Leducq Transatlantic Network on Clonal Hematopoiesis and Atherosclerosis

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Glossary. **BM**, bone marrow; **BMT**, bone marrow transplantion; **CH**, clonal hematopoiesis; **CVD**, cardiovascular disease; **JAK2^{VF}**, JAK2V617F; **DZHK**, German Center for Cardiovascular Research; **MI**, myocardial infarction; **NET**, neutrophil extracellular trap; **PLA**, platelet-leukocyte aggregates; **rHDL**, reconstituted HDL; **RBCs**, red blood cells.

Atherosclerotic cardiovascular disease (CVD) remains the major cause of death and disability in the US and Europe and is rapidly increasing in the emerging world. LDL lowering therapies, while effective in the treatment of CVD, leave a large burden of residual risk. The recent CANTOS trial has provided the first direct proof that in subjects with evidence of systemic inflammation, targeting inflammatory signaling can reduce CVD.[1] However, inflammation was broadly defined (elevated CRP), and anti-inflammatory therapy was associated with an increased risk of infection. Thus, there is an unmet clinical need for a more precise, genetically based approach to identify subjects who may benefit from novel therapeutic approaches.

With the support of the Leducq Foundation, seven teams from both sides of the Atlantic will join forces to address this challenge. Our Network is led by Alan Tall (Columbia University) in the U.S. and Andres Hidalgo (Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain) in Europe. The other project leaders are Ben Ebert (Harvard Medical School/Brigham), Sekar Kathiresan (Massachussets General Hospital/Broad Institute), Sidd Jaiswal (Stanford University), Ira Tabas (Columbia University), Oliver Soehnlein (Ludwig Maximilians University) and Steffen Massberg (Ludwig Maximilians University). Other key members include Nan Wang (Columbia University) and Christian Schultz (Ludwig Maximilians University). We are also most fortunate to have enrolled Peter Libby as senior adviser and collaborator.

Clonal hematopoiesis (CH) represents a clonal expansion of blood cells arising from somatic mutations in genes that confer a proliferative advantage to hematopoietic stem cells (HSCs).[2] Members from our Leducq Network have shown that CH, also known as Clonal Hematopoiesis of Indeterminate Potential (CHIP), increases in frequency from age 40 onwards, is present in >10% of people aged >70 and is associated with a 2-3 fold increased risk of myocardial infarction (MI) and stroke, suggesting that CH could be a major non-traditional risk factor underlying atherosclerotic CVD.[3] CH usually involves mutations in one of four genes producing loss of function in epigenetic modifiers (*TET2*, *ASXL1* or

DNMT3A) or increased hematopoietic signaling (*JAK2*). Intriguingly, the Kathiresan and Tall laboratories have shown that the most common mutation of the JAK2 gene (V617F; *JAK2*^{VF}) lowers LDL but increases atherosclerosis in both mice and humans,[4, 5] highlighting a potential dissociation between LDL levels and CVD risk in CH. A causal relationship between CH and atherosclerosis was shown in mice with *Tet2* deficiency and macrophage inflammation, including NLRP3 inflammasome activation, was identified as a key underlying mechanism.[3, 6] Even though *Tet2*-deficient clones expanded in the bloodstream, accelerated atherosclerosis occurred without increased numbers of leukocytes in the circulation. These findings suggested that targeting inflammation driven by mutant leukocytes might be an effective way to reduce CVD risk in CH

In addition to increased inflammation in myeloid cells, aberrant hematopoiesis may increase CVD risk in CH. Although prospectively studied CH subjects did not have abnormal total blood cell counts at baseline,[3] qualitative changes in blood cell function, as well as development of myelo-proliferation in some patients, appears likely. Our Leducq team has considerable expertise in studies of aberrant hematopoiesis and vascular disease. Tall and colleagues showed that hematopoiesis may be driven by hypercholesterolemia, thereby linking myeloid cell expansion to atherosclerosis.[7, 8] Hidalgo and co-workers have shown that platelet-leukocyte aggregates (PLA) are a hallmark of early atherosclerosis[9] and that polarized neutrophils bound to inflamed endothelium scan the bloodstream for activated platelets, thus promoting leukocyte activation and entry into affected tissues.[10] Soehnlein et al. further demonstrated the importance of neutrophils and NETs in early atherogenesis[11], and Massberg and colleagues have established that platelets and their interactions with myeloid cells play a critical role in lesion development and athero-thrombosis.[12, 13] Recent collaborative studies by these investigators with Wang, Tall, and Tabas at Columbia University using hypercholesterolemic *Jak2*^{VF} mice suggest that macrophage inflammation, erythrophagocytosis, failed efferocytosis and inflammation resolution, PLA formation and neutrophil NETosis, are all potential mechanisms promoting atherosclerosis and thrombosis in CH.[5] Moreover, there are likely distinctive

mechanisms for each of the four genetic variants underlying CH. In particular *JAK2*^{VF} mice display prominent features of a myeloproliferative state and the *JAK2*^{VF} variant predisposes to NETosis and thrombosis in mice and patients.[14] In contrast, the other CH mutations give rise to epigenetic changes that promote myeloid inflammation and failure of inflammation resolution. The understanding of mechanisms linking CH to athero-thrombosis is in its infancy. Therefore this network proposes a broad mechanistic approach drawing on the expertise in hematopoiesis and atherosclerosis represented by its members (Figure 1).

The overarching **goal** of our network is to elucidate mechanisms linking CH to atherosclerosis as well as other aspects of CVD, and to assess potential therapeutic interventions. The overall **hypothesis** is that CH promotes atherosclerosis and thrombosis, and that underlying mechanisms include macrophage inflammation, aberrant epigenetic regulation of inflammation, inflammasome activation, erythrophagocytosis, abnormal neutrophil and platelet functions leading to NET formation and thrombosis, increased PLA formation and failed inflammation resolution. The goals of our Network are encompassed by three major aims. First, we will explore mechanisms of accelerated atherosclerosis in humans with CH. The goal of these analyses will be to obtain insights into how CH affects cellular dynamics and gene expression in human atherosclerosis. We will use autopsy samples obtained at Stanford (Jaiswal) as well as a Biobank of carotid plaque tissue (Soehnlein) to develop methods that allow assessing gene expression and CH genotype at the single-cell level from human atherosclerosis specimens immediately after death, or from surgical patients. Similarly, blood samples from CH carriers with and without CVD, and non-CH controls will be analyzed for CH mutations and associated gene expression changes (Kathiresan). The Kathiresan team will develop a large, population-based cohort of individuals harboring CH, in order to identify factors that promote the association of CH with athero-thrombotic disease and to evaluate whether clinical factors (e.g., cigarette

smoking and diabetes) or germline genetic variation modulate the association of CH with atherosclerotic CVD.

A second major goal is to investigate mechanisms of atherogenesis and thrombosis using mouse models of CH. Our teams will investigate the hypothesis that CH variants increase atherosclerosis and thrombosis, and dissect the underlying mechanisms. Mouse models of the four common CH mutations will be extensively analyzed in experimental models of athero-thrombosis, myocardial infarction and stroke. We will use conditional mouse strains to understand the contributions of different cell lineages, including macrophages, neutrophils and platelets to these processes. We will also take advantage of CRISPR/Cas9 technology to evaluate the effects of deletion of specific genes, e.g. up-regulated inflammatory genes on atherosclerosis, in the CH mouse models. For example, the Ebert and Jaiswal teams will examine whether *Dnmt3a* and *Asxl1* mutations have effects on macrophage inflammation and atherogenesis similar to those first reported in *Tet2*$^{-/-}$ mice, and will explore the mechanisms linking these epigenetic modifiers to inflammation and atherosclerosis. An unexpected finding from our teams is an apparent dysregulation of macrophage-mediated erythrophagocytosis and inflammasome activation in mouse models of CH atherosclerosis,[5] which will be further explored by the Tall team. A role of neutrophils and NETosis in atherogenesis and its complications such as plaque erosion and thrombosis is an important emerging area and the Soehnlein and Libby/Ebert groups will assess the effects of CH on these processes. At the source of intravascular inflammation, activated platelets and neutrophils interact to propagate vascular damage;[10] potential dysregulation of these interactions and early vascular damage in the context of CH will be studied by the Hidalgo team using advance intravital imaging. The consequences of such vasculopathic effects will be also studied in mouse models of myocardial infarction and stroke. Because thrombosis is a common feature of myelodysplastic syndromes and CH, the Massberg team will evaluate alterations in thrombopoiesis and early platelet activation in the bone marrow, as well as during athero-thrombosis in mouse models of CH. Finally, the Tabas team has recently provided evidence for a key role of defective inflammation resolution

in the atherosclerotic process and will build on preliminary findings showing that these processes may be prominently involved in mouse models of CH.

The final aim of our Network will be to assess potential therapeutic Interventions to reduce atherosclerosis in CH using approaches in both humans and mouse models. In particular, we aim to explore whether therapies that reduce inflammation or LDL will have greater efficacy in CH subjects. To this end, the Kathiresan team will assess whether the CH status modifies treatment benefit in completed trials of statin and IL-1β therapy where baseline blood samples have been collected. Given the recent demonstration that inflammation drives clinical atherosclerosis[1], the Ebert and Jaiswal teams will examine whether targeting the inflammatory products of mutant macrophages, such as IL-1β, can inhibit atherosclerosis in mouse models of CH. An alternative approach will be to explore the ability of rHDL infusions and LXR activator treatment to suppress inflammasome activation, leukocyte recruitment and atherosclerosis, which will be assessed collaboratively by the Tall and Soehnlein team. Finally, mechanisms that restore homeostasis and have failed during atherosclerosis will be studied by Tabas, whose team will test whether treatment with the resolution mediator Resolvlin D1 (RvD1) ameliorates resolution and improves plaque progression in the context of CH.

In addition to our efforts in scientific discovery and therapeutic exploration in CH and atherosclerosis, the training and education of emerging investigators is an essential goal of our network. Thus, the interweaving of basic and clinical studies in network collaborations will provide excellent training for young scientists. Moreover, we have established an **early stage investigator** program, led by Dr Soehnlein. This program will feature a mentorship and training plan with a dedicated budget and structured review process to support travel and collaborations between network laboratories. It is our ardent hope that at the completion of our program we will have gained major new insights into the significance of CH in athero-thrombosis, the underlying mechanisms and will have

identified potential new treatments that may help to alleviate the major disability and death caused by atherosclerosis in our societies.

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Figure legend

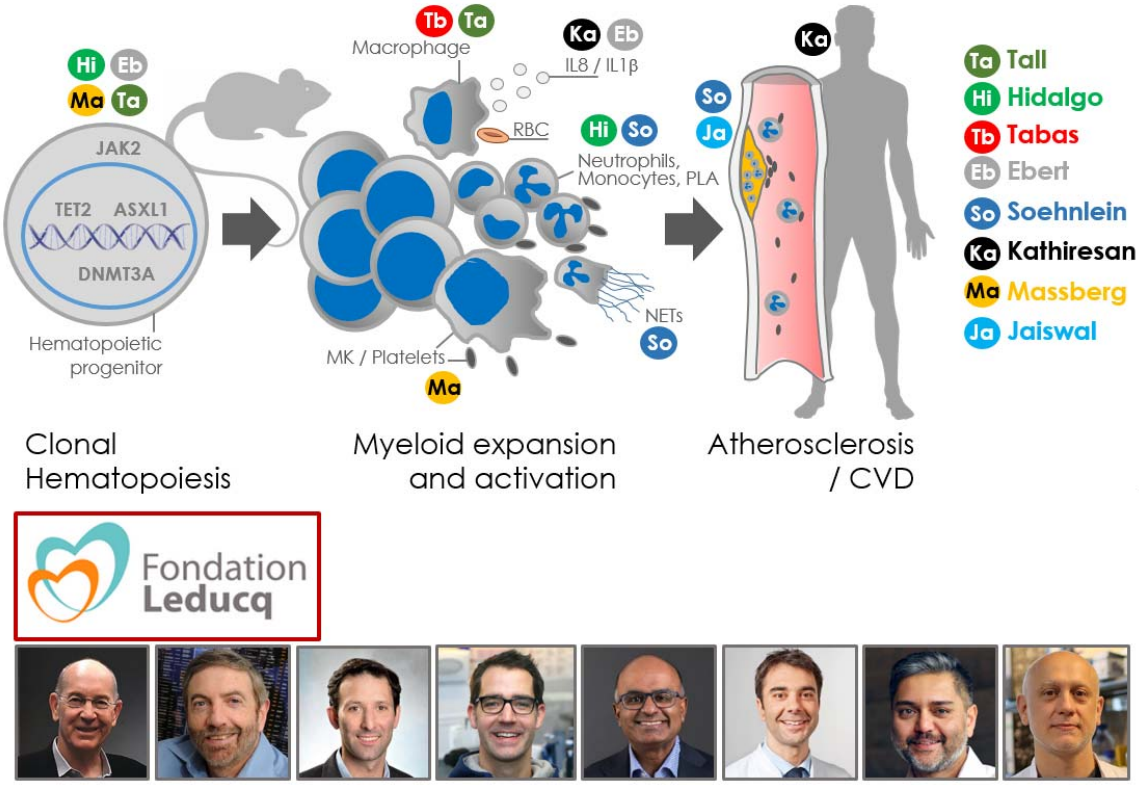

Figure 1. The "Clonal hematopoiesis in atherosclerosis" Leducq Transatlantic Network will study multiple aspects of CH-related immune dysregulation, with a focus on mutations most commonly associated with CH. We will dissect how these mutations alter the function of hematopoietic progenitors, megakaryocytes/platelets, macrophages,

monocytes and neutrophils in experimental models and in patients, and its consequences in inflammation, atherosclerosis and other forms of CVD. Below are the members of the Network, a multidisciplinary team of researchers and clinicians from across the US and Europe.