals with anemia or thrombocytopenia but not neutropenia.[236] The highest incidence and mortality of hematological malignancies were observed in individuals with >1 cytopenia.[236]

It is important to point out that the diagnosis of ICUS does not imply evidence of a clonal disorder. However, the exploration of ICUS subjects in various studies showed that a part of these patients displayed somatic mutations. Two initial investigations revealed that a significant proportion of ICUS subjects have somatic mutations, partly overlapping with those observed in myelodysplastic syndromes.[237-238] Kwok et al. explored 369 patients with ICUS by targeted NGS and observed that 28% of patients with ICUS displayed one or more somatic mutations: these patients were subdivided into a dysplastic (patients with rare dysplastic features), and non-dysplastic group and somatic mutations were observed in 62% of patients with rare dysplastic morphology and 20% of patients with no evidence of dysplasia.[237] Patients with ICUS displayed fewer mutations than those with myelodysplasia and less frequently two or more mutations; the spectrum of mutated genes in ICUS patients was similar to that observed in myelodysplastic syndromes, except for *SF3B1* mutations and other splicing factors that are less frequent in ICUS patients without dysplasia.[237] Cargo et al. showed that patients developing at later stages MDS or AML display in pre-diagnostic samples somatic mutations with a spectrum mirroring more than observed in MDS than in healthy individuals (CHIP).[238]

Malcovati et al. using a panel of 40 myeloid genes in a cohort of 683 patients with myeloid neoplasms, including 154 ICUS patients, showed that about 36% of ICUS patients displayed one or more somatic mutations; the number of somatic mutations per patient and their VAF was lower in ICUS patients than in patients with myeloid neoplasms.[239] The most recurrent mutations observed in ICUS patients involved *TET2* (14.9%), *ASXL1* (8.4%), *DNMT3A* (8.4%), *SRSF2* (7.1%), *SF3B1, ZRSF2, IDH2, RUNX1* (all with a frequency of 2%).[239] ICUS patients with clonal mutations (CCUS patients) displayed a probability of developing a myeloid neoplasm that was 14 times higher than that of patients with no evidence of clonal disease; ICUS patients with spliceosome gene mutations or co-mutated gene patterns involving epigenetic regulators had a risk of disease progression comparable to patients with a myeloid neoplasm.[239] In addition to somatic mutations, ICUSs, at least in some patients, display structural aberrations. Thus, Mikkelsen et al. explored the possible presence of structural aberrations (CNAs and CNLOHs) in a cohort of 153 patients with ICUS.[240] 23 of 153 ICUS patients displayed structural aberrations, excluding LOY; mutations in MDS-related genes were observed in 52% of these 23 patients; the CAN/CNLOH identified in the ICUS patients were similar to those observed in myeloid malignancies.[240] 10% of these ICUS patients progressed to myeloid malignancy in a median follow-up time of 25 months; all patients but one who progressed displayed somatic mutations. The presence of CAN/CNLOH was associated with reduced PFS and OS, while the presence of somatic mutations was associated with reduced PFS but not OS.[240] In the group of CCUS patients, after a follow-up of 24 months, the median overall survival was 67 months in the group of patients with CAN/CNLOH, compared to 104 months in the group with or without CAN/CNLIH.[240]

Cytotoxic therapies may worsen a condition of cytopenia associated with clonal hematopoiesis. Singh recently reported the study of 13 cases of endocrine cancer patients undergoing treatment with peptide receptor radionuclide therapy (PRRT). 62% of patients displayed CHIP at baseline; persistent cytopenias were

observed in 64% of the patients; PRRT exposure resulted in clonal expansion of the mutant DNA damage response genes *TP53, PPM1D,* and *CHEK2* and associated cytopenias in 75% of the patients.[241]

Some CCUS patients may develop transfusion-dependent anemia; no large clinical studies have explored these patients, but a recent single-center study showed that transfusion-dependent CCUS patients might benefit from MDS-type therapies, such as growth factors and hypomethylating agents.[242]

Mouse Models of Clonal Hematopoiesis. Several mouse models involving the main genes implied in human CHIP have been developed.

Many studies involved the analysis of *DNMT3A*, the gene most frequently mutated in CHIP. Knocking out *DNMT3A* in mice showed a consistent enhancement of LT-HSC self-renewal, with an expansion of the pool of these cells.[243] A sequential knocking experiment showed that: (i) *DNMT3A* mutation R878H (the equivalent of human R882H) induces an expansion of HSCs and MPPs; (ii) the induction of *NPM1* mutation in these cells causes progression to a myeloproliferative disorder.[244] The cooperativity of *DNMT3A* and *NPM1* mutations in inducing leukemia development is related to the capacity of the mutant gene to alter chromatin structure at the level of HSCs inducing increased accessibility of gene promoters of cell cycling, stem cell, transcription factors, and PI3K/AKT/mTOR signaling members.[245] In addition to the effects on HSC expansion, *DNMT3A* knockout also induced a decreased bone mass via increased osteoclastogenesis.[246] In line with this observation, subjects with *DNMT3A* mutant CHIP showed increased incident osteoporosis and reduced bone mineral density.[246]

A recent study systematically explored and characterized at a molecular level the different *DNMT3A* mutants observed in human hematopoietic cells and showed that 74% were loss-of-function mutations; half of *DNMT3A* variants exhibited reduced protein stability.[247]

Several recent studies based on the analysis of mouse knockout models or single human hematopoietic cells bearing R882H mutations have supported the conclusion that *DNMT3A* mutants induce a condition of selective hypomethylation, largely responsible for their pro-leukemic effect. Thus, Smith et al. developed a somatic, inducible model of hematopoietic *DNMT3A* loss and showed that inactivation of *DNMT3A* in murine hematopoietic cells induces a relatively slow loss of methylation of some DNA sites throughout the genome.[248] According to this observation, it was suggested that slow methylation loss might explain the long latent period required for clonal expansion and leukemic development in individuals with CHIP *DNMT3A* mutations.[248] Furthermore, Nam et al. reported a sing cell analysis of $CD34^+$ cells purified from individuals bearing CHIP bearing *DNMT3A* R882H mutation; multi-omics single-cell sequencing to detect the mutational status of individuals cells was applied together with downstream epigenetic and transcriptional information, thus enabling to compare mutated cells with their wild-type counterparts from the same individuals.[249] The results of this single-cell analysis showed that: (i) *DNMT3A* mutations resulted in myeloid over lymphoid imbalance of HSCs, and in an expansion of immature myeloid progenitors primed toward megakaryocytic-erythroid fate; (ii) *DNMT3A* R882H resulted in preferential hypomethylation of polycomb repressive complex two targets and in the dysregulated expression of lineage and leukemia stem cell markers and some key hematopoietic transcription factors.[249]

Other studies have explored the effects of *TET2* mutations on stem/progenitor cells. Two initial studies showed that the conditional *TET2* loss in the hematopoietic stem cell compartment leads to increased HSC self-renewal; in some animals, *TET2* loss may also lead to a myeloproliferative condition.[250-251] While *TET2* knockout mice exhibited expansion of HSCs and HPCs, *TET2* mutant mice, engineered with a catalytic inactive *TET2* variant, predominantly developed myeloid malignancies.[252]

Studies in *TET2*-mutant cells have characterized their methylation abnormalities. The conversion of 5-methylcytosine (5mC) to hydroxymethylcytosine (5hmC) is the key step of DNA demethylation catalyzed by TET2, which requires ascorbate. 5hmC maps at the level of specific DNA sites and is associated with active transcription and chromatin accessibility of key hematopoietic regulators. *TET2* mutations in primary human HSCs/HPCs determine an increased self-renewal activity, increased colony-forming activity *in vitro*, defective erythroid/megakaryocytic differentiation, and myeloid skewing, associated with a decrease of 5hmC at the level of erythroid-associated gene loci.[253] The study of the methylation profile of CHIP and CCUS subjects provided evidence that *TET2* mutations are associated with DNA hypermethylation at enhancer DNA sites: the hypermethylated sites are functionally related to genes involved in leukocyte function and immune response and to ETS-related and C/EBP-related transcription factor motifs. Most TET2-associated hypermethylation sites are shared between CHIP and AML; some AML-specific hypermethylation sites are located at active enhancer DNA elements in HSCs.[254]

In conclusion, the studies on CHIP hematopoietic cells bearing mutated *DNMT3A* and/or *TET2* display an altered phenotype resulting in specific changes in the DNA methylome, which in turn lead to altered regulation of specific genes or gene sets.

Other studies have characterized the biological effects elicited by the induction of *ASXL1* loss or of

ASXL1 mutants. *ASXL1* loss *in vivo* induces progressive multilineage cytopenias and dysplasia with an increased size of the pool of HSCs and impaired mature cell differentiation, resulting in several changes resembling human MDS.[255-256] A similar phenotype was observed by retroviral overexpression of mutant *ASXL1,* thus suggesting that *ASXL1* variants may inhibit the function of WT *ASXL1*.[257] Physiological expression of a C-terminal truncated *ASXL1* mutant *in vivo* using conditional knock-in resulted in HSC expansion, myeloid skewing, age-dependent anemia, thrombocytosis, and morphological dysplasia.[258] Using this knockin mouse overexpressing a-terminally truncated form of *ASXL1*-mutant, the effect of mutant *ASXL1* on physiological aging in HSCs was explored: HSCs expressing mutant *ASXL1* acquire clonal advantage during aging; mutant *ASXL1* cooperates with *BAP1* to deubiquitinates and activate AKT.[259]

An analysis carried out on a UK Biobank cohort provided evidence that *ASXL1* loss of function mutations are strongly associated with current and past smoking status; this finding strongly supports the hypothesis that the inflammatory environment induced by smoking may promote the outgrowth of *ASXL1*-mutant clones.[260]

Comments and Conclusions. The studies carried out in the last two decades have elucidated in part the mechanisms underlying the aging of HSCs and the development of clonal hematopoiesis.

The most relevant findings can be summarized as follows:

- Even though HSCs increase in number with age, they have a significantly decreased self-renewal capacity and reconstitution potential upon transplantation.
- By aging, human HSCs become more myeloid-biased in their differentiation potential, a phenomenon seemingly related to the increase of their clonality with age, selecting for myeloid-biased HSC clones.
- It was estimated that HSCs or MPPs accumulated a mean number of 17 mutations per year after birth and lost 30 base pairs per year of telomere length.
- Hematopoiesis in individuals under 65 years of age was largely polyclonal, implying a population of 20,000-200,000 HSC/MPPs. By contrast, in individuals aged 75 years, 30-60% of hematopoiesis is sustained for 12-18 independent clones. Thus, there is a progressive switch from polyclonal to oligoclonal hematopoiesis during aging.
- CHIP is characterized by the presence of some recurrently mutated genes, such as *DNMT3A, TET2,* and *ASXL1,* all three acting as epigenetic modifiers. However, less frequently are mutated genes involved in DNA damage response (*PPM1D, TP53, CHEK 2*), spliceosome (*SF3B1, SRSF2, U2AF1*), or epigenetic control (*IDH1, IDH2*).
- *DNMT3A*-mutant clones preferentially expanded early in life, while splicing gene mutations expanded only in old individuals, and *TET2*-mutant clones expanded at all ages.
- CHIP occurs with higher frequency in individuals with lymphoid or solid tumors and is associated with expexposure genotoxic stress. *PPM1D* mutations drive clonal hematopoiesis in response to cytotoxic chemotherapy. Clonal hematopoiesis confers a greater risk of developing therapy-related myeloid neoplasms.
- CHIP is associated with a consistently increased risk (about 10-fold) of hematopoietic malignancy; some CHIP features are associated with a higher risk of transformation, such as the presence of *TP53* or spliceosome gene mutations, a VAF> 10%, and the presence of multiple mutations.
- Some individuals display clonal hematopoiesis in association with peripheral blood cytopenia, a condition known as clonal cytopenia of undetermined significance (CCUS)
- Individuals with CHIP and CCUS have a markedly higher probability of leukemic transformation than those with cytopenia alone.

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