Nilotinib-induced vasculopathy: identification of vascular endothelial cells as a primary target site

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Abstract

The BCR/ABL1 inhibitor Nilotinib is increasingly used to treat patients with chronic myeloid leukemia (CML). Although otherwise well-tolerated, Nilotinib has been associated with the occurrence of progressive arterial occlusive disease (AOD). Our objective was to determine the exact frequency of AOD and examine *in vitro* and *in vivo* effects of Nilotinib and Imatinib on endothelial cells to explain AOD-development. In contrast to Imatinib, Nilotinib was found to upregulate pro-atherogenic adhesion-proteins (ICAM-1, E-selectin, VCAM-1) on human endothelial cells. Nilotinib also suppressed endothelial cell proliferation, migration and tube-formation, and bound to a distinct set of target-kinases, relevant to angiogenesis and atherosclerosis, including angiopoietin receptor-1 TEK, ABL-2, JAK1, and BRAF. Nilotinib and siRNA against ABL-2 also suppressed KDR expression. In addition, Nilotinib augmented atherosclerosis in ApoE-/- mice and blocked reperfusion and angiogenesis in a hind-limb-ischemia model of arterial occlusion, whereas Imatinib showed no comparable effects. Clinically overt AOD-events were found to accumulate over time in Nilotinib-treated patients. After a median observation-time of 2.0 years, the AOD-frequency was higher in these patients (29.4%) compared to risk factor- and age-matched controls (<5%). Together, Nilotinib exerts direct pro-atherogenic and anti-angiogenic effects on vascular endothelial cells, which may contribute to development of AOD in patients with CML.

Introduction

Chronic myeloid leukemia (CML) is a hematopoietic neoplasm characterized by expansion of myeloid stem- and progenitor cells in the bone marrow (BM), peripheral blood and spleen, and the chromosomal translocation t(9;22).[1,2] The resulting fusion gene-product, BCR/ABL1, acts as a dominant driver and major oncogenic tyrosine kinase (TK) in CML.[1,3] Correspondingly, the BCR/ABL1 TK inhibitor (TKI) Imatinib exerts substantial anti-leukemic effects on BCR/ABL1+ cells and is widely used to treat patients with chronic phase (CP) CML.[4,5] However, resistance to Imatinib develops in a subset of patients, often in association with *BCR/ABL1* mutations[6,7], which is an emerging challenge in clinical hematology.[8-10] For these patients, novel BCR/ABL1 TKI have been developed.[8-14]

Nilotinib is a second-generation BCR/ABL1 TKI that exerts 50-100 times more potent effects on BCR/ABL1 compared to Imatinib. Correspondingly, Nilotinib induces complete cytogenetic responses in a high proportion of patients with Imatinib-resistant CML.[15,16] Moreover, Nilotinib is a superior drug when used as first-line therapy in newly diagnosed patients with CML.[17,18] Similar to Imatinib, Nilotinib inhibits several additional kinase-targets in CML cells, including platelet-derived growth factor receptors (PDGFR) and KIT.[19-21]

In initial clinical trials, Nilotinib has been described as a well-tolerated drug.[15-18] Recurrently reported adverse events include an increase in pancreatic enzymes and an increase in fasting glucose levels.[15-18,22] Furthermore, Nilotinib-treated patients may develop hyperlipidemia.[23] More recently, several studies have reported on severe progressive arterial occlusive disease (AOD) during treatment with Nilotinib.[24-30] However, the exact incidence and etiology of AOD remain unknown.[31-33] In the current study, we analyzed cellular and molecular mechanisms underlying the development of AOD in patients receiving Nilotinib. Our data show that Nilotinib directly induces pro-atherogenic and anti-angiogenic effects on endothelial cells.

Materials and Methods

A more detailed description of methods is provided in the Supplementary Appendix.

Nilotinib-treated patients and controls

Thirty-six patients with BCR/ABL1+ CML receiving Nilotinib (initial dose: 800 mg/day; 16 females, 20 males, observation time: 2006-2013) were analyzed. The patients´ characteristics are shown in Table S1. During treatment, patients were examined for hematologic and molecular responses (Table S1 and S2) and occurrence of adverse events (Table S1). Serum tryptase levels were analyzed at diagnosis as well as before and during Nilotinib-treatment. The frequency of AOD was examined after a median observation time (MOT) of 2.0, 3.0, and 3.7 years. From July 2012, most Nilotinib-treated patients received prophylactic aspirin (100 mg/day per os). In case of hypercholesterolemia, statins were administered and in case of overt diabetes mellitus, the patients received anti-diabetic drugs. First-line therapy of CML and co-medications during Nilotinib are shown in Table S3, and metabolic parameters before and during treatment in Table S4. We also examined 4 age- and AOD-risk factor-matched control-cohorts (MOT: 2 years): 34 CML patients receiving Imatinib (400 mg/day), 34 with lymphoid neoplasms (LPN), 34 with JAK2 V617F+ myeloproliferative neoplasms (MPN) and 34 with myelodysplastic syndromes (MDS) (Table S5). The study was approved by the local ethics committee (IRB) of the Medical University of Vienna.

Evaluation of drug effects in a mouse model of lipid-induced atherosclerosis and a model of experimental vascular (arterial) occlusion

Twelve-week-old male ApoE-/- mice received Nilotinib (2x37.5 mg/kg/day orally) or Imatinib (2x50 mg/kg/day orally) and a hyper-lipid diet. After 8 weeks, mice were sacrificed and aortic specimens were examined for signs of atherosclerosis. Hind-limb ischemia was

induced in 12-14 week old C57BL/6 mice by arterial excision. Then, mice were treated with vehicle-control, Nilotinib (75 mg/kg/day) or Imatinib (2x50 mg/kg/day) for 28 days. Thereafter, vascular re-perfusion and hind-limb necrosis were measured. All protocols were approved by the local Ethics Committee and the Austrian Animal Care and Use Committee.

Examination of growth and function of endothelial cells

Cultured human umbilical vein endothelial cells (HUVEC), coronary arterial endothelial cells (HCAEC), vena saphena-derived endothelial cells (HSVEC), and the microvascular endothelial cell line HMEC-1 were examined. Cells were exposed to Nilotinib (0.01-20 μM), Imatinib (0.01-20 μM), or control medium for up to 48 hours. Drug-exposed cells were examined for viability, proliferation, migration, and capillary tube-formation. In target-validation experiments, endothelial cells were incubated with Nilotinib, Imatinib, Ponatinib, Bosutinib, or PF-114, a novel BCR/ABL1 TKI known to spare most vascular target-kinases.[34] In addition, antibodies or drugs directed against individual targets were applied.

Evaluation of drug effects on expression of pro-atherogenic, pro-thrombotic, pro-fibrinolytic and anti-fibrinolytic molecules in endothelial cells

Drug-exposed HUVEC were examined for protein- and mRNA expression levels of ICAM-1 (CD54), E-selectin (CD62E), VCAM-1 (CD106), and urokinase-type plasminogen-activator (uPA) receptor (CD87). In addition, drug-exposed HUVEC were evaluated for expression of tissue-type plasminogen-activator (tPA)-, urokinase(u)PA-, and PA inhibitor-1 (PAI-1) mRNA levels by qPCR.

Evaluation of drug targets expressed in endothelial cells

Binding of coupleable (c) Nilotinib (c-Nilotinib) and c-Imatinib to endothelial kinases was determined by examining lysates of HUVEC and HMEC-1 by chemical proteomics profiling

(CPP) and mass spectrometry (MS) following published techniques.[20] To investigate drug effects on receptor tyrosine kinase (RTK) activity in cytokine-stimulated HUVEC, phospho-RTK array- and Western blot experiments were performed. In these experiments, HUVEC were incubated with Nilotinib or Imatinib (each 1-10 μM) for 1 hour. In select experiments, the effects of ABL2-knockdown on expression of KDR were examined in HUVEC.

In vivo effects of Nilotinib on microvessel density

To assess Nilotinib effects on endothelial cells *in vivo*, BM sections of CML patients were examined for microvessel density before and after treatment with Nilotinib. Moreover, we analyzed tissue sections of C57BL/6 mice after treatment with vehicle control, Nilotinib or Imatinib. Tissue sections were examined for microvessel density (angiogenesis) as well as expression of ICAM-1, VCAM-1, and E-selectin.

Analysis of age-related clonal hematopoiesis in Nilotinib-treated patients

Samples of all 36 Nilotinib-treated patients were analyzed for the presence of age-related mutations, including mutations in *TET2*, *ASXL1*, *DNMT3A*, *ZRSR2*, *CEBPA*, and *SF3B1*, using the Myeloid Solution Kit (Sophia Genetics, Saint Sulpice, Switzerland) according to the manufacturer's recommendations.

Evaluation of TKI effects on platelets

Effects of Nilotinib and Imatinib on platelet adhesion and –aggregation were measured in *in vitro*- and *ex vivo* experiments. In *in vitro* experiments, Nilotinib and Imatinib (20 μM each) or vehicle control (DMSO) were added to hirudin-anticoagulated blood obtained from normal donors. In *ex vivo* experiments whole blood, obtained from CML patients treated either with Nilotinib or Imatinib, was analyzed. Platelet adhesion and plug formation were measured with

the platelet function analyzer PFA-100. Platelet aggregation was measured using the multi-plate electrode aggregometer Multiplate.

Evaluation of TKI effects on pancreatic cells and mast cells

In ^{3}H-thymidine uptake experiments, PANC-1 and 1.4E7 cells were incubated with control, Nilotinib (0.1-1.5 μM) or Imatinib (0.1-1.5 μM) for 48 hours. Normal human islet cells were incubated in control, Nilotinib (1-10 μM) or Imatinib (1-10 μM) for 24 or 48 hours before survival and insulin production was measured. MC were cultured from cord blood mononuclear cells (MNC).[35] Cultures were maintained with or without various concentrations of Nilotinib (0.001-1 μM). After 4 weeks, cells were examined for the percentage of MC. To determine the *in vivo* effects of Nilotinib on tissue MC, we examined MC numbers in BM sections in our CML patients before and after treatment with Nilotinib using immunohistochemistry.[36] In a separate set of experiments, the effects of Nilotinib on tPA production in HMC-1 cells and cord blood-derived human MC were examined by ELISA.

Results

Nilotinib promotes atherosclerosis in ApoE-/- mice and inhibits vascular re-perfusion and angiogenesis after arterial stenosis

Treatment of ApoE-/- mice (under hyper-lipid diet) with Nilotinib (37.5 mg/kg twice daily) for 8 weeks resulted in an augmented lipid-accumulation and plaque-formation in the aortic wall compared to control mice, whereas Imatinib showed no comparable pro-atherogenic effect (Figure 1A). Vascular repair and re-perfusion following arterial occlusion were analyzed in a murine model of hind-limb ischemia. In this model, Nilotinib suppressed re-perfusion and augmented hind-limb necrosis, whereas Imatinib showed no significant effect (Figure 1B,C). Plasma Nilotinib levels measured during treatment were 2.1±2.7 μM and plasma Imatinib levels 1.39±0.55 μM. As assessed by immunohistochemistry, Nilotinib-treatment decreased the numbers of NG2+ capillaries and thus suppressed neo-angiogenesis after arterial occlusion in mice (Figure 1D). In patients with CML, treatment with Nilotinib was followed by a substantial decrease in the BM microvessel density compared to pre-treatment values (Figure 1E). Together, treatment with Nilotinib is associated with pro-atherogenic changes and reduced angiogenesis (reduced vascular repair) after arterial ligation.

Evidence for direct growth-inhibitory effects of Nilotinib on endothelial cells

Nilotinib was found to inhibit *in vitro* growth and viability in primary cultured human vascular endothelial cells and in the human microvascular endothelial cell line HMEC-1, whereas Imatinib showed no comparable effects (Figure 2A). The effects of Nilotinib were seen in all human endothelial cell types examined, including HMEC-1, HUVEC, HCAEC, and HSVEC. The effects of Nilotinib were dose-dependent, with IC_{50} values ranging between 0.5 and 1 μM (Table S6). Growth-inhibitory effects of Nilotinib were seen in the presence and absence of VEGF (Figure S1A), and were accompanied by increased expression of active

caspase 3 and 7 (Figure 2B and Figure S1B) suggesting drug-induced apoptosis. Again, no significant effects were seen with Imatinib. Moreover, in contrast to Imatinib, Nilotinib suppressed endothelial cell migration in a wound scratch assay (Figure 2C,D) as well as VEGF-induced tube formation (Figure 2E).

Nilotinib induces a pro-atherogenic phenotype in endothelial cells

An increased expression of cytoadhesive cell surface molecules (CAM) on endothelial cells has been implicated as a critical event in early stages of atherosclerosis.[37,38] In this study, we examined the effects of Nilotinib and Imatinib on CAM expression on endothelial cells. Nilotinib was found to promote expression of the pro-atherogenic surface antigens ICAM-1, VCAM-1, and E-selectin in HUVEC whereas no comparable effects were seen with Imatinib (Figure 3). The effects of Nilotinib on CAM expression were demonstrable at the mRNA level (Figure 3A) and surface-protein level (Figure 3B). In addition, microvascular endothelial cells expressed increased levels of VCAM-1 in Nilotinib-treated mice compared to control-mice (Figure 3C). We also examined the effects of Nilotinib on expression of pro- and anti-fibrinolytic molecules in HUVEC. However, no significant effects of Nilotinib on expression of tPA-, uPA-, PAI-1-, or uPAR mRNA levels were seen (data not shown).

Nilotinib interacts with multiple molecular targets in endothelial cells

Using chemical proteomics profiling (CPP) and mass spectrometry (MS), we identified a number of Nilotinib-targets in human endothelial cells. Major targets identified by Nilotinib but not Imatinib in HUVEC and HMEC-1 were Tie-2/TEK, ABL2, JAK1, BRAF, and several MAP kinases (MAPK) (Figure 4 and Table S7). As assessed by phospho-array profiling and Western blotting, Nilotinib was found to inhibit the phosphorylation of KDR, TEK, FGFR3, and ERK (MAPK-signaling) in HUVEC and HCAEC (Figure S2). Moreover, we found that a drug- or siRNA-induced downregulation of ABL2 leads to a decreased expression of KDR in

HUVEC and HCAEC (Figure S3). In control experiments, we also analyzed the effects of a polyclonal anti-KDR antibody. In these experiments the anti-KDR antibody (50 ng/ml) produced a significant inhibition of HUVEC migration (Figure S4). In validation experiments, Ponatinib, a TKI recognizing most ´vascular´ Nilotinib-targets suppressed endothelial proliferation, with even lower IC50 values compared to Nilotinib (Figure S5A) whereas PF-114, a BCR/ABL1 TKI sparing most ´vascular´ targets, showed no comparable effects on HUVEC (Figure S5B). In an attempt to identify kinases responsible for the observed Nilotinib effects, we applied a series of more specific kinase-blockers, including MAPK14-, KDR-, TEK-, BRAF-, and JAK1 inhibitors. Whereas the individual drugs tested did not suppress endothelial growth at the concentrations applied, a mixture of all drugs suppressed HUVEC growth in the same way as Nilotinib (Figure S5C), suggesting that multiple Nilotinib targets may be involved in the observed drug effects. IC_{50} values for all compounds tested are shown in Table S6.

Delineation of additional Nilotinib effects potentially contributing to vascular damage and atherosclerosis

A number of previous data suggest that mast cells (MC) play an essential role in the vascular repair accompanying thromboembolic events.[39,40] Most importantly, MC are well known as unique source of heparin and uncomplexed tPA, and these cells accumulate around the thrombosed vessels and release their tPA and heparin which facilitates thrombolysis and the consecutive repair in the affected organ.[39,40] We found that Nilotinib inhibits stem cell factor (SCF)-dependent growth of human MC in a long-term culture system (Figure S6A). Correspondingly, BM MC numbers and serum tryptase levels decreased in our CML patients during Nilotinib therapy (Figure S6B,C). Moreover, Nilotinib suppressed SCF-independent and dependent secretion of tPA in MC (Figure S6D,E). No significant effects of Nilotinib or Imatinib on platelet adhesion or aggregation were seen (Figure S7). Finally, we asked whether

Nilotinib affects pancreatic cell function or insulin production. Specifically, we examined the effects of Nilotinib on proliferation of the human pancreatic cancer cell lines PANC-1 and 1.4E7, and insulin production in primary human pancreatic islet cells. We found that Nilotinib but not Imatinib inhibits proliferation in PANC-1 and 1.4E7 cells (Nilotinib IC50: 0.25-0.5 μM versus Imatinib IC50: >1.5 μM). Human islet cells were incubated in control medium or medium supplemented with either Nilotinib or Imatinib (1-10 μM) for 24 or 48 hours before survival and insulin production were measured. In these experiments, no effects of Nilotinib or Imatinib on insulin production or survival of islet cells were seen (data not shown).

AOD events accumulate over time in patients receiving Nilotinib

CML patients receiving Nilotinib were examined after a median observation time (MOT) of 2.0 years (n=34), 3.0 years (n=36) and 3.7 years (n=36). After 2.0 years, 10/34 patients (29.4%) had developed one or more AOD events. Of these, 7 (20.6%) had peripheral AOD, one a myocardial infarction, one a coronary syndrome requiring bypass surgery, and one a spinal infarction. The frequency of AOD in age- and risk factor-matched controls (Imatinib-treated CML, lymphoproliferative neoplasms, *JAK2* V617F+ myeloproliferative neoplasms, and myelodysplastic syndromes; n=34 each) after 2 years was significantly lower (<5%) than that in the Nilotinib-cohort ($p<0.05$) (Figure 5A). In Nilotinib-treated patients, AOD events were found to accumulate over time. After a MOT of 3.0 years, the frequency of AOD+ patients increased to 38.9%, and after a MOT of 3.7 years, it reached 47.2% (Figure 5B). However, the frequency of patients with severe AOD did not increase after 3.0 years (Figure 5B). In 8 patients, one or more pre-existing risk factor(s) for atherosclerosis were identified. A substantial number of our patients (n=36) were found to be at high risk according to the European Society of Cardiology (ESC) score (Figure 6A, Table S8). We also counted the total number of events in all patients. After 3.7 years, 39 symptomatic AOD events were recorded in our 36 patients, resulting in 32 AOD events per 100 patient-years which exceeds by far the

incidence of symptomatic AOD events in the general population of high-income countries[41] with a 1-year hazard ratio of 4.72 (95% confidence interval: 2.82-7.90) ($p<0.01$) (Figure 6B). The number of severe AOD events per 100 patient years in our Nilotinib-treated CML patients was 17.4. Interestingly, however, no venous thromboembolic events were recorded in the CML patients examined during treatment with Nilotinib. Metabolic parameters, including fasting glucose levels and cholesterol levels before and during Nilotinib therapy, are shown in Table S4. Confirming the available literature, an increase in fasting glucose levels and cholesterol levels was found in a subset of our Nilotinib patients.

The impact of the somatic background on AOD development

Recent data suggest that somatic mutations in *TET2*, *ASXL1*, *DNMT3A*, *ZRSR2*, *CEBPA*, and *SF3B1*, are associated with an increased risk of healthy individuals to develop a myeloid neoplasm but also with an increased risk to develop a cardiovascular disease.[42] In the present study, we found that these mutations, especially mutations in *TET2*, *ASXL1*, and *DNMT3A*, are more frequently detectable in CML patients who developed AOD during treatment with Nilotinib compared to those who did not develop AOD (Figure 6C). This pattern did not change when excluding patients with elevated BCR/ABL1 (>1%) (Figure S8). We also screened for other well-established molecular risk factors. However, no Factor V Leiden mutation, and no prothrombin mutations were detected. However, in one patient (#31) low levels (1.6% allele frequency) of *JAK2* V617F were detected.

Discussion

In most patients with CML, BCR/ABL1 TKI induce major molecular responses and long-term relapse-free survival.[4-6,9,10] Therefore, long-term safety during treatment with BCR/ABL1-targeting TKI is an important issue. Initially, Nilotinib was considered a well-tolerated drug.[15-18] However, recent studies reported on an increased risk of AOD in patients receiving Nilotinib.[24-30,43] We analyzed potential mechanisms underlying AOD-development and found that Nilotinib augments atherosclerosis in ApoE-/- mice and inhibits vascular reperfusion and angiogenesis in a model of hind-limb ischemia. Moreover, Nilotinib was found to exert pro-atherogenic and anti-angiogenic effects on human endothelial cells. We hypothesize that these effects as well as the metabolic impact of Nilotinib act together to trigger atherosclerosis and vascular occlusion in patients with CML (Figure 7).

We and others have recently shown that AOD occur in Nilotinib-treated CML patients.[24-30] However, the observation-time was relatively short and no robust control-cohorts were examined. In the current study, we analyzed all Nilotinib-treated patients in our center over time, and included several control cohorts. The frequency of AOD in Nilotinib-treated patients exceeded the AOD rates recorded in age-matched and risk factor-matched controls, including CML patients receiving Imatinib and patients with other hematopoietic neoplasms. Even in patients with *JAK2* V617F+ MPN, in whom the disease-related risk of vascular events is high, the frequency of AOD was lower compared to our Nilotinib-treated patients. Together, these data strongly argue for a relationship between Nilotinib-intake and the occurrence of AOD. An alternative explanation would be that previous treatment with Imatinib provided a protective effect against AOD-development and that the switch to Nilotinib was associated with a rebound facilitating AOD evolution. Indeed, Imatinib has been described to exert protective effects against atherosclerosis *in vivo*.[44] However, AOD

events were found to accumulate steadily over time in our Nilotinib-treated patients, with a maximum of 47.2% after a MOT of 3.7 years, which argues against a simple rebound-effect.

The etiology of atherosclerosis is complex and includes genetic, epigenetic, metabolic, and other factors.[37,38] A key-event in early atherosclerosis may be an altered endothelial function with increased expression of CAM and consecutive pro-atherogenic changes in the vessel wall.[37,38] We found that Nilotinib promotes atherosclerosis in ApoE-/- mice and induces the expression of pro-atherogenic adhesion-molecules in endothelial cells. In addition, Nilotinib was found to inhibit angiogenesis and thus the vascular repair in a model of arterial stenosis in mice, and to block proliferation, migration, and tube-formation in endothelial cells *in vitro*. In each assay, Imatinib was tested in parallel, but did not affect endothelial cell function *in vitro* or vascular parameters *in vivo* in mice.

As mentioned above, an increased CAM expression on endothelial cells is considered a critical event in the evolution of atherosclerosis.[37,38] Once overt AOD is present, additional factors may count, one of them being the vascular repair with proper revascularization that is dependent on angiogenesis. Drugs that act pro-atherogenic as well as anti-angiogenic may thus have a substantial influence on AOD development. Based on the data generated in this study, Nilotinib appears to be such a drug that acts pro-atherogenic as well as anti-angiogenic at the same time.

We next explored molecular Nilotinib-targets expressed in endothelial cells. As assessed by CPP+MS, Nilotinib was found to bind to a number of kinase-targets relevant to vascular repair and/or atherosclerosis. Several of these target-kinases, including TEK, BRAF, JAK1, and several MAPKs, were only recognized by Nilotinib, but not by Imatinib. We were also able to show that Nilotinib blocks the activity of several target-kinases *in vitro*. At higher concentrations, Nilotinib also suppressed KDR activity. However, unexpectedly, KDR was not identified as a kinase-target in the proteomics-approach which may have several explanations. First, the binding of Nilotinib to KDR may be too weak to show up in a

proteomics-approach. Another possibility may be that KDR is an indirect target of Nilotinib, a hypothesis that was supported by the finding that siRNA against ABL2, a major target of Nilotinib, resulted in down-regulation of KDR expression in HUVEC and HCAEC. In this regard it is noteworthy that ABL2 has recently been described as a mediator of TEK-expression in vascular cells.[45] In consecutive validation experiments, Ponatinib, a BCR/ABL1 TKI that identifies a similar set of vascular targets compared to Nilotinib and also promotes vasculopathies[46], blocked the proliferation of human endothelial cells *in vitro*. By contrast, PF-114, a novel BCR/ABL1-blocker sparing several vascular targets, did not inhibit endothelial proliferation below 1 μM. In a next step, we tried to identify those targets that are involved in Nilotinib effects on endothelial cells. In these experiments, a series of specific kinase blockers were applied. Most of these kinase inhibitors did not exert major growth-inhibitory effects on endothelial cells (IC50 >1 μM). However, a mixture of these more specific kinase blockers directed against individual endothelial targets, was found to mimic the growth-inhibitory effects of Nilotinib on endothelial cells. These data suggest that Nilotinib exerts specific effects on endothelial cells via multiple kinase targets.

In almost all patients in whom AOD developed during Nilotinib-therapy, one or more risk factors for atherosclerosis were recorded. On the other hand, even patients with very low risk according to the ESC-score were found to develop AOD. Since age is a major risk factor for AOD development, we also screened for age-related clonal hematopoiesis which has recently been associated with an increased risk of cardiovascular disorders.[42] Indeed our data show that risk-related mutations (*TET2*, *ASXL1*, *DNMT3A*, *ZRSR2*, *CEBPA*, *SF3B1*) cluster preferentially in those Nilotinib-treated patients who developed AOD. These data may have clinical implications and may help select/exclude those patients who have a low/high risk of AOD development during treatment with Nilotinib in the future. We also screened for other molecular risk factors, such as Factor V Leiden or prothrombin mutations. However, no other genetic risk factors were detectable in our Nilotinib-treated AOD patients. An unresolved

question is why our patients developed arterial stenosis selectively, but no venous thromboembolic events. One explanation for this phenomenon may be that Nilotinib primarily exerts pro-atherogenic effects on vascular cells but does not affect pro-thrombotic or anti-fibrinolytic molecules.

An interesting observation was that the numbers of AOD-positive patients increased steadily during a MOT of 3.7 years. However, whereas the total number of affected patients increased steadily, the number of severe events requiring surgery and hospitalization remained rather stable. This may be explained by increased awareness, better patient-selection, early management of AOD-events, and discontinuation of the drug in those who developed severe events. In addition, based on the obvious relationship between drug intake and AOD, we started to administer prophylactic aspirin in our Nilotinib-treated patients. Moreover, we screened for AOD before starting with Nilotinib. Finally, all patients who developed hyperlipidemia received a statin, and all patients with diabetes mellitus were advised to check and adjust their fasting glucose levels. Whether these management will be sufficient to keep AOD rates within an acceptable range during Nilotinib (or Ponatinib) therapy remains to be determined in prospective clinical trials.

Together, Nilotinib exerts direct pro-atherogenic and anti-angiogenic effects on endothelial cells. Together with its well-known metabolic impact, these effects of Nilotinib may explain the rapid evolution of clinically overt AOD in Nilotinib-treated patients with CML.

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Authorship

Contribution: E.H., K.A.S., G.E., I.S., C.K. and J.W. performed experiments on cultured endothelial cells. K.H., F.G., U.R. and G.S.F. performed proteomics and MS studies. W.S., M.T. and R.K. performed experiments in mice. G.H. performed molecular studies. B.J. performed studies on platelet adhesion and aggregation. S.H., G.H.S., W.R.S. and P.V. provided patients and analyzed the clinical and laboratory results. E.H., D.W., R.K. and P.V. drafted parts of the manuscript. E.H. and P.V. established the study concept and wrote the final paper-draft.

Supplementary information is available at Leukemia's website.

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Figure Legends

Figure 1. Nilotinib exerts pro-atherogenic and anti-angiogenic effects *in vivo*. (A) ApoE-/- mice were fed with a high-caloric lipid-diet and were treated either with vehicle control (Control, 9 mice), Imatinib (50 mg/kg twice daily, 9 mice) or Nilotinib (37.5 mg/kg twice daily, 8 mice) for 8 weeks. Then, mice were sacrificed and their aortic root was evaluated for plaque-formation. Results are expressed as percent of aortic areas affected by plaque-formation per mouse and show all mice in each group as well as median values (black bars). (B-D) Hind-limb ischemia was induced in C57BL/6 wild-type mice by ligation of their left femoral artery. Thereafter, mice (13 mice per group) were treated with vehicle control, Imatinib (50 mg/kg twice daily) or Nilotinib (75 mg/kg/day) for 28 days. (B) Ratio of blood flow reconstitution in the ischemic versus non-ischemic limb as assessed by Laser Doppler Perfusion Imaging (LDPI). Results are expressed as mean±S.D. of all mice in each group. (C) Clinically overt (macroscopic) limb necrosis on day 28 provided as mean±S.D. of all mice in each group. (D) Microscopic evaluation of vessel density in hind-limb muscle sections in control mice and Nilotinib-treated mice (ischemic hind-limb) on day 28. Capillary density was determined by immunohistochemistry using an antibody against the pericyte-antigen NG2. Results are expressed as NG2 positive capillaries per microscopic high power field (HPF) and represent the mean±S.D. of all mice in each group. (E) Bone marrow sections were obtained from 7 CML patients before treatment with Nilotinib and after these patients had been treated for more than one year with Nilotinib. Sections were stained using an antibody against CD34 to assess microvessel density. Results show microvessels per HPF and represent the mean±S.D. of all patients. Statistics: (B) Kruskal Wallis test - ANOVA; (C-E) unpaired, two-way t-test - ANOVA; *: $p<0.05$.

Figure 2. Nilotinib inhibits growth, survival and migration of endothelial cells *in vitro*. (A) Human umbilical vein endothelial cells (HUVEC), the human endothelial cell line HMEC-1, and human coronary artery endothelial cells (HCAEC) were incubated with control medium or various concentrations of Imatinib or Nilotinib at 37°C for 48 hours. Thereafter, proliferation was examined by measuring ^{3}H-thymidine uptake. Results are expressed as percent of control and represent the mean±S.D. of at least 3 independent experiments. (B) HCAEC were cultured in control medium or in medium containing VEGF (50 ng/ml). Thereafter, Nilotinib or Imatinib (1 μM each) were added for 16 hours and apoptosis was evaluated by measuring active caspase 3 and 7 by luminescence assay. Results show the mean±S.D. of triplicates. (C) After producing a scratch, HUVEC were cultured in the absence or presence of Imatinib (10 μM) or Nilotinib (10 μM) at 37°C for 18 hours. Migration was assessed by measuring the relative amount (percent) of closed (repopulated) scratch-area. Results are expressed as percent of control (no drugs) and represent the mean±S.D. of 3 independent experiments. (D) Dose-dependent effect of Nilotinib on migration of HUVEC. (E) HUVEC were incubated in matrigel with VEGF (50 ng/ml) in the absence (Control) or presence of Nilotinib (0.1 μM) or Imatinib (0.1 μM) at 37°C for 6 hours. Then capillary tubes were counted. Results show tube formation relative to control and represent the mean±S.D. of 3 independent experiments. Statistics: (A-E) unpaired, two-way t-test - ANOVA; *: $p<0.05$.

Figure 3. Effects of Imatinib and Nilotinib on expression of pro-atherogenic cell surface molecules in vascular endothelial cells. (A) HUVEC were incubated in control medium or medium containing 5 μM of Nilotinib or Imatinib for 24 hours. Then, ICAM-1, VCAM-1 and E-selectin mRNA expression levels were determined by qPCR. Results show mean±S.D. values relative to control of 3 independent experiments. (B) HUVEC were treated with control medium or 7.5 μM of Nilotinib or Imatinib for 4 hours. Thereafter, expression of cell surface adhesion-molecules (CAM) was evaluated by flow cytometry. Results show

mean±S.D. values from 3 independent experiments. (C) Tissue sections of mouse hind-limbs (animals treated with vehicle control, Nilotinib or Imatinib) were examined for expression of VCAM-1 by immunofluorescence-staining. Results show the mean±S.D. of VCAM-1 positive cells per HPF. Statistics: (A-C) unpaired, two-way t-test - ANOVA; *: $p<0.05$.

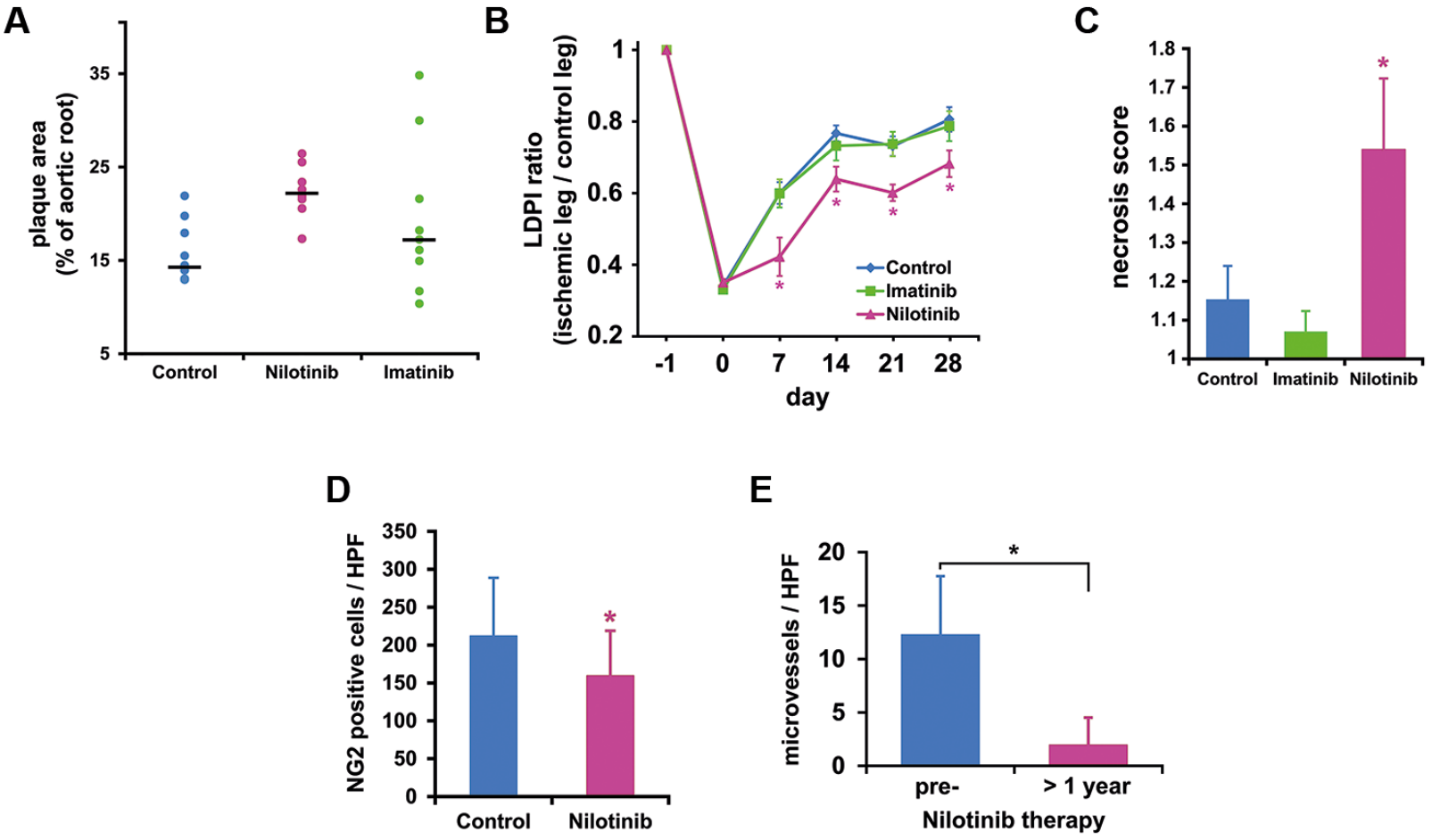
Figure 4. TKI target expression profiles in endothelial cells. Target expression profiles for Nilotinib and Imatinib as determined by chemical proteomics profiling (CPP) and mass spectrometry (MS) performed with lysates obtained from HUVEC (A,B) and HMEC-1 (C,D). (A,C) The figures show targets bound by Nilotinib (red color), Imatinib (green) or both drugs (blue). Kinase targets are indicated by colored ellipses and non-kinase targets by grey ellipses. (B,D) Major kinase-binders in HUVEC and HMEC-1 identified by CPP and MS are shown in form of kinome-maps adapted from Cell Signaling Technology. Binding intensity (spectral counts) is indicated by circle color. Kinases relevant for angiogenesis are marked by a dashed red circle.

Figure 5. Events of arterial occlusive disease (AOD) during treatment with Nilotinib. (A) Five age-matched groups of patients (n=170) were followed in our center and examined for the occurrence of AOD. AOD events (blue bars) and severe AOD events requiring hospitalization and/or intervention (red bars) were recorded retrospectively. Patients included 34 CML patients receiving Nilotinib, 34 CML patients receiving Imatinib, 34 patients suffering from lymphoproliferative neoplasms (LPN), 34 patients with *JAK2* V617F+ myeloproliferative neoplasms (MPN), and 34 patients with myelodysplastic syndromes (MDS). The median observation time in all groups was 24 months. All four control cohorts were matched for age and risk factors for AOD development. (B) The diagram shows the accumulation of AOD events (blue bars) and severe AOD events (red bars) in CML patients treated with Nilotinib in our center.

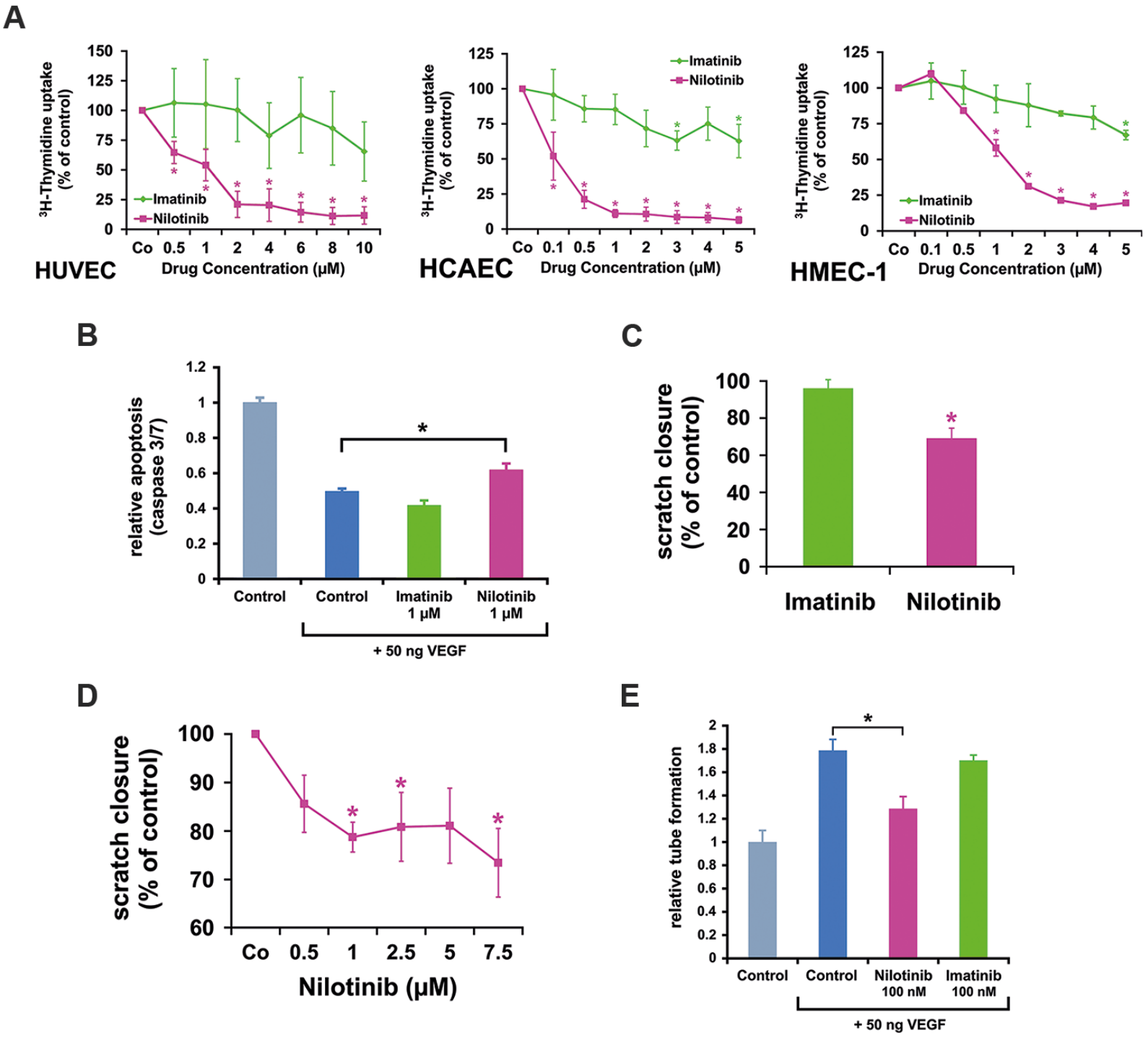
Figure 6. Risk factors for vascular events in TKI-treated patients. (A) Our Nilotinib-treated patients were separated into three risk-groups according to the European Society of Cardiology (ESC) score. The numbers of patients in each ESC-cohort were: 17 in ESC 0-1; 13 in ESC 2-4; and 5 in ESC ≥5. The percentage of patients without AOD (open bars) and with AOD (blue and red bars) is shown for each group. The red color in the blue and red bars depicts the percentage of severe AOD relative to total AOD. (B) The curves show the reduction of the cumulative fraction of individuals without AOD in our Nilotinib-treated patients (red line) and a risk-factor-matched population in high-income countries described in a meta-analysis by Fowkes et al.[41] (blue line). The difference in reduction between the two groups is statistically significant: unpaired, two-way t-test: $p<0.05$. (C) Overview of myeloid neoplasm/CHIP-related gene variants in all 36 Nilotinib-treated patients. Peripheral blood samples were analyzed for the presence of age-related, somatic mutations at the time of best response. White numbers indicate the variant allele frequencies. The right column shows the BCR-ABL1 mRNA levels (% of ABL1 according to IS) at the time of sampling; levels lower than 1% are depicted in green, levels above 1% in red. The difference in the percentages of mutations between the two groups of patients (AOD patients versus patients without AOD) was significant ($p<0.05$) as assessed by the chi-squared test. AOD: Arterial occlusive disease; CHIP: clonal hematopoiesis of indeterminate potential indicating the presence of at least one myeloid neoplasm/CHIP-related mutation.

Figure 7. Potential etiology of vascular events in TKI-treated patients. Proposed mechanisms underlying Nilotinib effects on AOD development: Nilotinib acts on a number of different target kinases expressed in endothelial cells and presumably also in many other cell types. Vascular targets that may play a pathogenetic role include TEK, KDR, MAPKs and JAK1. Nilotinib exerts pro-atherogenic and growth-inhibitory effects on vascular endothelial cells, presumably via these and other (unknown) target structures. However, Nilotinib also

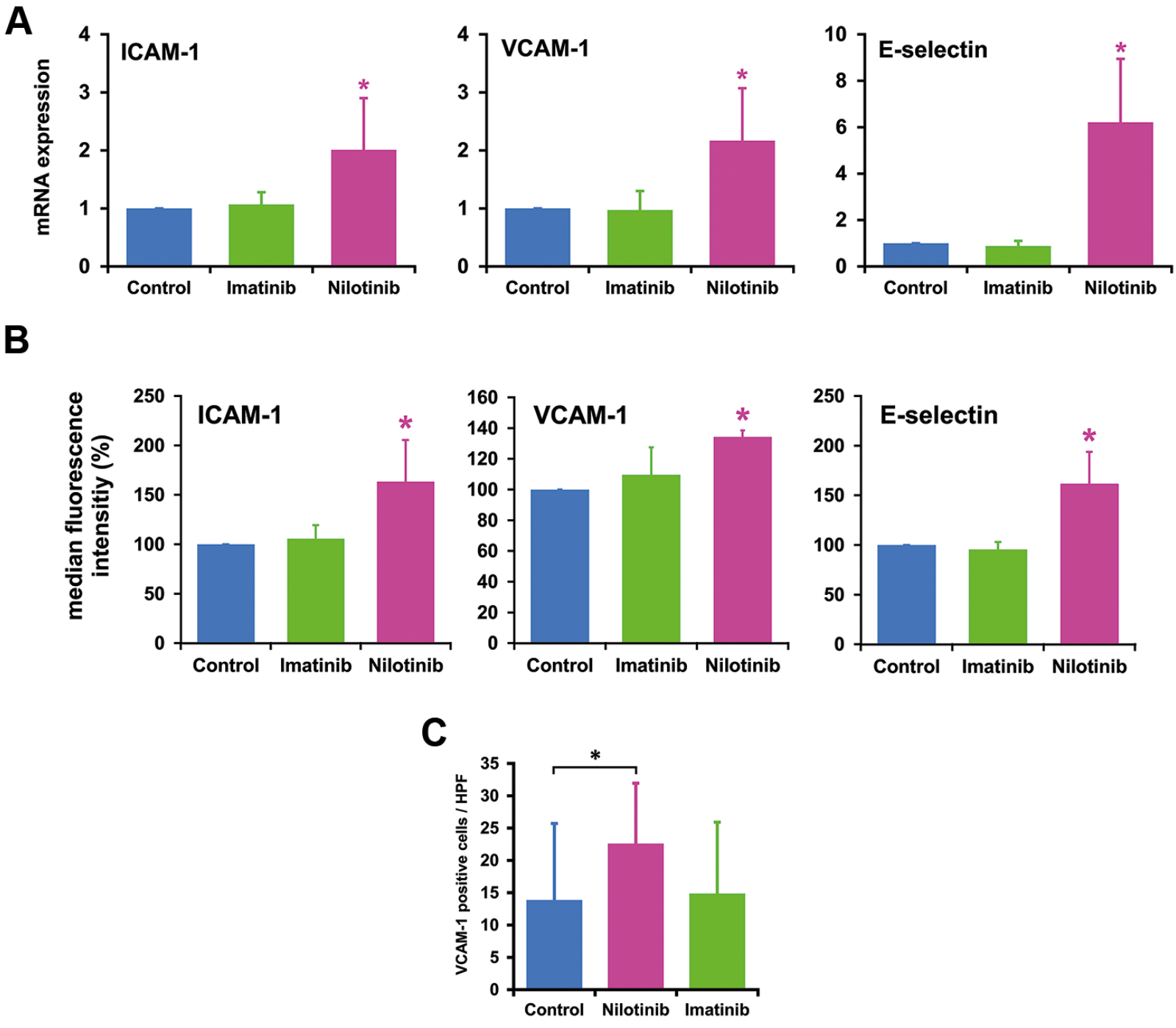
673 induces peripheral insulin resistance with subsequent elevations in blood glucose levels and
674 hypercholesterolemia. All these factors as well as pre-existing (patient-related) risk factors
675 including acquired somatic mutations may act together to trigger atherosclerosis and thus
676 AOD development in Nilotinib-treated patients with CML. AOD: Arterial occlusive disease;
677 JAK1: Janus kinase 1; KDR: Kinase insert domain receptor; MAPKs: Mitogen-activated
678 protein kinases; TEK: Angiopoietin-1 receptor.



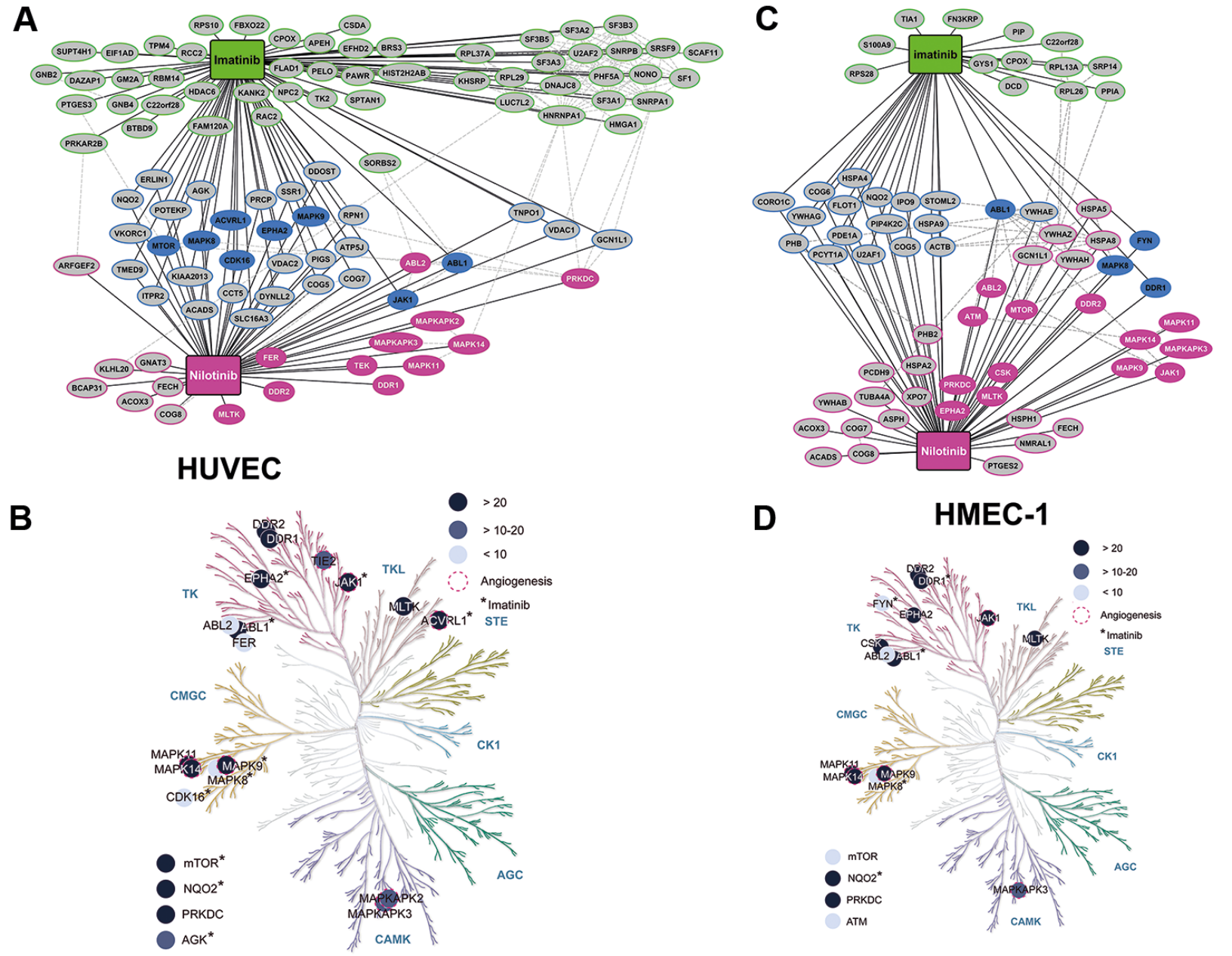
Hadzijusufovic et al., Figure 1



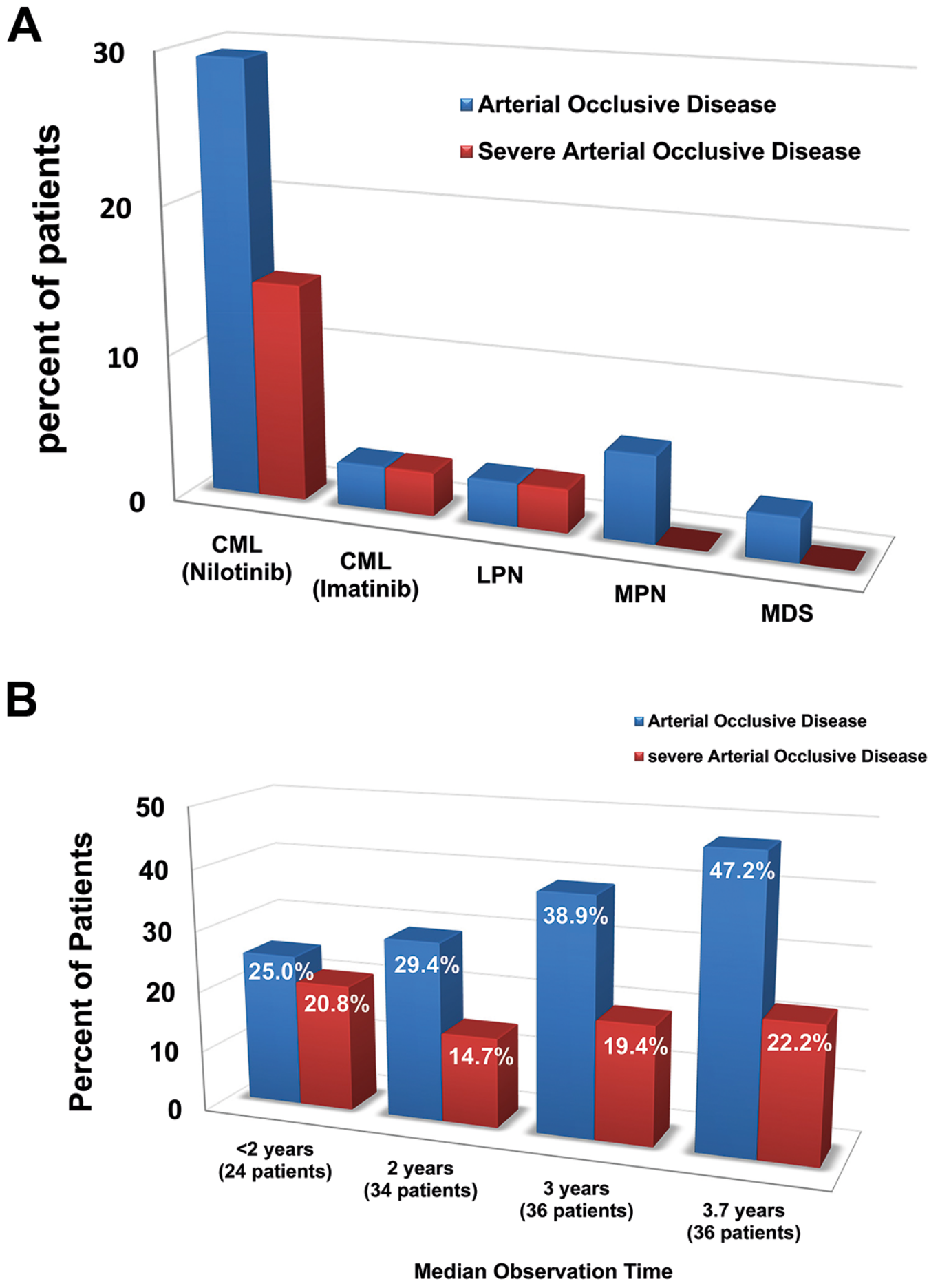
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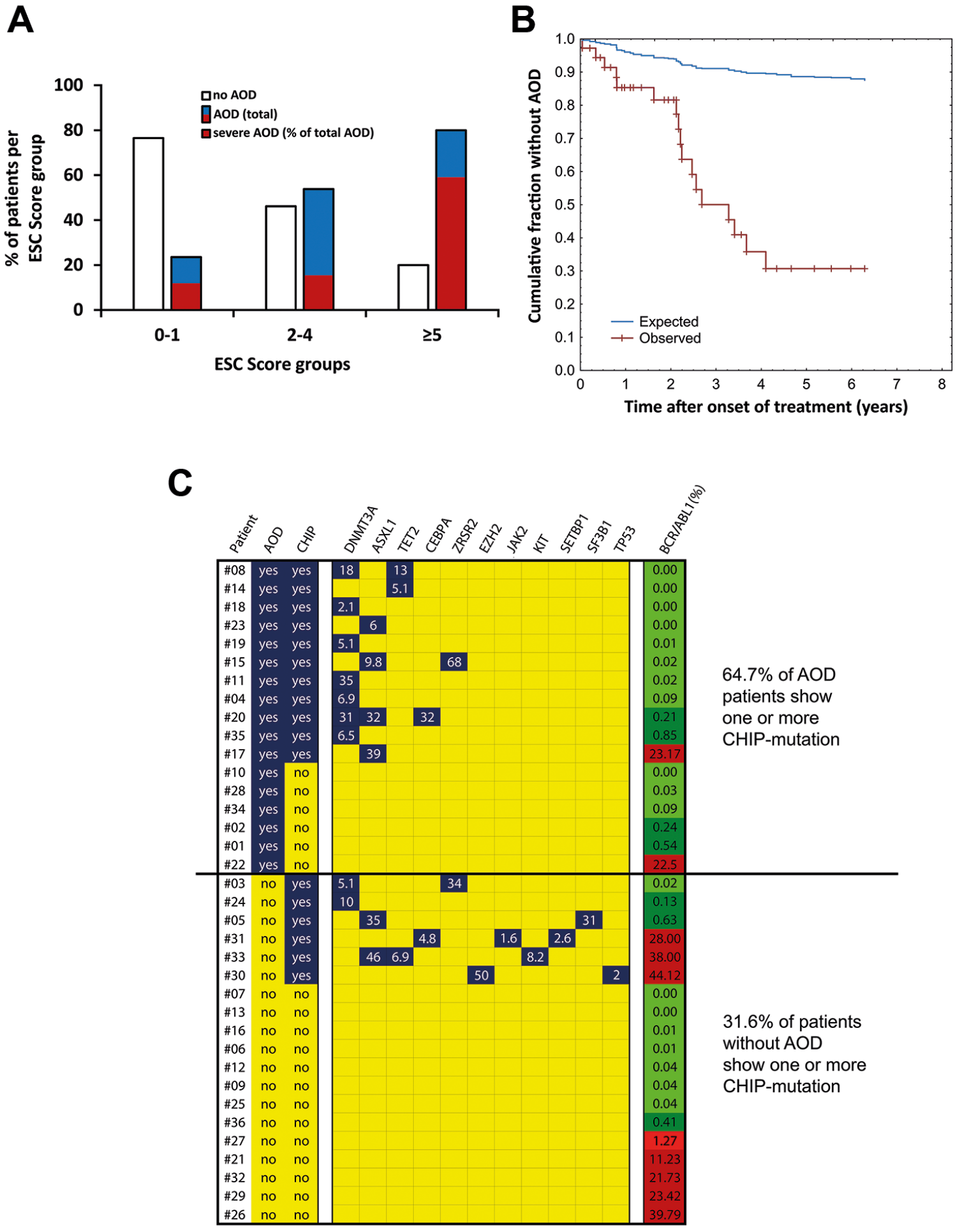
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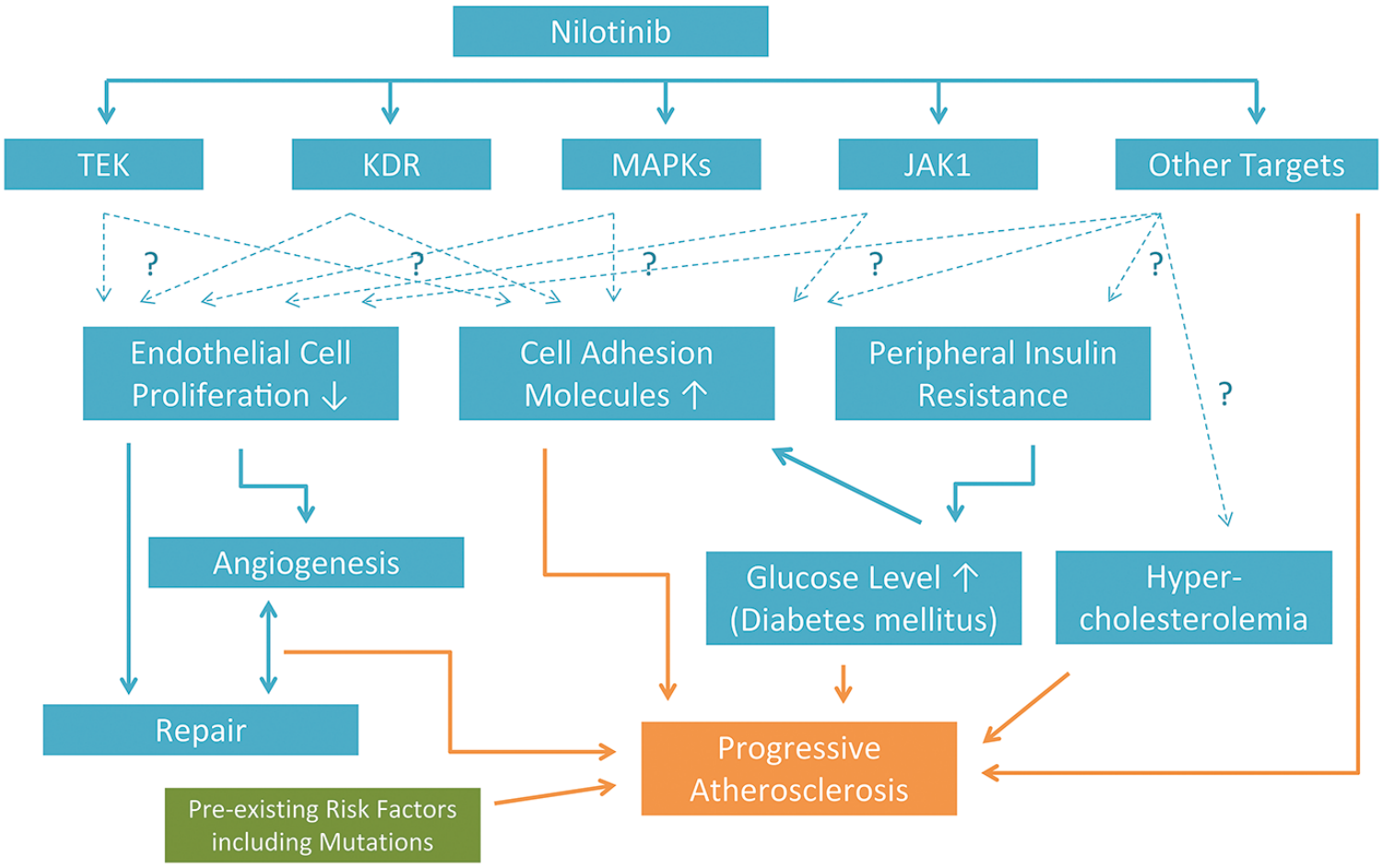
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Hadzijusufovic et al., Figure 5



Hadzijusufovic et al., Figure 6



Hadzijusufovic et al., Figure 7