The Immunophenotype of Mast Cells

The Search for Diagnostic & Prognostic Features within Systemic Mastocytosis

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Word of thanks

I am grateful for the opportunity that has been given to me to work on this project. I would like to thank my supervisors, Andre Mulder, Kees Meijer and Marije Nijk for their positive input and feedback and Rene Mulder for his help. I would like to thank my father, and my whole family for what they have given me.

And all praise is due to my Lord.

Hereby, I present to you a modest piece of research on the disease of mastocytosis.

Contents

Abstract

Systemic mastocytosis is a clonal disorder that is defined by the neoplastic proliferation of MCs in one or more organ systems where the cKIT D816V activating point mutation plays a predominant role. Constitutive activity of the KIT receptor, excessive proliferation, increased cell survival and an aberrant expression of a series of proteins is induced. The WHO 2017 classification of SM is clinically subdivided into different forms; ie. indolent systemic mastocytosis (ISM), smouldering systemic mastocytosis (SSM), aggressive systemic mastocytosis (ASM), systemic mastocytosis with an associated hematological neoplasm (SM-AHN) and mast cell leukemia (MCL). In some cases ISM disease progression to ASM or MCL occurs. The immunophenotype of MCs aids in the determination of the diagnose of SM. Signalizing specific patterns in the immunophenotype of MCs that associate with the development of an indolent form of SM to a non-indolent form of SM or an AHN could be of great prognostic value. In this research the aim is to discover SM subtypes on immunophenotypical grounds and to detect associations between immunophenotype and clinical features of added value.

In this study, a series of immunophenotypical patterns within SM versus non-SM cases have been characterized. For this, a flow cytometric gating strategy has been developed. A frame of parameters consisting of clinical features with implications for clinical treatment and decision making is set. It is of importance that the data for this frame is collected, and coupled to the immunophenotypical database. Thereby extrapolating our immunophenotypical findings to the clinical setting, and thus finding associations that may help considering time and choice of therapy.

Introduction

Mast Cell Function

Mast cells (MCs) are part of the myeloid cells, have a widespread distribution in skin tissue and other peripheral organs and play a key role in the body immune system. Their ability to recognize various stimuli at the interface between host and the external environment, and thereupon release specified biological mediators, allows them to play a master regulator role in the innate as well as the adaptive immunity. [1] [2] MCs contain secretory granules with cytokines, chemokines, growth factors, lysosomal enzymes and other proteins, with a spectrum of functions. It is widely accepted that the high-affinity IgE receptor (FceRI) on MCs plays a central role in acute allergic reaction, and is triggered through cross-linking with IgE. [3] This explains the unique role of mast cells in allergic inflammation. In figure below, potential MC interactions of which among others inflammatory activation, recruitment of other immunocytes and bidirectional signaling with the nervous system are depicted.

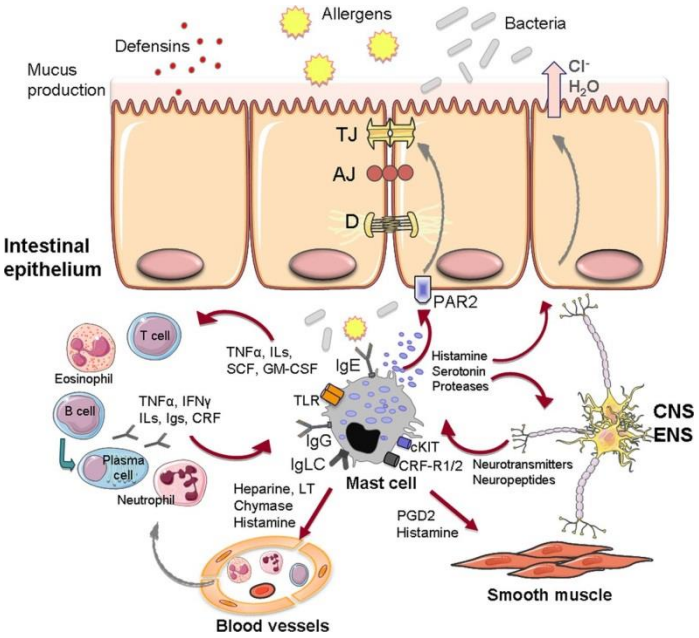


Figure 1 Potential mast cell functions and interactions in intestinal mucosa. [4]

Consequently, MC pathology leads to an array of diseases that may be classified as mainly allergic disorders and clonal disorders. [1] [5] From clinical aspect, the specialism of allergology is most involved in mast cell activating diseases (MCADs), whereas the specialism of hematology is more focused on myeloproliferative neoplasms (MPNs) of the MC and others. Worthy of mention, patients with allergic disorders typically do not exhibit organ dysfunction, while patients with clonal disease may do.

Mast Cell Maturation and Pathogenesis

Maturation of MCs starts with the differentiation of a $CD13^+$, $CD34^+$, $CD117^+$ and $FceRI^-$ pluripotent hematopoietic stem cell to the common myeloid progenitor as a result of IL-1, IL-3, IL-6, stem cell factor (SCF) and granulocyte-macrophage colony-stimulating factor (GM-CSF). [6] [7] [8] Thereafter the common myeloid progenitor maturates towards an MC as a result of SCF and IL-3. [6] Early MC committed progenitors are $CD13^+$, $CD33^+$, $CD34^+$, $CD45^+$, $CD117^+$, $CD123^+$ and $FceRI^-$. CD13, CD45

and CD117 are up- and CD34 and CD123 are downregulated during maturation. [9] The location of the mast cell in the classical model of hematopoiesis and marker expression during MC maturation are shown below.

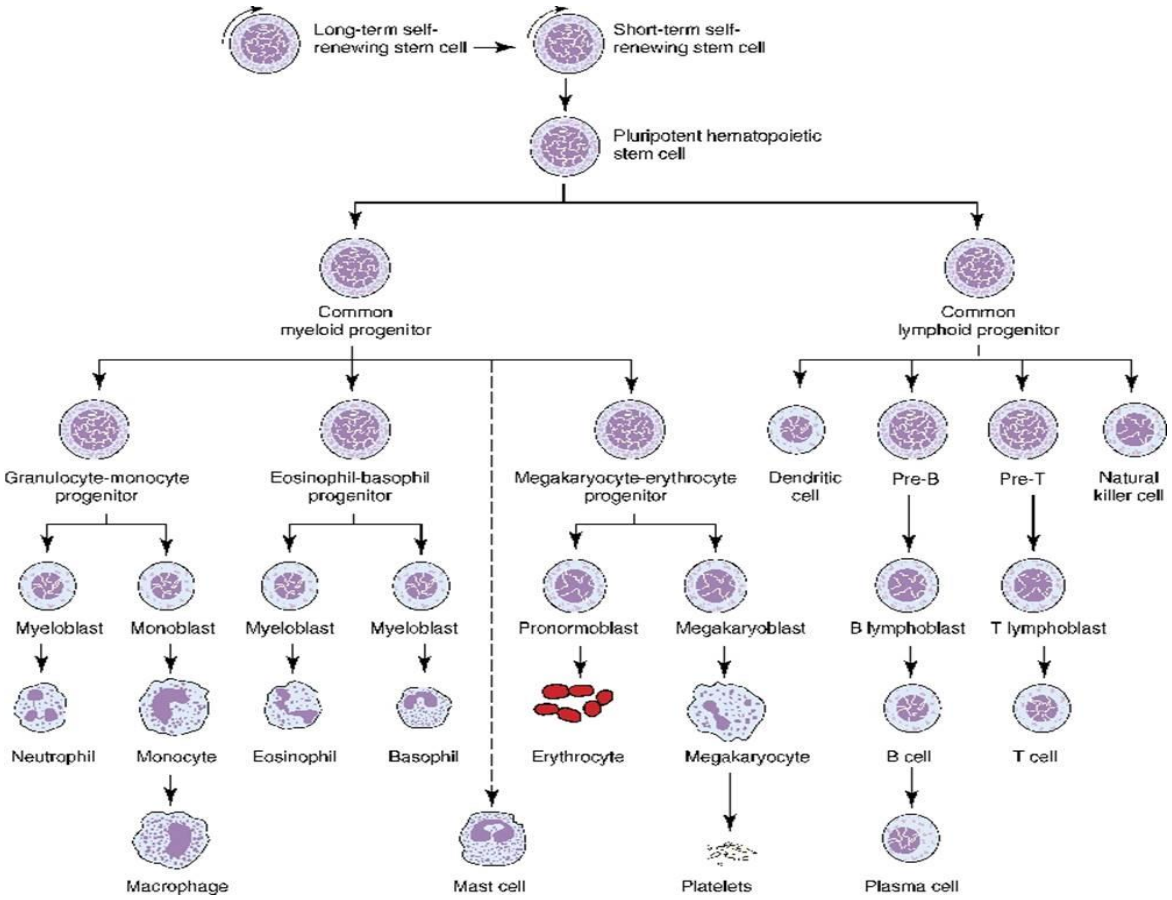


Figure 2 Classical model of hematopoiesis. Image taken from Doctorlib.info [10]

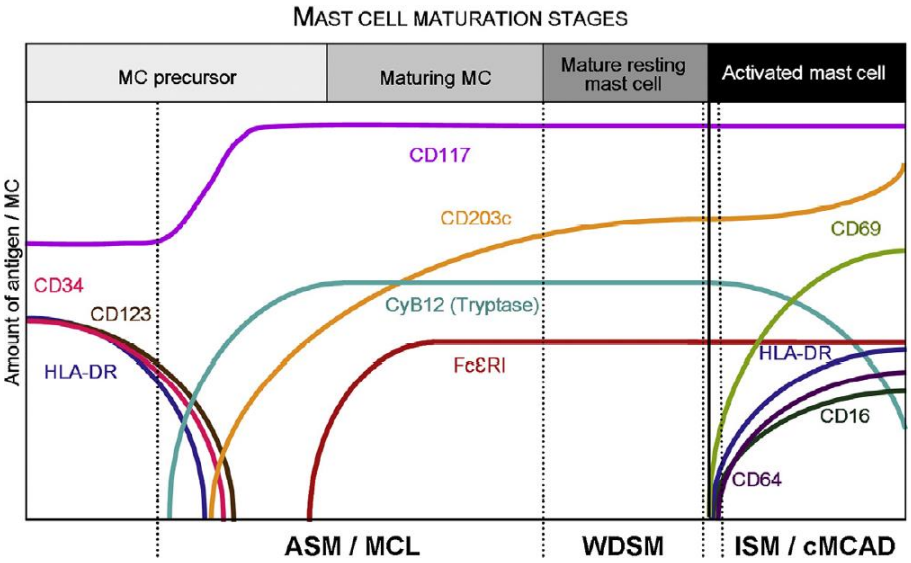


Figure 3 Maturation diagram of MCs. Image taken from Sánchez-Muñoz et al [9]

Mastocytosis is a clonal disorder that is defined by the neoplastic proliferation of MCs in one or more organ systems where the cKIT D816V activating point mutation plays a predominant role. [11] The KIT gene consists of 21-exons and is located on human chromosome 4q12. It is a proto-oncogene that plays the central role in the maturation of an MC and leads to the expression of stem cell growth factor receptor (SCFr or CD117), a tyrosine kinase membrane receptor. The KIT receptor (SCF receptor or CD117) is a 976 amino acid protein with a molecular weight of 145 kDa. [12] [13] KIT ligand (or SCF) enhances development, proliferation, maturation, survival and mediator release from MCs. In normal conditions, regulation of KIT receptor is tightly controlled by KIT ligand. In contrast, if cKIT D816V activating point mutation occurs, constitutive activity of the KIT receptor, excessive proliferation, increased cell survival and an aberrant expression of a series of proteins is induced. [14] [15] [16] In figure below the mutation location on CD117 is illustrated.

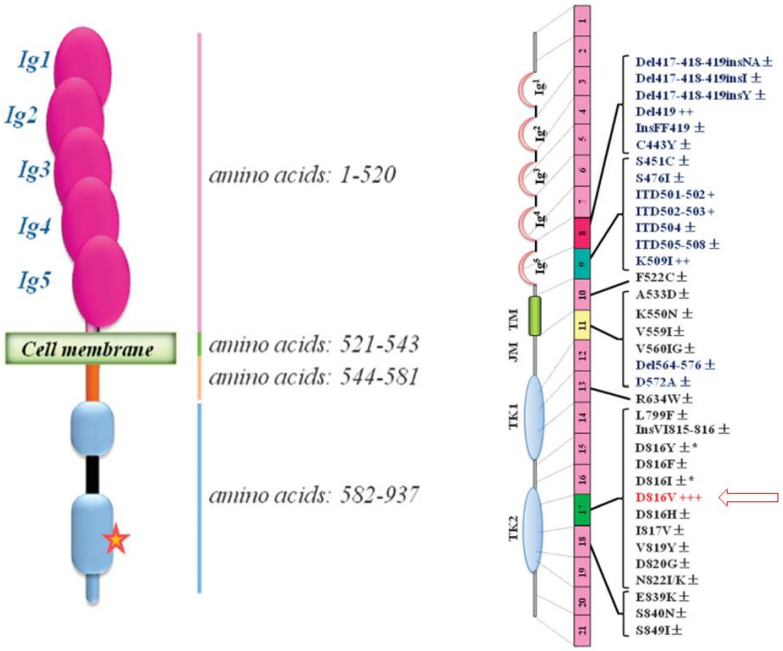


Figure 4 Location of D816V KIT mutation on tyrosine kinase receptor. Image taken from Leukemia (2015) 1223-1232

Diagnosis of Mastocytosis

The diagnosis of mastocytosis as handled in the World Health Organization (WHO) depends on several clinical, morphological, molecular and genetic observations. One of the most evident observations in mastocytosis includes topical involvement. [17]

Additionally, mastocytosis may present systemic involvement. The presence of more than one multifocal dense infiltrate consisting of at least 15 MCs in aggregate in a bone marrow (BM) biopt is considered a major criterion for systemic involvement in mastocytosis. If additionally one of the following minor criteria, or three of them alone are found positive, the diagnosis of systemic mastocytosis (SM) is established. The minor criteria are first, more than 25% of MCs in BM or other extracutaneous organs being spindle-shaped or having atypical morphology. Secondly, detection of the activating point mutation at codon 816 of the KIT-gene in BM or other extracutaneous organs. Thirdly, expression of CD25 on the cell surface of MCs in BM or other extracutaneous organs. Fourthly, serum total tryptase being persistently higher than 20 ng/mL in the absence of an associated hematological neoplasm (AHN). [18]

Classification of Systemic Mastocytosis

The WHO 2017 classification of SM is clinically subdivided into different forms; ie. indolent systemic mastocytosis (ISM), smouldering systemic mastocytosis (SSM), aggressive systemic mastocytosis (ASM), systemic mastocytosis with an associated hematological neoplasm (SM-AHN) and mast cell leukemia (MCL). The subclassification of SM into its' different forms is bound to "burden of disease" B-findings and "cytoreduction-requiring" C-findings which correlate with the graveness of the disease and indicate organ involvement with or without organ toxicity respectively. Detection of an MC burden that reaches up to more than 30% infiltration on BM biopsy with serum total tryptase > 200 ng/mL is considered a B-finding. Signs of dysplasia in non-MC lineages with the absence of an AHN likewise. Lastly, a palpable hepato- and/or splenomegaly is also considered a B-finding. C-findings are confined to first, BM dysfunction caused by neoplastic MC infiltration, manifested by one or more cytopenia with cut off values for absolute neutrophil count < 1,0 x 10^9 /L, hemoglobin level < 10 mmol/L and platelet count < 100 x 10^9 /L. Secondly, hepatomegaly with impairment of liver function which may –in its most severe forms- cause ascites or portal hypertension. Thirdly, skeletal involvement in the form of osteolytic lesions (osteoporosis is a diffuse form of skeletal degeneration and does not qualify as a C-finding). Fourthly, splenomegaly with hypersplenism. Fifthly, malabsorption with weight loss caused by gastrointestinal MC infiltration. [18] For the readers' convenience table 1 summarizes the stated classification.

Table 1 Classification of SM based on B and C findings and provisional subtypes. Data from Horny HP, Akin C, Arber D, et al. Mastocytosis. In: WHO classification of tumors of haematopoietic and lymphoid tissues. Revised 4th edition. Lyon (France): IARC Press; 2017.

	Criteria for SM	**Provisional subtype**
ISM	No C findings	Bone marrow mastocytosis
SSM	>1 B findings & no C findings	
SM-AHN	Criteria for SM MDS/MPN, AML, lymphoma or other AHN apply	
ASM	1 or more C findings	Transformational: 5%<BMMCs<20% Untransformational: BMMCs<5%
MCL	BMMCs ≥20%;	Classic/aleukemic: ≥10%/<10% Chronic/acute: no C findings, ≥1 C finding

SM can be manifested with an AHN. In most cases an AHN is presented as a myelodysplastic syndrome (MDS), myeloproliferative neoplasm (MPN), MDS/MPN, acute myeloid leukemia (AML) or other hematologic neoplasms. [18]

In the WHO classification system the following criteria are held for the subclassification of SM: No C-findings and no more than one B-finding meets the criteria for ISM. No C-findings but more than one B-finding corresponds with SSM. Having one or more C-findings, irrespective of the B-findings, meets the criteria for ASM. Furthermore, for the verification of MCL no B- or C-findings are taken into account, instead if >20% MCs are found in BM the diagnosis of MCL is confirmed. [18]

Provisional Subtypes of Systemic Mastocytosis

Different SM subtypes are further classified. Within ISM a rare form which is associated with better prognosis is reported, namely isolated bone marrow systemic mastocytosis. [19] ASM can be divided into a transformational state (ASM-t) if 5%<MC<20% in BM is found or into an untransformational state if

MC<5% in BM is found. [20] MCL can be either classic or aleukemic MCL, where MCs make up ≥10% or <10% of the peripheral blood (PB) white blood cells count respectively, and chronic or acute MCL, where respectively solely organ enlargement or organ toxicity is observed. [21]

One of the less well-described subtypes of SM is well differentiated systemic mastocytosis (WDSM). It is reported to typically show compact MC infiltrates in BM, have a round morphology, having no expression of CD25 or CD2 and showing no KIT mutation. It may present as any category of SM considering B- and C-findings.

Prognosis of Systemic Mastocytosis

SM shows clinically distinctive subtypes that bear significantly different prognoses. This is important considering time and choice of therapy.

ISM has a low disease progression rate and survival in patients with ISM is superior to that of patients with ASM and MCL and does not significantly differ from overall healthy patients. [22] Median age within ISM patients at time of first symptoms is lower compared to other types of SM. ISM patients show anaphylactic reactions and skin involvement in greater extent. It is estimated that around 95%, 50% and less than 50% of ISM, ASM and MCL patients respectively show skin involvement. [23] Also, the risk of anaphylactic reactions in children correlates with skin involvement and serum tryptase levels. [24] [25]

Nonetheless in some cases ISM disease progression to ASM or MCL occurs. SM may present as smoldering neoplasm and slowly progress to ASM, but may also present as rapidly progressing ASM or MCL. Additionally, a patients' disease may begin as ISM only to develop more aggressive associated hematological neoplasms such as acute myeloid leukemia (AML). [26]

Multilineage involvement in the KIT mutation and increased serum β2-microglobulin are so far known the most powerful independent parameters for predicting transformation into a more aggressive form of the disease. [27]

In general, significant inverse associations between patient survival and WHO subtype, advanced age, weight loss, anemia, thrombocytopenia, hypoalbuminemia, and increased bone marrow blasts have been found. [27]

Immunophenotype of Mast Cells

The immunophenotype of MCs aids in the determination of the diagnose of SM. Different biomarkers have been described in literature to be involved in SM. Prominently, if bone marrow mast cells (BMMCs) show positive CD25 expression then one of the minor criteria for SM is fulfilled. Additionally, biomarkers like CD2, CD52, CD22 and CD123 show up- or downregulation in variable patterns in SM. Signalizing specific patterns in the immunophenotype of MCs that associate with the development of an indolent form of SM to a non-indolent form of SM or an AHN could be of great prognostic value.

In this research, a selection of a series of biomarkers has been made for measurement in a heterogeneous cohort of patients suspected of mastocytosis in an attempt to discover subgroups on immunophenotypical grounds and to find associations between immunophenotype and clinical features of added value.

Biomarker Function

A background understanding of the biological function of all markers used in the protocol is essential.

CD117 is highly expressed on hematopoietic stem cells (HSCs), multipotent progenitors (MPPs) and the common myeloid progenitors (CMPs). In contrast, the common lymphoid progenitors have low CD117 expression. During maturation CD117 disappears from most hematopoietic cells. Among the matured hematopoietic cells, CD117 is expressed on MCs, melanocytes and the interstitial cells of Cajal. When SCF binds to CD117, receptor dimerization occurs, and an intracellular signaling cascade is induced to augment cell survival, proliferation and differentiation.

CD45 is member of the protein tyrosine phosphatase (PTP) family. It is a pan leukocyte marker. It is known for its' function in regulating B- and T cells antigen receptors, and as a negative regulator of cytokine receptor signaling, thus generating a negative feedback. [28] Furthermore, it has a regulatory role in hematopoiesis. Indeed, enhanced CD45 expression on BM leukocytes induces higher motility in response to stress signals. [29]

CD71 is the transferrin receptor 1, and is involved in the iron uptake from transferrin through endocytosis. [30]

CD2 is a cell adhesion molecule and possesses a co-stimulatory function. It is normally expressed on T cells and NK cells. [31] [32]

CD30 is a tumor necrosis factor receptor. It is specifically expressed on activated B- and T cells. When TRAF2 and TRAF5 bind to this receptor, apoptosis is enhanced, and proliferation of CD8 effector T-cells is inhibited. [33]

CD13, or alanine aminopeptidase is located in the small-intestinal and renal microvillar membrane and involved in peptide digestion by gastric and pancreatic proteases. It is further expressed on proximal tubular epithelial cells, where its' function is not known. [34]

CD25 is the alpha chain of IL-2, a receptor on T cells with a regulatory function to prevent T cell autoimmunity. It is mainly expressed on activated B and T cells, thymocytes, myeloid precursors and oligodendrocytes. However, resting T-cells also exhibit constitutive activity of CD25. [35] [36]

CD52 is mainly expressed on mature lymphocytes, monocytes and dendritic cells, it is a sialic acid-binding Ig-like lectin 10 (SIGLEC10). [37] It is bound to the immunoreceptor tyrosine-based inhibitory motif (ITIM) intracellularly, thus decreasing activation of molecules involved in cell signaling. [38]

FceRI is the high-affinity receptor for antibody IgE, which is primarily involved in inducing allergic reaction. FceRI is expressed on MCs, basophils and eosinophils. [39] Crosslinking of FceRI with IgE triggers an intracellular cascade that leads to degranulation of cytokines, interleukins, leukotrienes and prostaglandins. [40]

CD33 is SIGLEC3 and mediates the same effect as CD52. Except, it is mainly expressed on myeloid cells [41], though it has been detected on lymphoid cells. [42]

CD56 is a neural cell adhesion molecule (NCAM), expressed on neuron, glia and skeletal muscle, however, also found within hematopoietic lineages. It is an adhesion molecule and known for its' role in

the morphogenesis, and in later stages for adhesion of neuron-neuron and neuron-muscle junctions. [43] CD56 expression is also found in the hematopoietic system where it seems to be confined to activated immune cells exhibiting cytotoxicity. [44]

CD123 is a heterodimeric cytokine receptor expressed on pluripotent progenitor cells and it induces proliferation and differentiation of hematopoietic cell lines. An upregulation of CD123 is associated with a series of leukemic disorders. [45]

CD22 belong to the SIGLEC family of lectins. In general, CD22 has a negative feedback role on the activation of the immune system and development of autoimmunity. [46] [47]

Immunophenotype SM vs non-SM

All nucleated hematopoietic cells express CD45. [48] MCs express more CD117 than all other normal $CD45^+$ nucleated hematopoietic cells. This allows for their identification on a sensitivity level of 10^{-4} to 10^{-5} within nucleated hematopoietic cells. [49] Additionally, in case of SM, BMMCs show slight abnormal downregulation of CD117 (100%) and CD45 (100%). [50] [51] However, in one other study CD45 is mentioned to be upregulated. [52]

Furthermore CD71 (38%) and FceRI ($P \leq .03$ vs normal BM) are also downregulated in SM. [51] [52]Specifically, patients with ISM with hepatomegaly and/or splenomegaly and increased tryptase levels have lower BMMC expression of FceRI. Also, BMMCs from ASM, ASM-AHN, and MCL, show aberrantly low sideward light scatter (SSC) and FceRI expression. [52]

An algorithm for the classification of SM patients with multilineage cKIT mutation vs patients with KIT mutation restricted to BMMCs was built. Using this algorithm, patients with multilineage KIT mutation were identified based on aberrant expression of CD25 by BMMC and absence of a coexisting normal BMMC population. [53] Consistently, lower light scatter values were significantly associated with an increasing number of mutated BM cell compartments. [53]

Also, CD25 (92%) and CD2 (72%) are strongly upregulated, and CD33 (100%) and CD123 (73%) are slightly upregulated in SM. CD13 and CD22 show variably dim to strong expression on BMMCs, but are more often upregulated in SM (33% vs 75% and 60 vs. 96% respectively). [51]

Interestingly, CD52 has been identified as a marker that is abundantly expressed on neoplastic MCs in ASM. In contrast patients with normal MCs or with ISM exhibited negative to dim expression of CD52. [54]

CD30 expression is positive in most SM patients (80%), whereas CD30 expression in non-mastocytosis cases is significantly lower (4,8%). Using CD30 as a diagnostic marker additional to CD25 has shown to increase the accuracy of SM diagnosis, in specific for WDSM where a $CD30^+/CD25^-$ immunophenotype is expressed. Otherwise, CD30 did not show added value in prognostic stratification of ISM or differential diagnosis between ISM and ASM. [55]

Three distinct immunophenotypes within SM that corresponded to patients with (1) ISM/cMCAD: $CD25^+/CD2^+$/CD45+/$CD203c^+/CD123^+$; (2) WDSM: $CD25^-/CD2^-/CD22^+$ and (3) ASM and MCL: $CD25^+/CD2^-/CD123^+$/$tryptase^{low}/SSC^{low}$ have been identified. Furthermore, SM-AHN cases showed

variable immunophenotype patterns that corresponded to the SM component subtype. This gives the impression that SM and the AHN develop parallel to eachother within a patient. [52]

Association of Immunophenotype with Clinical Features

Mastocytosis exhibits an array of clinical symptoms that may be classified as allergy-like symptoms or rather resemble the features of a neoplasm. Among allergic symptoms are elevated methylhistamine (Mhis), methylimidazole acetic acid (MIMA) and total serum tryptase levels. The shift is made to a truly neoplastic disorder when symptoms of organ involvement, or even organ dysfunction appear. They are represented by the previously described B- and C-findings, typically involving elevated bone marrow blasts, high MC infiltration rates and dysplasia, an increased red blood cell distribution width (RDW), cytopenias, and eosinophilia being at the border of the two classes.

Research Goals

In this research the aim is (1) to work out a gating strategy to assess the factual immunophenotype expressions in a large cohort of SM patients along with laboratory findings; (2) to discover SM subtypes on immunophenotypical grounds and (3) to detect associations between immunophenotype and clinical features of added value.

Materials & Methods

Principle of Flow Cytometry

For our analysis BMMCs are acquired. BM smears are taken from the patient and 'standard operating procedure (SOP) for bulk lysing erythrocytes in cell suspensions with lysis buffer' is performed. In this, erythrocytes are selectively lysed with ammonium chloride to retain cell suspension with purified leukocytes.

For MC parameter acquisition flow cytometry is applied, and for this purpose the Beck and Dickinson FACSCANTO 2 flow cytometer is used. Flow cytometry depends on the principle of stained individual cells passing by a laser beam, detecting certain parameters. The laser beam is obstructed as the cell passes by. This is detected by a frontal detector and time of light obstruction is logged and translated to a forward scatter (FSC) signal. Synchronously, the laser beam is scattered by inner particles of the cell proportional to its' inner granularity. This scatter is detected by a side detector and is converted to a side ward scatter (SSC) signal. Cells are also stained with fluorochromes bound to specific membrane proteins. Fluorescence of these biomarkers is detected by a series of other detectors, for each color one detector. With this, a series of variables is logged per event. Every event passing the detector should in principle equal one cell.

An output of plots is created, where biomarker expression intensities and scatter properties of all events of one measurement can be variably plotted against each other. For data analysis INFINICYT (Cytognos SL, Salamanca, Spain) was used.

Data Assessment

Gating Methods

MC population from all events is defined: (1) Cell debris is ruled out, considering intact cells cannot have an area < 50.000 on FSC-A; (2) from all hematopoietic cells, nucleated hematopoietic cells fraction is defined as $CD45^{+}$; (3) MCs are selected as the most positive $CD117^{+}$ cell cluster from the remaining events and lastly, (4) events that come from doublet cells are discarded, which are identified by their abnormal forward height to forward area scatter ratios. To define effective marker expression of a defined marker on MCs, a correction for autofluorescence must be made. This is calculated as (median crude marker expression) - (median autofluorescence intensity).

First, populations are analyzed and false populations and artifacts are identified with the principle of identifying recurring patterns, while taking into account biological variation.

CD25 is bound to fluorochrome PE, and may present one positive and one negative population in a patient. However, in a significant number of cases autofluorescence signal PE also presents the two respective populations. This is a false CD25 positivity case marked by the presence of highly autofluorescing eosinophils that are not filtered in the $CD117^{+}$ gate. Their crude expression in comparison with $CD25^{-}$ MCs is typically FSC-A^{high}/FSC-H^{high}/SSC-A^{high}/HV500-A^{high}/FITC-A^{same}/PE-A^{high}/PerCP-Cy5-5A^{high}/$CD45^{high}$/$CD117^{low}$/$CD2^{same}$/$CD25^{high}$/$CD33^{low}$/$CD30^{high}$/$CD52^{high}$/$CD22^{high}$/$CD56^{high}$/$CD138^{high}$/$CD13^{high}$/$FceRI^{low}$/$CD34^{high}$/$CD123^{high}$. More importantly, their expression compared to $CD25^{+}$ MCs is most distinct on the CD45 and CD71 channel, as they show greater positivity on these channels than $CD25^{+}$ MCs do. Furthermore, if no distinction on one of the backbone markers can be made, yet a double

PE population is observed, then eosinophils may be cleared in tube 2 where CD25 resides with the CD33 negative gate. The CD71 gate is the most distinctive gate in this aspect in the current protocol. The corrected expression for autofluorescence of eosinophils is: $CD2^{+/-}/CD25^{-}/CD33^{-}/CD30^{+/-}/CD52^{-}/CD22^{-}/CD56^{-}/CD138^{+/-}/CD13^{+/-}/FceRI^{-}/CD34^{-}/CD123^{-}$

An artifact on HV500-A autofluorescence channel is regularly observed. It consists of a small cluster of around 10 events characteristically and shows high SSC properties and high autofluorescence. It is seen on several other channels, but can be best gated out using the HV500-A channel.

Different cell clusters show overlapping expression with doublets and are considered false MC populations. The right side lines on CD138; CD13; CD34; CD123 with high SSC consist of the same cell type and are considered a false population. This population also variable recurs as $CD45^{low}$ relatively to true MCs.

Merging is not performed as it cannot give accurate estimations of cell expression with low quantities. Moreover, the merge algorithm that is used in infinicyt spreads out SSC, giving loss of information about SSC properties of MCs. Also, loss of information is created in the aspect distribution of CD25 subpopulations on other channels.

For extensive gating instructions and data assessment methods see appendix annex 1.

Laboratory and Clinical Data

Laboratory and clinical data for all cases is collected. Laboratory data includes Urinary N-methylhistamine (Mhis) and methylimidazole acetic acid (MIMA), total serum tryptase levels, Beta2-microglobulin, cell counts including leukocytes, erythrocytes, hemoglobin, thrombocytes, erythroblasts, neutrophils, lymphocytes, monocytes, eosinophils, immature granulocytes, MCs in BM smears and biopts with dysplasia rates, blasts and eosinophils in BM and cell properties including hematocrits (Ht), mean corpuscular volume (MCV) and red blood cell distribution width (RDW). Clinical data includes gender, age at date of first symptoms, performance status, skin involvement, diagnose, B- and C-findings and allergy.

Statistical Methods

Variables

For all continuous variables from MC populations cell counts, percentages from total events, medians and first and third quartiles were calculated. Clinical data includes continuous, ordinal and categorical variables.

Statistical Calculations

1. To test for the presence of groups within one distribution (because we do not assume a normal distribution) Test … must be used.
2. For the correlation of two continuous variables (e.g. CD25 with CD52) (no normal distribution assumed) Kruskall Wallis test is used to compare overall equality of groups, then Mann-Whitney U-test is used to compare group pairs.
3. For the correlation of a continuous variable with dichotomous variable (all markers with cKIT) the point-biserial correlation is used (it’s equivalent to computing the Pearson correlation).
4. Continuous with categorical (for correlating markers with diagnose) Test … must be used.

5. For correlating two dichotomous variables use the chi squared test or the phi test
6. For correlating two categorical variables (more than dichotomous) use the Cramer's V (or Cramer's Phi) test.
7. Spearman's test*
8. Univariate analysis (which marker has biggest influence on variable x) (only for continuous variables)
9. Multivariate analysis (which combinations of markers show greatest influence on variable x?) (only for continuous variables?) Difference between uni/multivariate analysis and the previous tests is that here you are not defining the variable beforehand.
10. I need a multivariate analysis where I can test for effects of continuous variables on categorical variables. (very important!)
11. Sensitivity/specificity of markers for cKIT:
    a. true positive (TP)/(TP1false negative)
    b. specificity as true negative (TN)/(TN 1 false positive)
    c. positive predictive value (PPV) as TP/(TP 1 false positive)
    d. and negative predictive value (NPV) as TN/(TN 1 false negative).
12. Parallel coordinates are used as a means to swiftly test for associations between variables on multiparametric level in one comprehensive representation.

Results

Gating methods

Recurring patterns have been identified. Populations are characterized based on empirical, and partially analytical evidence. First, it is stated MCs are defined as the population with most positive CD117 expression. It is found that two populations may interfere with the MC cluster on the CD117 channel:

(1) A population that has been identified as eosinophils, with high SSC and a crude expression in comparison with $CD25^{-}$ MCs of typically FSC-A^{high}/FSC-H^{high}/SSC-A^{high}/HV500-A^{high}/FITC-A^{same}/PE-A^{high}/PerCP.Cy5.5.A^{high}/$CD45^{high}$/$CD117^{low}$/$CD2^{same}$/$CD25^{high}$/$CD33^{low}$/$CD30^{high}$/$CD52^{high}$/$CD22^{high}$/$CD56^{high}$/$CD138^{high}$ /$CD13^{high}$/$FceRI^{low}$/$CD34^{high}$/$CD123^{high}$. And a corrected expression for autofluorescence of: $CD2^{+/-}$/$CD25^{-}$/$CD33^{-}$/$CD30^{+/-}$/$CD52^{-}$/$CD22^{-}$/$CD56^{+}$/$CD138^{+/-}$ /$CD13^{+/-}$/$FceRI^{-}$/$CD34^{-}$/$CD123^{-}$. In figure below exemplary eosinophils clusters are shown.

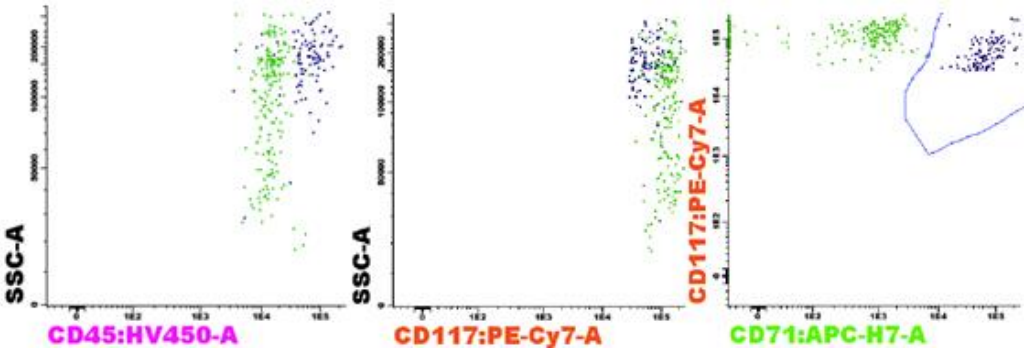


Figure 5 Expression pattern of eosinophils and interference with MCs.

Furthermore, an immunophenotyping of this population has been performed with CD18, CD3/CD56, CD64 and CD3/CD56 and B-cells, T-cells, monocytes and natural killer cells were ruled out respectively.

(2) A population with low SSC, that has yet to be identified with $CD45^{high}$ expression compared to MCs, an FSC-H and FSC-A expression that is very similar to the expression of normal MCs – and in a great variety of cases an even more confined FSC-H/FSC-A expression then normal MCs – exhibit and a scattered expression pattern on other channels. Below, exemplary figures for this populations' expression are shown.

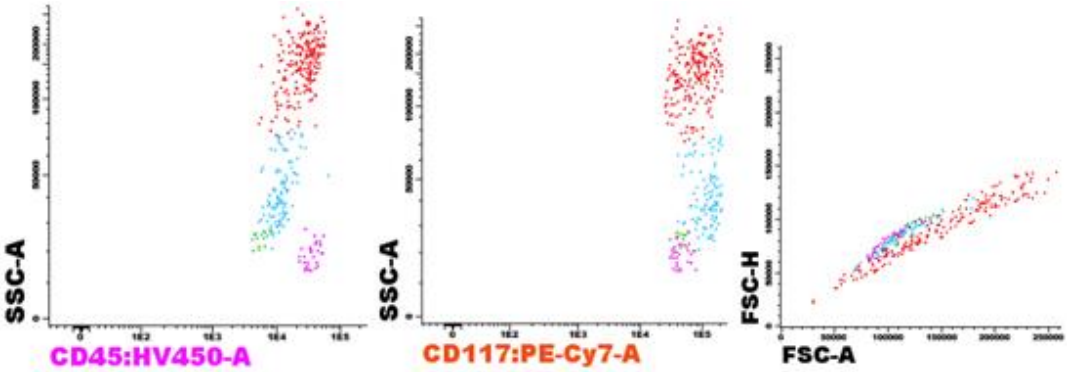


Figure 6 Expression pattern of $CD117^{+}$ low SSC population and interference with MCs.

Additionally, specific patterns in the protocol that may be artifacts have been detected. It is observed in virtually all cases where CD71 is present in the protocol, CD203c is upregulated. In contrast, whenever CD71 is absent, no CD203c is detected. This is illustrated in figure below.

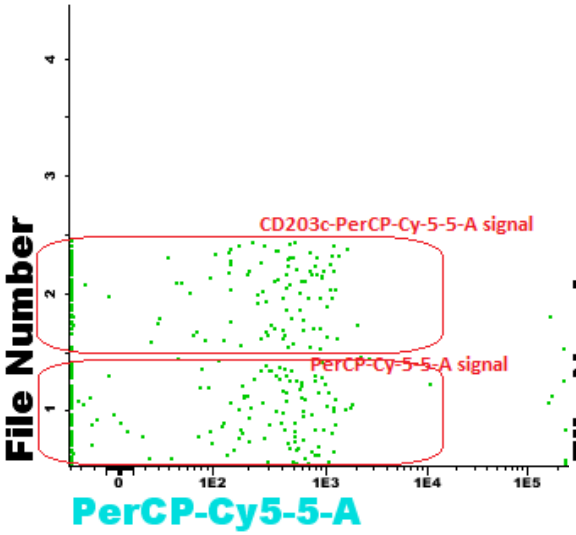


Figure 8 CD203c signal in absence of CD71 biomarker, measurement type number 170478.

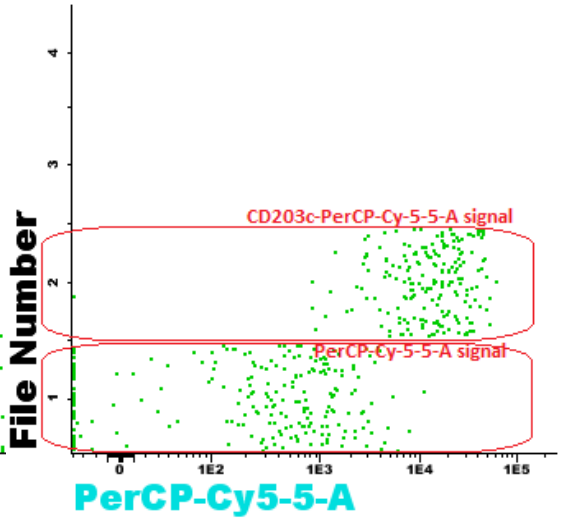


Figure 7 CD203c signal in presence of CD71 biomarker, measurement type number 170914.

Additionally, a HV500-A very bright, very low FSC-H/FSC-A small population with dubiously similar expression on several channels is occasionally observed.

A population that occupies the right side lines on CD34-PerCP-Cy5-5-A, CD123:APC-A and CD22-PerCPCy-5-5-A, exhibits negative FceR1-PE-A expression and positive CD13-FITC-A expression is regularly observed. This population shows negative for CD45, and doublets have partially been associated with negative CD45 expression. This population does not fully fall below the FSC-H/FSC-A diagonal on the areaplot.

Commonly, expression of all biomarkers with PerCP-Cy5-5 and HV500 as fluorochromes was spreaded towards the left side line. This phenomenon is also regularly observed for fluorochrome APC and incidentally detected for fluorochromes PE and FITC. Events from the left side line in these cases did not show abnormal expression on any of the other channels.

Marker Correlations with cKIT

In this series of results correlation of marker expression with KIT mutation is shown in (1) boxplots; (2) histograms, where in the first diagram the whole patient cohort is plotted, and in the second diagram the the cKIT positives are split from the cKIT negatives for comparison and (3) ROC-curves are plotted to assess the diagnostic utility of the markers with sensitivity and specificity outcomes.

A comprehensive overview of biomarker expression is presented and significant correlations of primary interest are depicted. The complete results set is included in appendix annex 2.

CD117

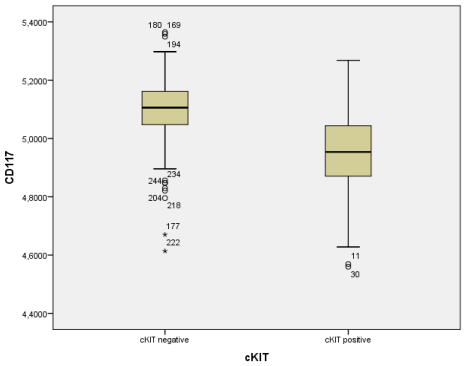


Figure 9 CD117 boxplots showing CD117 is downregulated in cKIT positive cases.

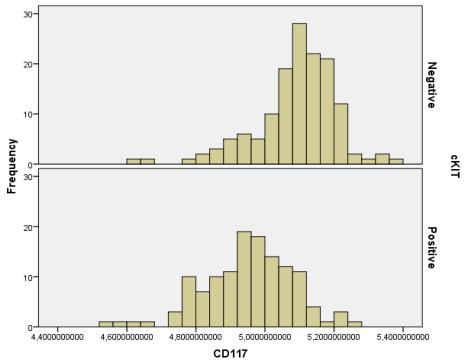


Figure 10 CD117 histograms showing CD117 is downregulated in cKIT positive cases.

CD117 downregulation in cKIT^{+} cases is observed. In the histograms expression seems to follow a well-defined lognormal curve with great overlap between the two peaks and possibly a small CD117^{-} subpopulation within cKIT^{-} cases.

CD45

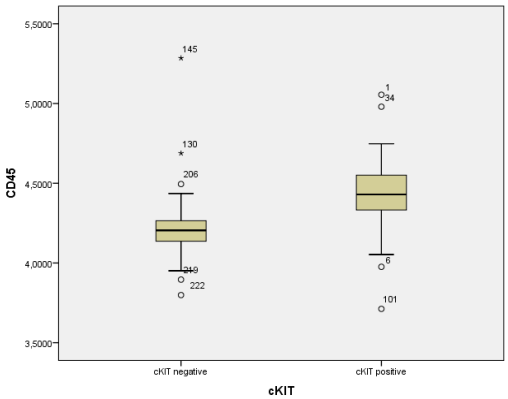


Figure 11 CD45 boxplots showing CD45 is upregulated in cKIT positive cases.

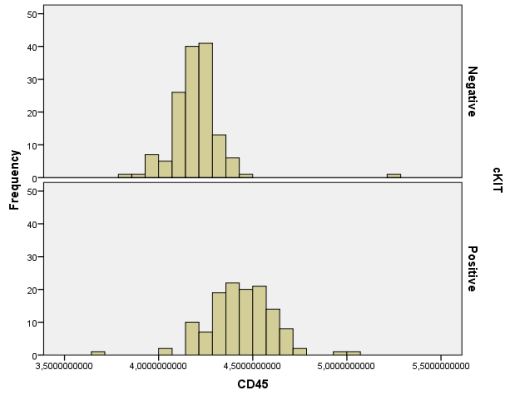


Figure 12 CD45 histograms showing CD45 is upregulated in cKIT positive cases.

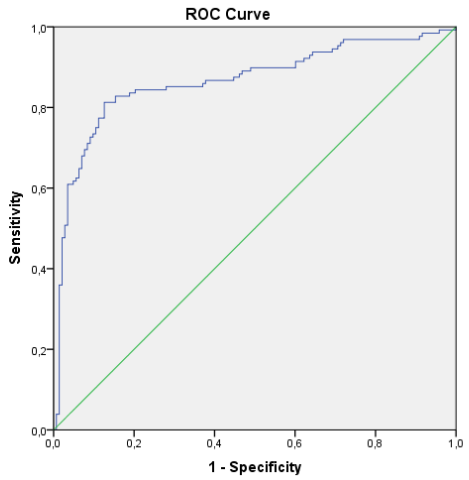


Figure 13 CD45 ROC curve showing sensitivity to specificity rate for cKIT mutation for all possible cut off values.

CD45 upregulation with cKIT mutation occurs. In the histogram a clear differentiation between two peaks can be seen. In the ROC curve it is observed that with CD45 it is possible to determine cKIT mutation with a sensitivity to specificity ratio of approximately 82% to 80% respectively.

CD2

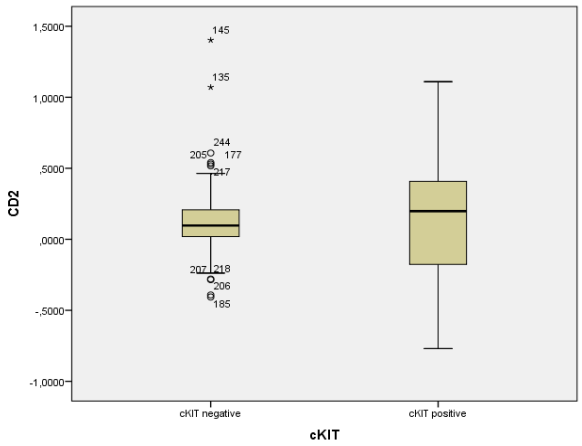


Figure 15 CD2 boxplots showing CD2 is upregulated in cKIT positive cases.

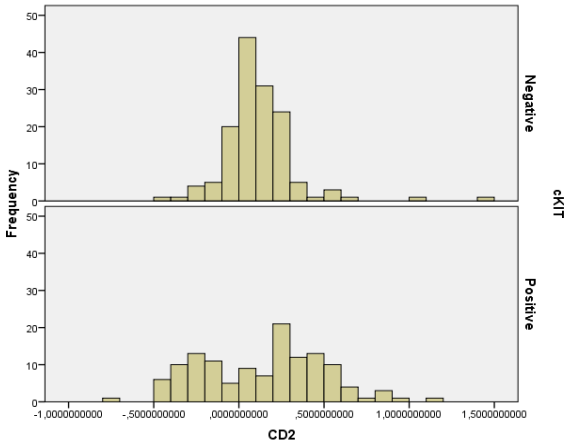


Figure 16 CD2 histograms showing CD2 has two populations within poisitive cases and one population within negative cases.

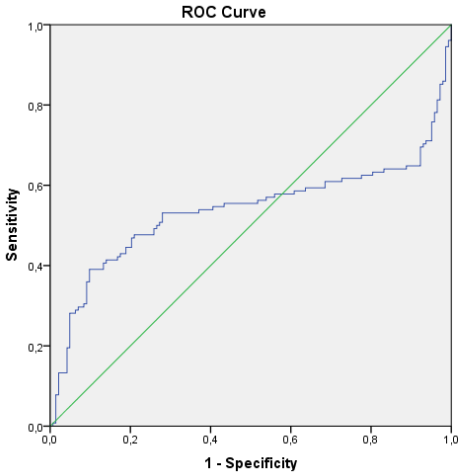


Figure 14 CD2 ROC curve showing sensitivity to specificity rate for cKIT mutation for all possible cut off values.

In figure 12 it is observed that with CD2 it is possible to determine cKIT mutation with a sensitivity to specificity ratio of approximately 50% to 75% respectively. This ratio is lower than ratios for CD45 and CD52. It is seen that CD2 reverses direction on higher cut-off values. This is caused by the presence of intensely bright $CD2^{+}$ cases within cKIT negative population.

CD30

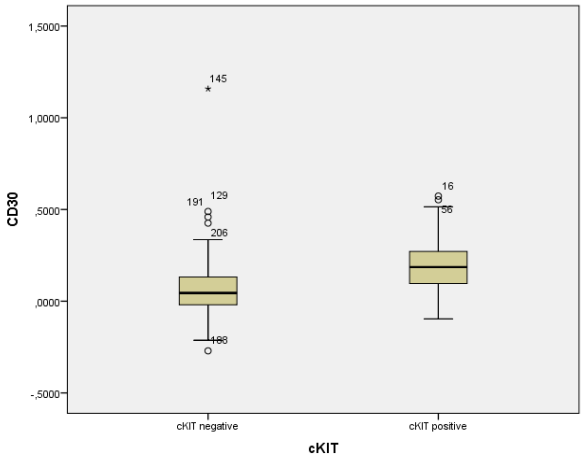


Figure 17 CD30 boxplots showing CD30 is upregulated in cKIT positive cases

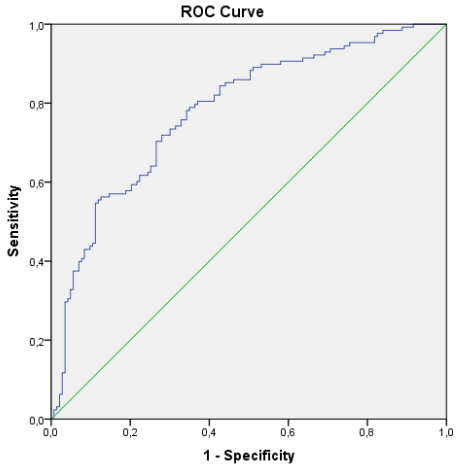


Figure 18 CD30 ROC curve showing sensitivity to specificity rate for cKIT mutation for all possible cut off values.

An upregulation of CD30 within cKIT^{+} cases is observed. In figure 14 it is observed that with CD30 it is possible to determine cKIT mutation with a sensitivity to specificity ratio of approximately 57% to 80% respectively.

CD25

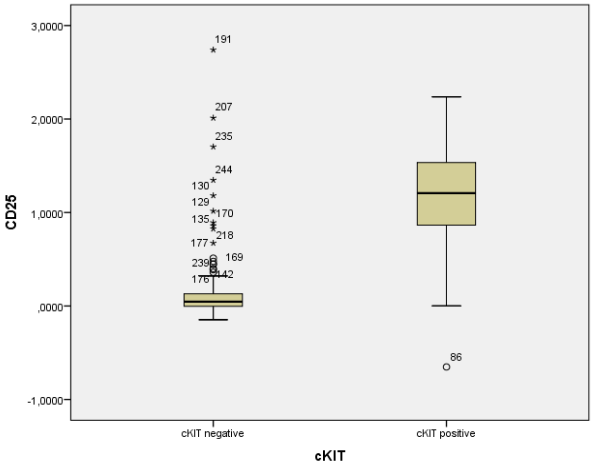


Figure 19 CD25 boxplots showing CD25 is upregulated in cKIT positive cases

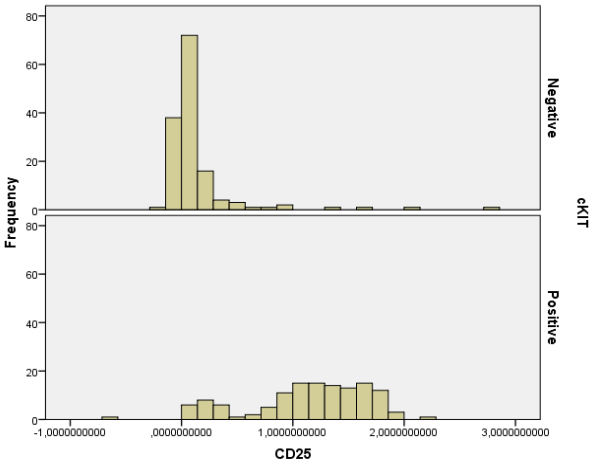


Figure 20 CD25 histograms showing CD25 has two populations within poisitive cases and one population within negative cases, with arguably also a CD25 positive population with cKIT negative cases.

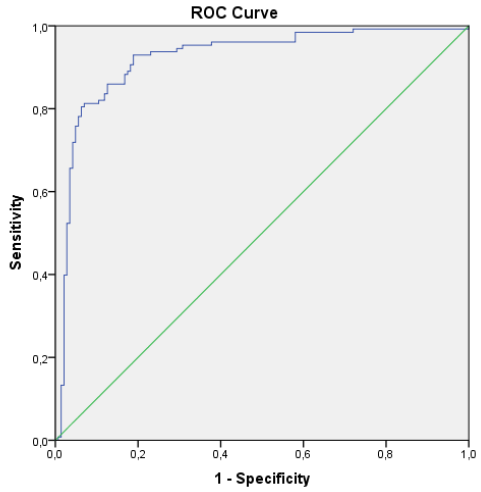


Figure 21 CD25 ROC curve showing sensitivity to specificity rate for cKIT mutation for all possible cut off values.

A CD25 upregulation is observed within cKIT^{+} cases. A group of outliers within cKIT^{-} cases is specified. This can be seen in the boxplots as well as the histograms. It is observed that with CD25 it is possible to determine cKIT mutation with a sensitivity to specificity ratio of approximately 82% to 87% respectively.

CD52

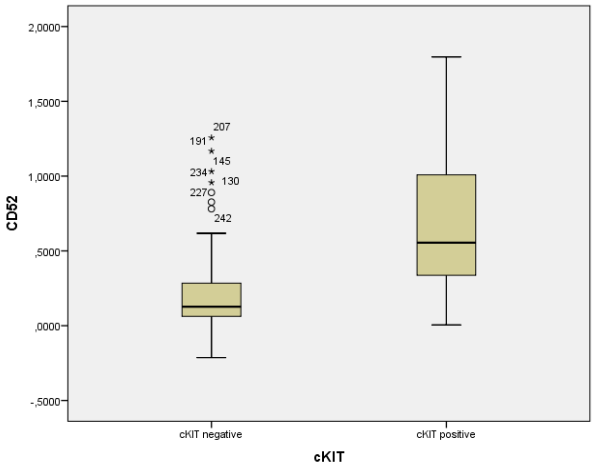


Figure 22 CD52 boxplots showing CD25 is upregulated in cKIT positive cases

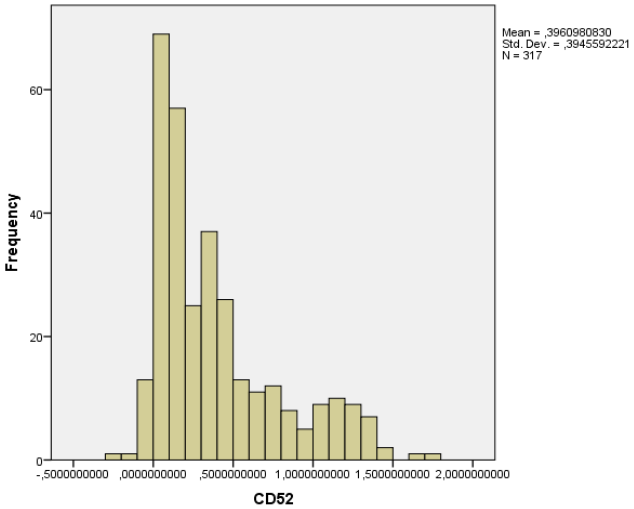


Figure 23 CD52 histogram showing CD52 presents three peaks for the total cohort.

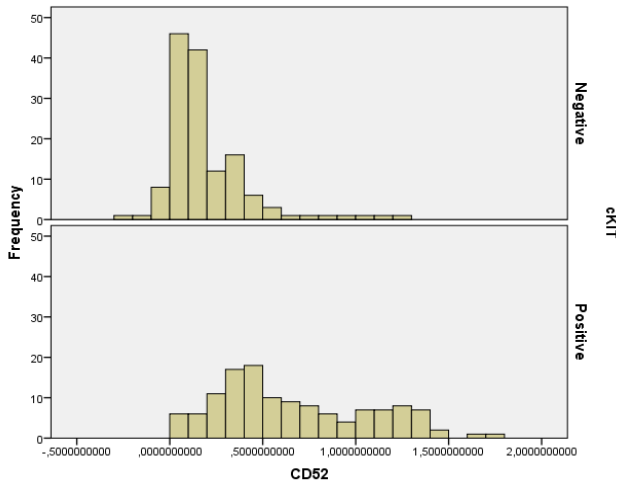


Figure 24 CD52 histograms split into cKIT positive and negative cases.

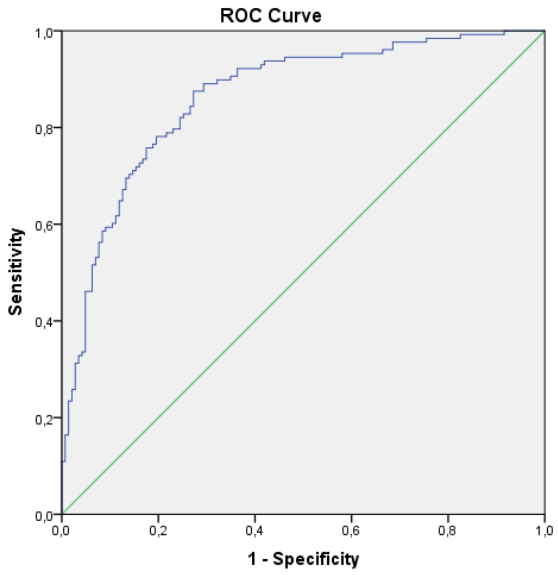


Figure 25 CD52 ROC curve showing sensitivity to specificity rate for cKIT mutation for all possible cut off values.

In the boxplots, CD52 exhibits an upregulation for cKIT$^+$ cases. More specifically, in the histogram for the total cohort three peaks are observed. When these histograms are split for cKIT$^+$ and cKIT$^-$ cases it is seen that the first peak and partially the second peak represent the negative cases, whereas the positive cases are represented by the other part of the second peak plus the third peak. Furthermore, it is observed that with CD52 it is possible to determine cKIT mutation with a sensitivity to specificity ratio of approximately 79% to 80% respectively.

CD123

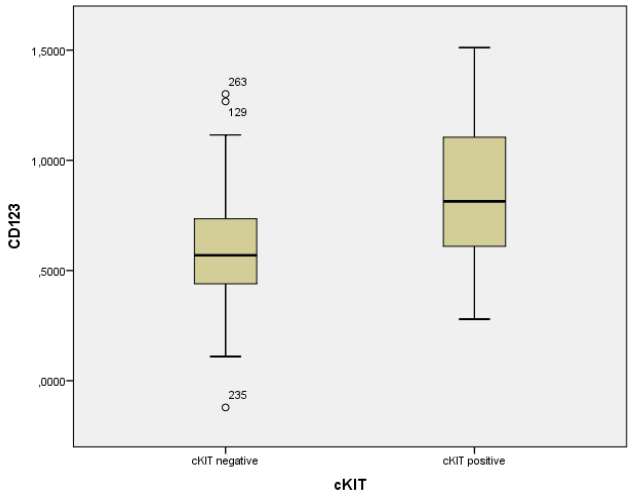


Figure 26 CD123 boxplots showing CD123 is upregulated in cKIT positive cases

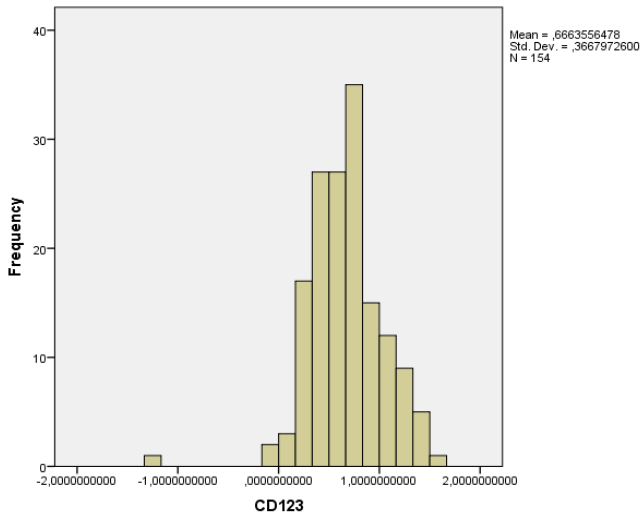


Figure 27 CD123 histogram for the total cohort.

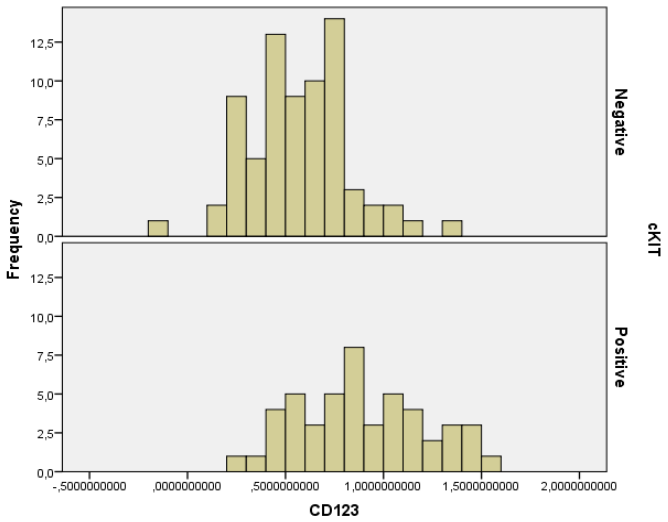


Figure 29 CD123 histograms split into cKIT positive and negative cases.

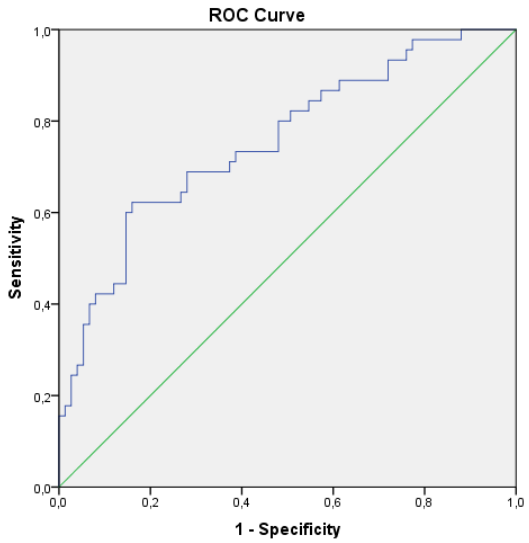


Figure 28 CD123 ROC curve showing sensitivity to specificity rate for cKIT mutation for all possible cut off values.

In the boxplots an upregulation of CD123 for cKIT^{+} cases is exhibited. In the histogram of the total cohort one peak of which the right tail is longer than the left tail is observed. In the cKIT-based split histograms it can be seen that the right shift of positive cases is present, but shows great overlap with the negative peak.

Dendrogram

In this dendogram cohort variables are clustered based on similarity. Pearson correlation is used as a distance measure, because no N-distribution is assumed, and cluster method is 'between-groups linkage'.

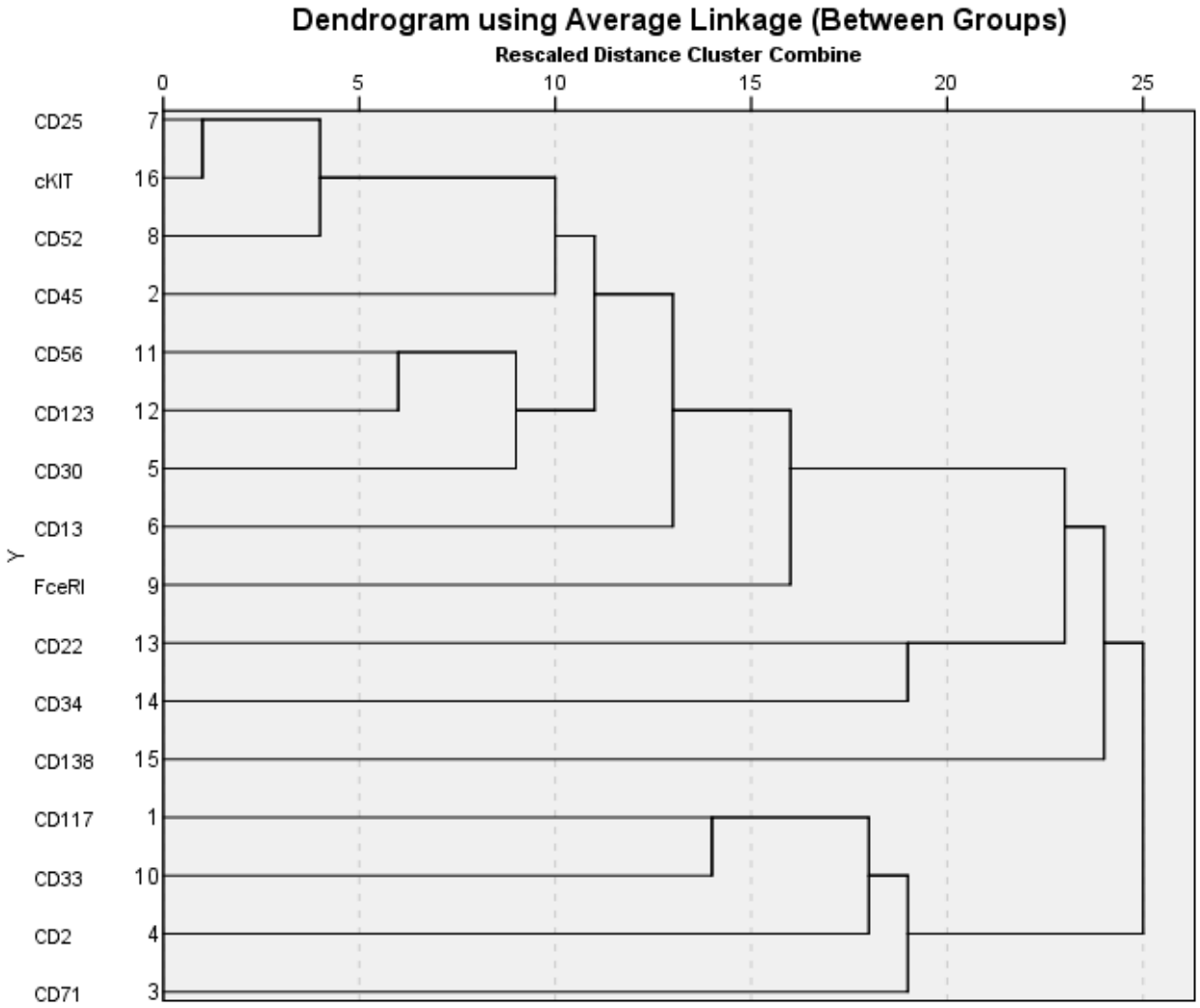


Figure 30 Dendrogram of total cohort presenting interrelatedness between biomarkers using distance measures.

CD25 is most linked to cKIT, next CD52, and next CD45, this follows the expectation. CD56 is most linked to CD123. CD56 is linked to multilineage involvement, and CD123 is a maturation marker. Both markers are brought into relation with more aggressive forms of SM. This is interesting. CD117 is most related to CD33 and then CD2. This is interesting and needs to be explored. CD34 is described as 'never expressed on MCs' and consistently it is not clustered with any other marker. CD138 likewise.

Correlation of all Markers with in a Proximity Matrix

In this table it is shown for every marker how much it correlates with all other markers. This is of specific interest to reveal correlations and a possible interplay between markers other than the correlation of markers with centrally the cKIT mutation.

Table 2 Proximity matrix showing correlation between all possible combinations of markers

Proximity Matrix

	Correlation between Vectors of Values															
	CD117	CD45	CD71	CD2	CD30	CD13	CD25	CD52	FceRI	CD33	CD56	CD123	CD22	CD34	CD138	cKIT
CD117	1,000	,008	,156	,206	-,212	-,333	-,546	-,392	-,155	,376	-,139	-,118	-,174	-,291	-,198	-,583
CD45	,008	1,000	,121	-,070	,479	,414	,626	,433	,146	,234	,505	,430	,133	-,105	,121	,472
CD71	,156	,121	1,000	,184	,194	,078	-,010	-,011	,300	,234	,155	,212	-,056	-,374	-,066	-,166
CD2	,206	-,070	,184	1,000	,127	,050	-,278	-,150	,075	,259	,197	,093	,010	-,073	-,063	-,133
CD30	-,212	,479	,194	,127	1,000	,369	,578	,355	,292	,247	,576	,500	,151	-,197	,127	,456
CD13	-,333	,414	,078	,050	,369	1,000	,460	,369	,340	-,043	,473	,302	,050	-,110	,058	,407
CD25	-,546	,626	-,010	-,278	,578	,460	1,000	,779	,384	,104	,491	,497	,245	,025	,074	,824
CD52	-,392	,433	-,011	-,150	,355	,369	,779	1,000	,505	,098	,445	,406	,157	,075	,007	,662
FceRI	-,155	,146	,300	,075	,292	,340	,384	,505	1,000	,166	,326	,245	,091	-,092	-,023	,192
CD33	,376	,234	,234	,259	,247	-,043	,104	,098	,166	1,000	,496	,389	,126	-,228	-,276	,007
CD56	-,139	,505	,155	,197	,576	,473	,491	,445	,326	,496	1,000	,645	,250	-,113	-,257	,384
CD123	-,118	,430	,212	,093	,500	,302	,497	,406	,245	,389	,645	1,000	,092	-,136	-,053	,412
CD22	-,174	,133	-,056	,010	,151	,050	,245	,157	,091	,126	,250	,092	1,000	,204	-,055	,173
CD34	-,291	-,105	-,374	-,073	-,197	-,110	,025	,075	-,092	-,228	-,113	-,136	,204	1,000	,118	-,022
CD138	-,198	,121	-,066	-,063	,127	,058	,074	,007	-,023	-,276	-,257	-,053	-,055	,118	1,000	,154
cKIT	-,583	,472	-,166	-,133	,456	,407	,824	,662	,192	,007	,384	,412	,173	-,022	,154	1,000

This is a similarity matrix

It is seen CD117 with CD45 has 0,008, and it is known that CD117 is downregulated in cKIT+ and CD45 is upregulated in cKIT+. So CD117 should have a significant strong correlation with CD45. Is this number so low because there is an inverse relation? How can SPSS interpret this aspect differently?

It is skewed because SPSS interprets the numbers as scales, would it solve the problem if the variables set as ordinals?

Probably multiple way anova as a solution?

Expression Patterns

In this section, matrices showing combinatorial expression patterns are presented. A blue box indicates the positivity for the concerning marker, a white box indicates the marker is negative. It is seen that CD33 is virtually always upregulated in cKIT^{+} as well as in cKIT^{-} cases. CD25 upregulation is accompanied by CD2 and CD52 positivity. Interestingly, in some cases CD25 occurs without CD52 upregulation, likewise for CD2. However no CD52 upregulation is observed where CD25 is not upregulated within cKIT positive cases, whereas it is sporadically observed within cKIT negative cases. In this way, observations are made to detect subpopulations within the total cohort.

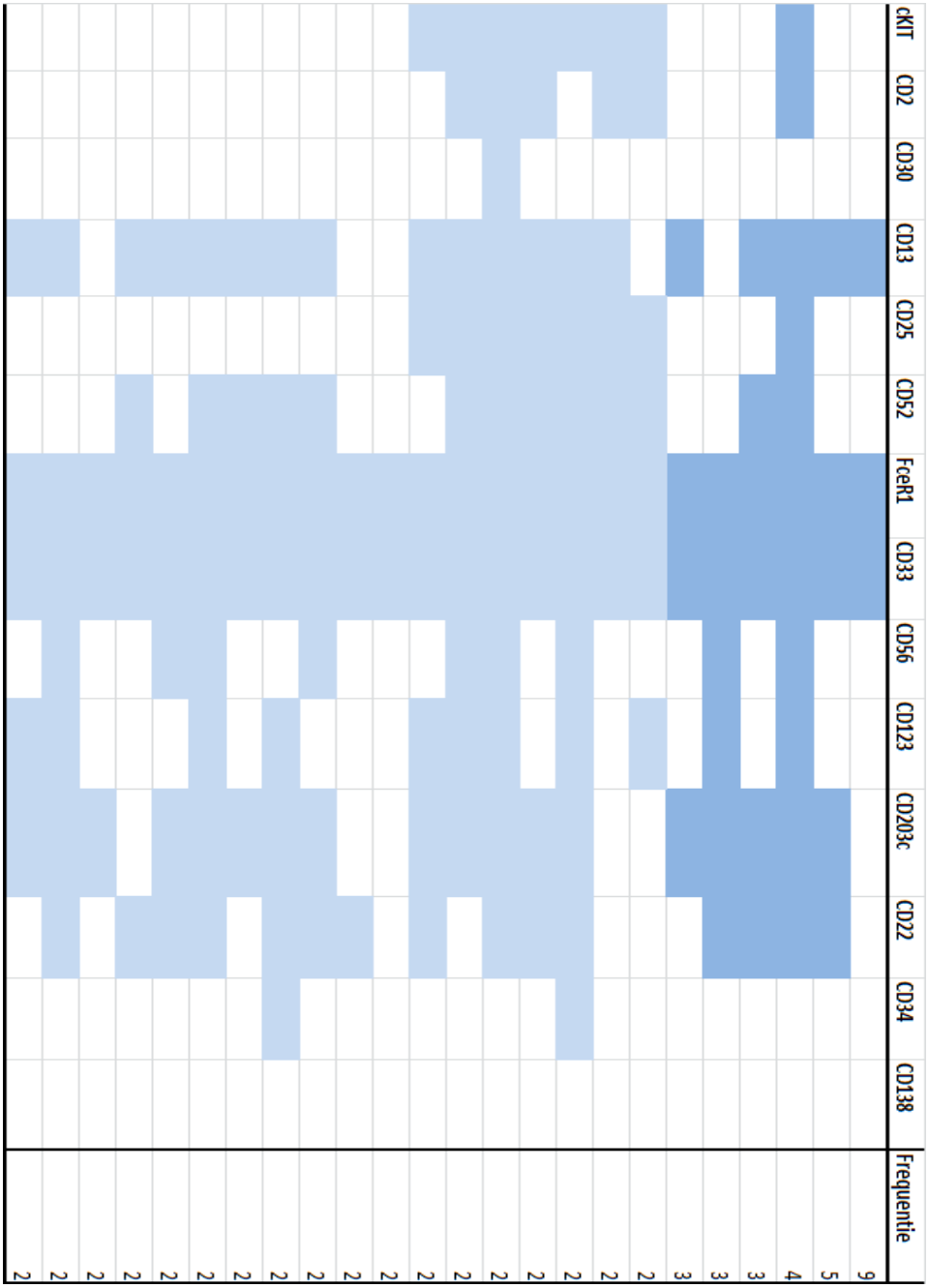
Table 3 Matrix showing the combinatorial expression patterns within the cohort for cKIT negative cases.

cKIT	CD2	CD30	CD25	CD52	CD33	CD22	Frequency
							78
							24
							6
							5
							6
							3
							1
							1
							1
							1
							1
							1
							1
							1
							1

Table 4 Matrix showing the combinatorial expression patterns within the cohort for cKIT positive cases.

cKIT	CD2	CD30	CD25	CD52	CD33	CD22	Frequency
							17
							26
							19
							12
							9
							5
							4
							4
							2
							2
							2
							1
							1
							1
							1

Table 5 Matrix showing the combinatorial expression patterns within the cohort for cKIT positive and negative cases on the whole set of markers.



Comparison Autofluorescence Signal with Marker Signal

In these plots an impression is created of the autofluorescence variance relative to marker expression in order to make an estimation of the validity of marker expressions where no autofluorescence signal was available. Additionally, information about the comparison of autofluorescence between cKIT^{+} and cKIT^{-} cases also has added value.

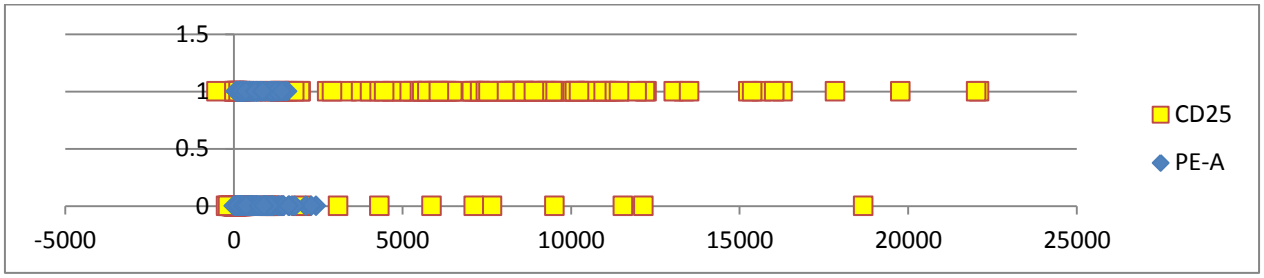


Figure 31Autofluorescence variance of PE-A relative to CD25 expression variance.

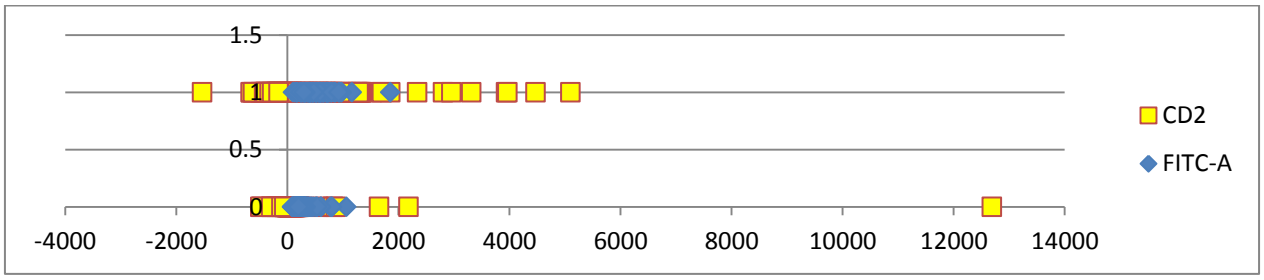


Figure 32Auto fluorescence variance of FITC-A relative to CD2 expression variance.

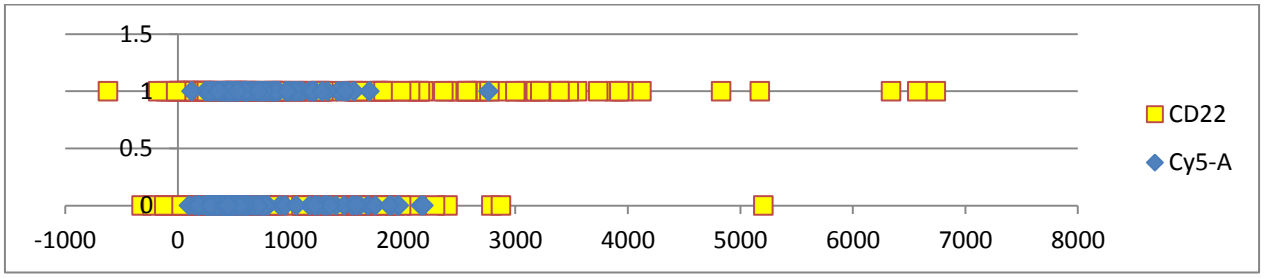


Figure 33Autofluorescence variance of PerCP-Cy5-5-A relative to CD22 expression variance.

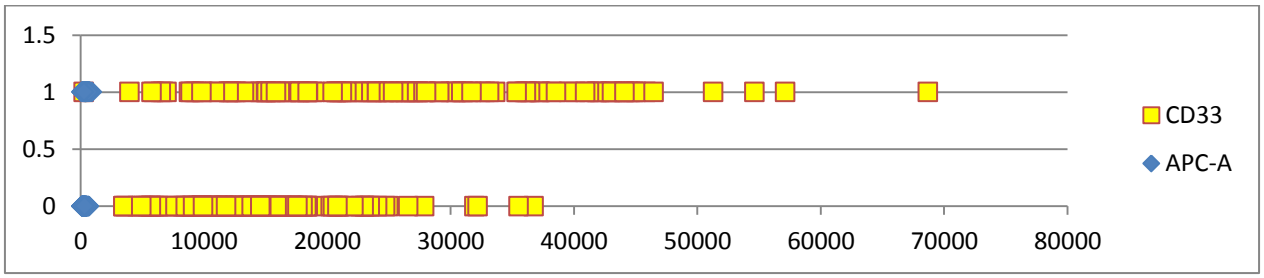


Figure 34Autofluorescence variance of APC-A relative to CD22 expression variance.

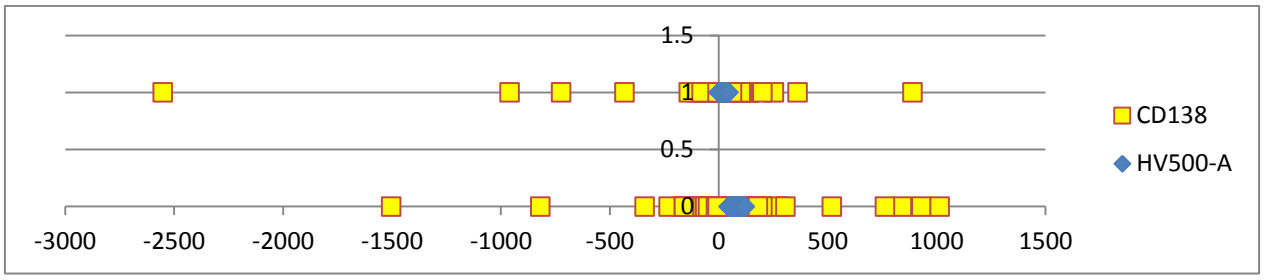


Figure 35Autofluorescence variance of HV500-A relative to CD138 expression variance.

Discussion

Gating Strategy

An efficient MC gating is established with the four-step selection: discarding debris; selecting hematopoietic nucleated cells; defining MCs and cleaning doubletts. [9] However, in order to assess accurate marker expression for association studies, false populations and artifacts need to be cleared. For the mastocytosis protocol that is used in the Universitary Medical Centre of Groningen (UMCG), clinical chemistry a need for the following corrections were affirmed.

In literature CD71 is stated to be downregulated in SM. [51] However, no significant relation between cKIT and CD71 was found. Rather, CD71 showed a double population in a great number of cases, where the first population was clustered on the left side line. This side line consisted of normal MCs on other channels and did not seem to be an artifact. Moreover, CD203c also showed no relation with SM while it is reported to be upregulated in SM. [51] Instead, in virtually all cases where CD71 was present, CD203c was strongly upregulated, while in absence of CD71, CD203c showed no upregulation. CD71 is used as a backbone marker in our protocol, which means that the two fluorochromes do reside in the same tube. Furthermore, fluorochromes APC-H7 and PerCP-Cy5-5 were used for CD71 and CD203c respectively. These two fluorochromes have great overlap in their emission peaks. Therefore, this could be an artifact caused by spectral overlap for which spectral overlap compensation settings are not correctly set.

CD25 needs to be put in backbone so that the distribution of MCs against CD25 expression can be accurately determined. Until now, if distribution of MCs against CD25 expression for a specific marker that is not in the same tube as CD25 is, needs to be determined, either all tubes need to be merged, or the CD25 positive versus negative MCs need to be defined in the backbone. In the first case artifacts come up because of the algorithm that is used for merging. In the latter case a mere approximation to define MCs according to their CD25 expression is made, which in some cases is far from ultimate.

A population that expresses very low SSC and -in comparison with normal MCs- is $CD117^{--/+}$ and $CD45^{-/++}$, is observed. Its' expression on FSC-H/FSC-A corresponds very well with the population of normal MCs. However, the expression pattern on all other channels seems very scattered and needs to be further explored. If it seems to be an MC population, then it means there may be a subpopulation within normal MCs. If it seems not to be an MC population then its' identity has yet to be defined.

A population with CD117 positivity that in some cases is undistinguishable from MCs has been detected. It is considered very likely that the population consists of eosinophils, because of several findings. First, they express a characteristic highly positive profile on autofluorescence channels. [56] Secondly, an immunophenotyping of this group has been performed with CD18, CD3/CD56, CD64 and CD3/CD56 to rule out B-cells, T-cells, monocytes or natural killer cells respectively. Thirdly, a correlation was found with the cases where this population had a high number of events and where an eosinophilia was microscopically diagnosed.

Immunophenotype

Findings of individual marker expression are consistent with literature statements to great extent. CD117 downregulation and CD45, CD2, CD25, CD52, CD33, CD13l, CD13, CD22 and CD123 upregulation in SM had been previously found. [51] [52] For CD34 it is found no up- or downregulation in SM occurs. [51] [52] These findings are confirmed in our dataset. CD71 is downregulated in SM. [52] In our dataset this could not be confirmed, which is probably due to an artifact in our data. CD138 is used as a negative control to exclude multiple myeloma and is ought not to be involved in SM. [57] Indeed, no up- or downregulation of CD138 has been found in the cohort.

For some diagnostic relevant antigens, of which among others CD34 and CD56 belong, it has been determined that they are never expressed on MCs. Such markers can be used to determine multilineage involvement of the cKIT mutation, for example in cases of SM-AHN. [58] It is of our interest to determine the correlation of such markers with multilineage involvement of the cKIT mutation, and consistently, with the risk of disease progression. Worthy of mentioning is that CD34 did indeed not correlate with SM in our analysis. CD56 however, did show slightly positive correlation with SM cases.

In some cases we further defined expression patterns from literature statements, or had contradictory findings. CD25 and CD2 are upregulated in 92% and 72% of SM cases respectively. [51] However, CD25 and CD2 upregulation in non-SM cases, has not been described yet. In our dataset sporadic CD25 and CD2 positivity within non-SM cases is found.

It has been determined that FceRI is not significantly altered in SM [51], though in specific for patients with ASM, ASM-AHN and MCL FceRI downregulation was found. [52] However, in our dataset we found a general upregulation of FceRI in SM versus non-SM cases.

$CD30^{+}/CD25^{-}/cKIT^{-}$ phenotype is characteristic for WDSM. [59] This phenotype could not be found within our dataset. Furthermore, WDSM correlates with CD22 upregulation. [52] This is not reviewed yet and may potentially reveal a diagnostic marker for WDSM.

It is suggested CD25 is upregulated in ASM and downregulated again in MCL. [52] This interplay between ASM and MCL for CD25 could be reviewed. Aditionally, marker expression in the transition from ASM to MCL could be determined for all available markers in the dataset, and most interestingly for CD2, CD52 and CD123.

Lower light scatter values, and in specific the side ward scatter are associated with ASM, ASM-AHN and MCL (P<,02). [52] This could be researched, and statistically elaborated.

In case of SM, BMMCs show slight abnormal downregulation of CD45 (100%). [50] However, in one other study CD45 is mentioned to be upregulated. [52] In our data we see indeed how well-confined CD45 expression for healthy and aberrant MCs is , but it is upregulated instead.

CD52 is abundantly expressed by MCs in patients with ASM. In contrast, patients with ISM and normal MCs exhibit only negative to dim CD52 expression. [54] Interestingly, plotting a histogram of CD52 expression for the whole cohort reveals three distinguishable peaks. Also, CD52 correlates better with the cKIT mutation then CD2 after CD25. Therefore, CD52 may be used as the marker next to CD25 instead of CD2.

Healthy MCs usually express a very confined small bandwith forward scatter (FSC), while aberrant MCs show a broader scatter on FSC. Furthermore, healthy MCs express in some cases an SSC that starts very low and reaches very high, but are in some cases delimited to low SSC rates. While, aberrant MCs show in the vast majority of cases high SSC, though in very few cases they show low SSC. Aberrant MCs with low SSC may correlate with MCs that are developing towards ASM or MCL. More research to define this relation is needed..

Expression patterns within cKIT negative as well cKIT positive cases were found. The found patterns may possibly open a gate to a classification of SM that shows great overlap with the classification of the WHO 2017, and also may show a classification that associates specific characteristics coming from different subtypes with eachother. For example associating the subgroup from ISM that would progress towards an aggressive form and the already aggressive forms. Such associations may possibly reside within combinatory expression patterns, and may also be found in individual marker transitions.

Moreover, if a marker appears to give strong correlation with the more aggressive forms of SM, then still time-relationship of the expression of that marker with progress of disease must be considered before accrediting it as a prognostic marker. In other words: If it seems that the marker only starts to express when ASM clinical features have already manifestated in the patient, the marker cannot be designated as a marker of prognostic value, but rather as a marker to affirm the presence of the aggressive disease as is being clinically observed.

For CD45, CD117 and CD71 no autofluorescence could be determined. To estimate marker expression validity of CD45, CD117 and CD71, an estimation of autofluorescence variance relative to marker expression variance must be made. All available autofluorescence signals were separately plotted along with one of their coupled markers on linear scale. For autofluorescence of MCs for PE, FITC, HV500 and APC no significant variance in autofluorescence for marker expression was observed. For autofluorescence for PerCP-Cy5-5 substantial variation within autofluorescence is observed, such that without correction for autofluorescence per case, validity of marker expression assessment cannot be assured. Fluorochromes HV450, PE-Cy7 and APC were used for CD45; CD117 and CD71 respectively. From within the tested fluorochromes the nearest fluorochrome in wavelength to HV450 is HV500, the nearest to PE-Cy7 is PerCP-Cy5-5, and the nearest to APC is APC itself. This would initially mean that by estimation CD45 and CD71 expressions are valid, and CD117 cannot be guaranteed to be. However, MCs have such a high expression that circulates around five logarithmical values. This causes the variation that is observed on PerCP-Cy5-5, and -by extrapolation- on PE-Cy7, to be insignificant after all.

Histograms are plotted to see the distribution of marker expression in the cohort. ROC curves for sensitivity and specificity determination per marker are plotted. This allows for a rational choice for a cut off value to set positivity for CD25. Dendrograms are plotted to see the most neighboring markers. A proximity table is created to test for the correlation of all markers with eachother. This is useful to determine which markers affect which ones in significant rates, and thereby speculate about biological associations between the markers. For example, CD123 and CD56 seem to be significantly affected by each other. CD123 is a maturation marker, and upregulation of this protein indicates an immature phenotype of SM, and CD56 is associated with multilineage involvement, which means that the cKIT mutation has occurred in one of the earlier myeloid progenitors.

The cohort consists of 319 cases of which 28 immunophenotypical variables are logged, and 60 variables with clinical and laboratory data included. The dataset is too complex for automated statistical correlation studies. Additionally, some variables may rationally have associations with each other, whereas some do not. Additionally some variables are inherent to others. Therefore, performing directed associations tests is more appropriate. With parallel coordinate plots the user is allowed to shift cut-off values of one or more variables, and instantly see what distribution the selected region has on all other parameters.

Correlations and Associations for Future Research

For future research we advise to research correlation of disease outgrow with multilineage involvement, and the possibility to detect multilineage involvement through (1) detection of KIT mutation in PB with a cDNA assay; (2) mapping other cell types that show upregulation of CD117 through an improved assay, and in specific for eosinophils ;(3) defining association with $CD25^{+}/SSC^{low}$ MCs and (4) analysis of the relation of double CD25 population with better prognosis.

Consequently, an increased number of eosinophils should exclude the presence of a double CD25 population, and an increased CD123 likewise.

We advise to research Mhis/MIMA as a second criterion analogous to the serum tryptase measurements to bring into association with immunophenotype and clinical aspects.

CD52 should be revised as correlator with disease progression as our results showed strong resemblance concerning CD52 distribution as to what is stated by Hoermann et al.

WDSM remains a relatively unknown subtype of SM for which no fixed criteria for diagnosis –especially based on immunophenotype- yet exist. Association of CD22 and CD30 with WDSM should be investigated. Moreover, a good scaffold to define WDSM on base of clinical findings is built. This should be used to search for any immunophenotypical characteristics that associate with WDSM.

Individual marker expressions on MCs are commonly studied. Distribution of MC subgroups within other MC subgroups is less well explored. Plotting multiple markers on one channel is a multidimensional immunophenotyping approach and will reveal MC subtypes in a new way that have not yet been described. For instance, it is frequently observed that $CD25^{-}$ in a case of a double CD25 cluster is confined to $CD52^{-}$, while $CD25^{+}$ from the same case is distributed over $CD52^{-}$ and $CD52^{+}$. Based on this given, MCs can be classified into three groups in total, instead of two groups per marker. This principle can be elaborated by taking more markers into account, hence the multidimensionality of this approach.

A frame of parameters consisting of clinical features with implications for clinical treatment and decision making is set. It is of importance the data for this frame is collected and coupled to the immunophenotypical database for further associations. This will allow for the extrapolation of our immunophenotypical findings to the clinical setting.

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Appendix

Annex 1:

Assessment of flowyctometric data with Infinicyt™

Goal

In this header a description is given for the assessment of the marker expression of MCs for the marker panel as later defined.

Gating instructions

1. Infinicyt™ 2.0 is started up.
2. Analysis is started by clicking on 'Analysis'.
3. The 'Typ…' folder of interest is opened.
4. All measurements from the different tubes are selected, except for the tube that contains IgE as a marker, and opened. They are named following the systematic: 'Last name patient' 'UMCG no. patient' bm bulk 'ID number'.fcs
5. 'Profile' > 'Load profile from folder …' is clicked.
6. For the old panel of markers 'oud protocol profile 4.inp' and for the new panel of markers 'nieuw protocol profile 3.inp' is chosen.
7. On the SSC-A/FSC-A channel the right mouse button is clicked > 'Configure data visualization' > under 'Density Configuration' 'Total Density' is checked.
8. The SSC-A/FSC-A channel is put on full screen. On low SSC-A height three or four density cluster will be observed. The first, or the first two clusters that fall largely below 50.000 FSC-A are gated out by drawing a contour around the rest of the clusters. See fig.

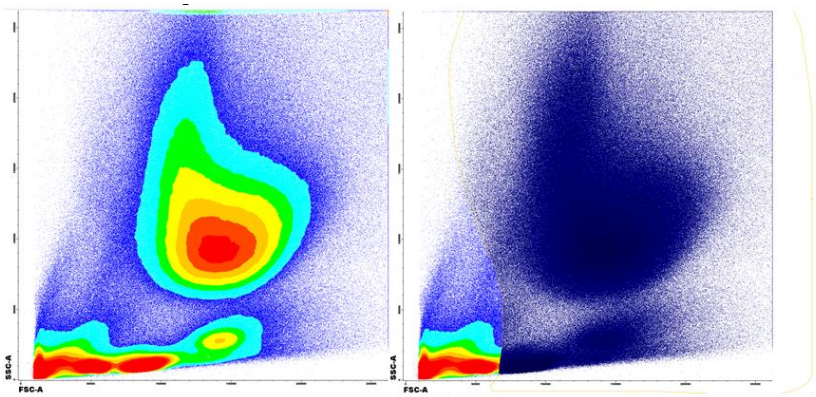


Figure 36

9. The SSC-A/FSC-A channel on full screen is minimized. The SSC-A/CD45:HV450-A channel is put on full screen. All nucleated cells are gated as seen in fig.

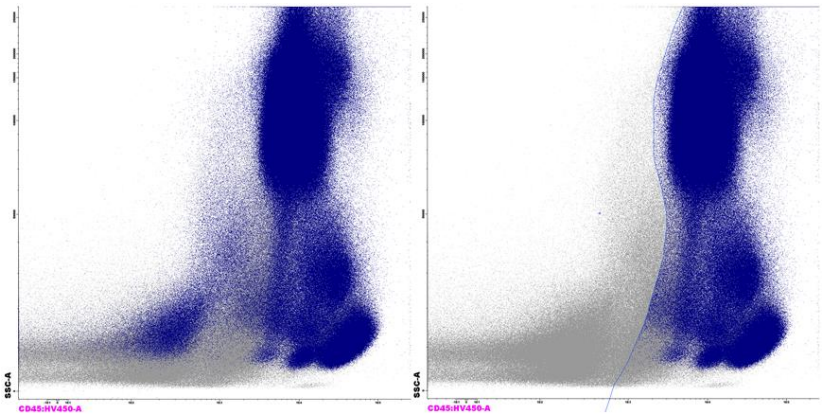
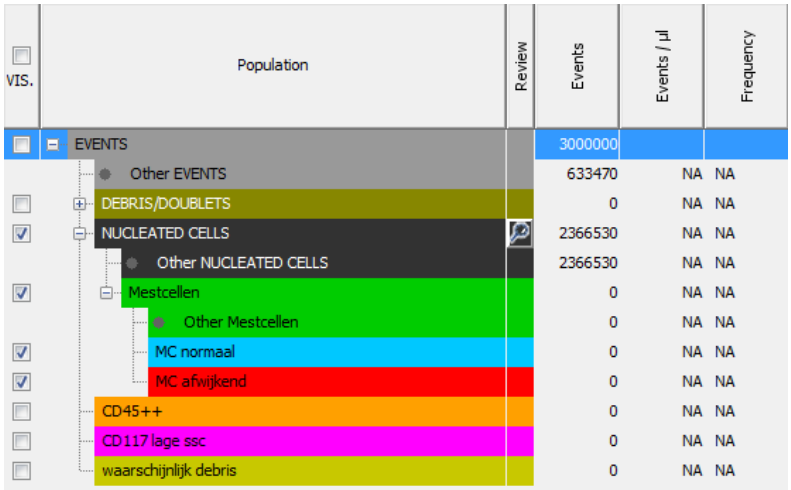


Figure 37

10. The selection is defined as nucleated cells by clicking on 'NUCLEATED CELLS' under the population tab. See fig.



VIS.	Population	Review	Events	Events / µl	Frequency
☐	EVENTS		3000000		
	Other EVENTS		633470	NA	NA
☐	DEBRIS/DOUBLETS		0	NA	NA
☑	NUCLEATED CELLS		2366530	NA	NA
	Other NUCLEATED CELLS		2366530	NA	NA
☑	Mestcellen		0	NA	NA
	Other Mestcellen		0	NA	NA
☑	MC normaal		0	NA	NA
☑	MC afwijkend		0	NA	NA
☐	CD45++		0	NA	NA
☐	CD117 lage ssc		0	NA	NA
☐	waarschijnlijk debris		0	NA	NA

Figure 38

11. The 'EVENTS' tab is unchecked and the 'NUCLEATED CELLS' tab is checked.
12. Mast cells are gated on the SSC-A/CD117:Pe-Cy7-A channel by selecting the most-positive CD117 cluster of cells. See fig.

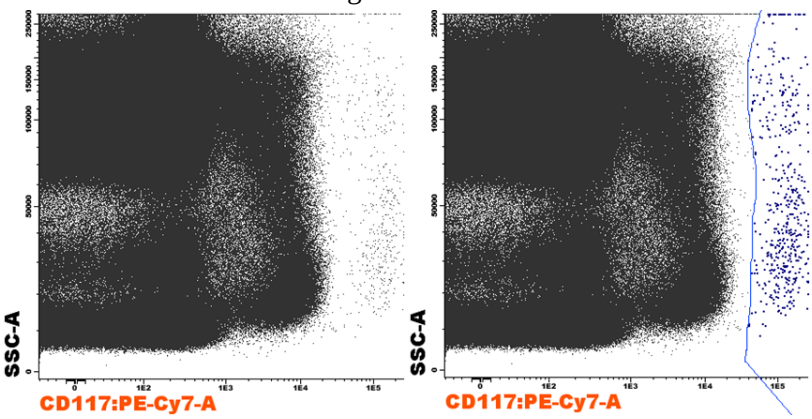


Figure 39

13. The mast cells are defined as mast cells by clicking on ‘Mestcellen’ under the population tab.
14. Under the population tab ‘NUCLEATED CELLS’ is unchecked and ‘Mestcellen’ is checked.
15. The upper side line on the CD117 channel is selected and defined as ‘DEBRIS/DOUBLETS’ under the population tab.
16. The right side line on the CD117 channel is selected and defined as ‘DEBRIS/DOUBLETS’ under the population tab.
17. On the FSC-H/FSC-A channel the events that fall below the diagonal cluster and the right side line are selected and defined as ‘DEBRIS/DOUBLETS’ under the population tab. See fig.

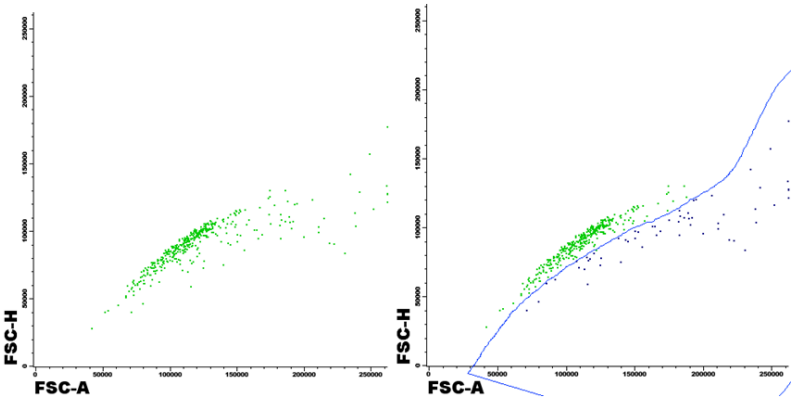


Figure 40

18. On the CD45 channel if an SSC-A^{low}/CD45^{high} population presents it is selected and defined as ‘CD117 lage ssc’ under the population tab. See fig.

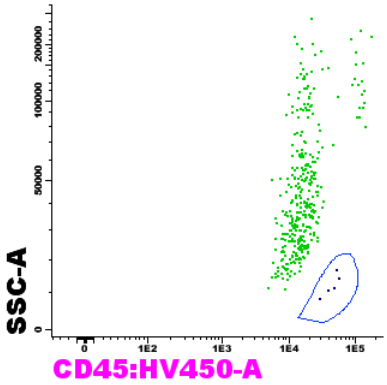


Figure 41

19. On the CD45 channel if an SSC-A^{high}/CD45^{high} population presents it is selected and defined as ‘CD45++’ under the population tab. See fig.

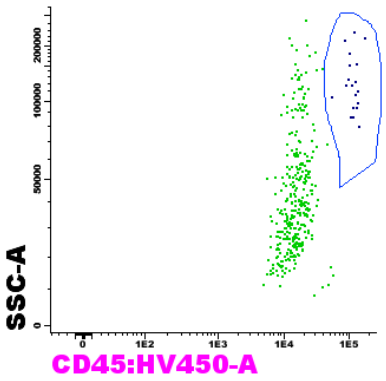


Figure 42

20. In the new protocol CD71 is also measured. There you may perform the previous step by gating the concerning population as seen in fig.

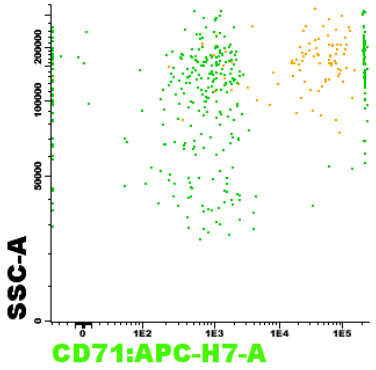


Figure 43

21. If there is no CD45++ population clearly distinguishable on neither CD45 nor the CD71 channel, and there is a double population on PE-A then select PE-A^{high} and define it as CD45++, and select the SSC-A^{high}/CD33^{low} population and define it as CD45++ under the population tab. See fig.

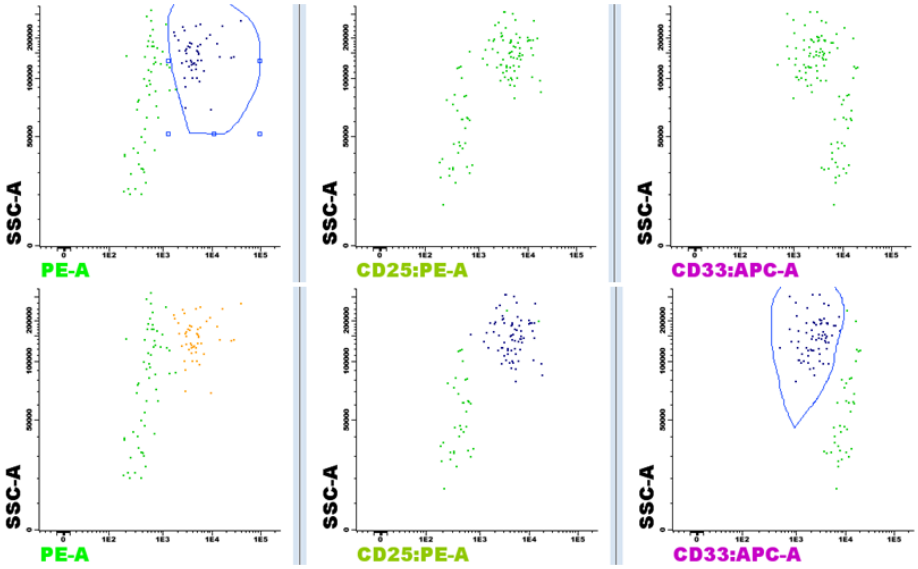


Figure 44

22. If you cannot make a clear distinction between ‘CD45++’ and the mast cells on neither one of the backbone channels nor on the CD33 channel then you may not exclude that a certain mast cell population contains CD25 false positivity. Beware CD25 positive mast cells never show negative compared to the CD25 negative subgroup on the CD30 channel within a patient! The CD45++ subpopulation most often shows a clearly distinguishable negativity for CD30 though intermittently it overlaps with the CD25 positive the mast cells.
23. In the new protocol CD71 is also measured. CD71 right line is removed. See fig.

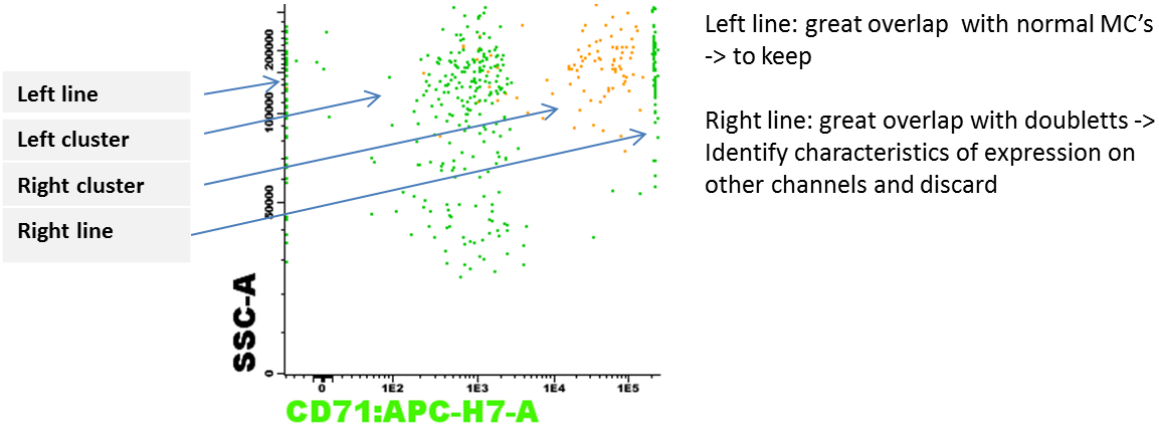


Figure 45

24. If after completing the gating a cluster in the right upper corner of the HV500-A channel is observed this cluster is removed. This is an artifact.
25. The gating is saved in concerning folder as ‘gating’!

Acquiring statistical data

1. If there are two CD25 populations CD25- and CD25+ from the backbone on channel CD117 and channel FSC-H/FSC-A is defined as ‘Normale MCs’ and ‘Afwijkende MCs’ under population tab respectively. See fig.

…

2. If there are two CD25 populations the CD25$^-$ population is deleted from the backbone and the CD25 population is changed back to ‘Mestcellen’
3. After gating is completed the ‘Open Report’ icon is clicked. See fig.

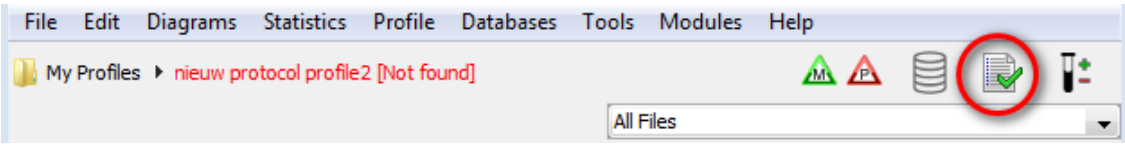


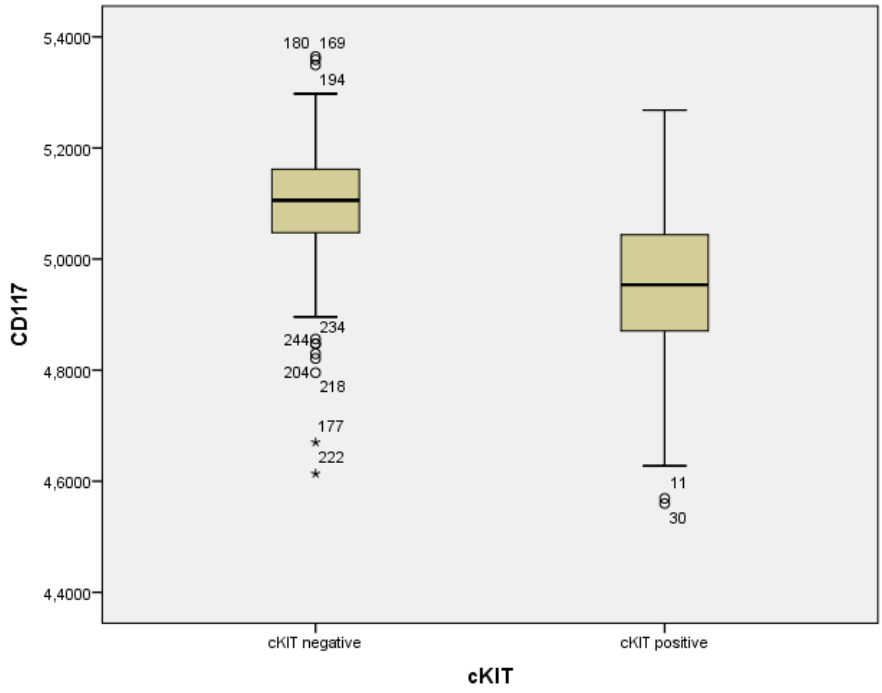
Figure 46

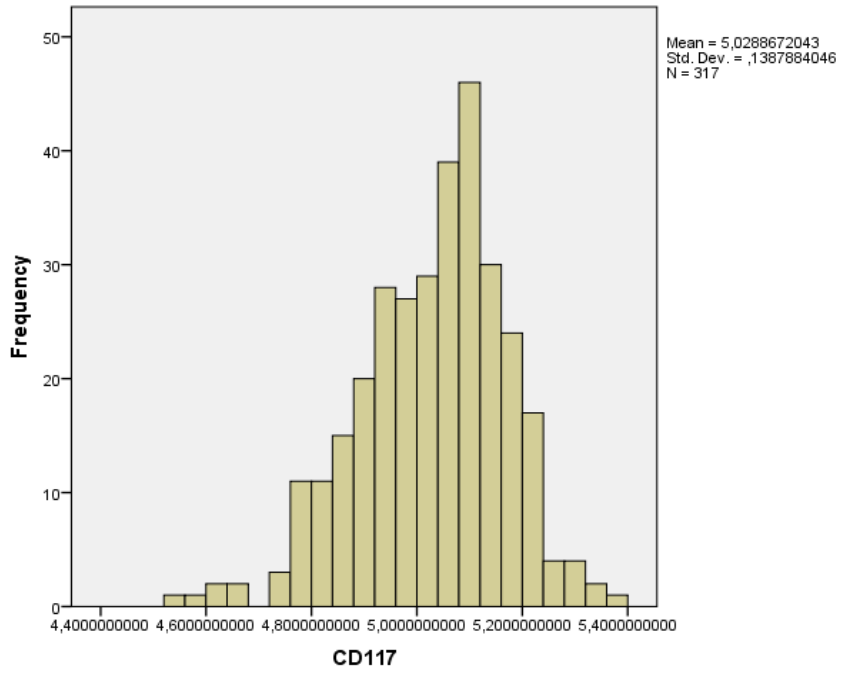
4. Delete table on third sheet by selecting it and pressing ‘Del’ on your keyboard.
5. Right mouse click the same sheet, choose ‘Add Component’ > ‘Statistics’.
6. Under ‘Populations/Parameter’ tab uncheck all populations, and check ‘Mestcellen’ with all its’ belonging parameters, uncheck all subpopulations. Under ‘Stats’ tab check ‘Median’. Click ‘OK’.
7. Copy tab and export to excel.

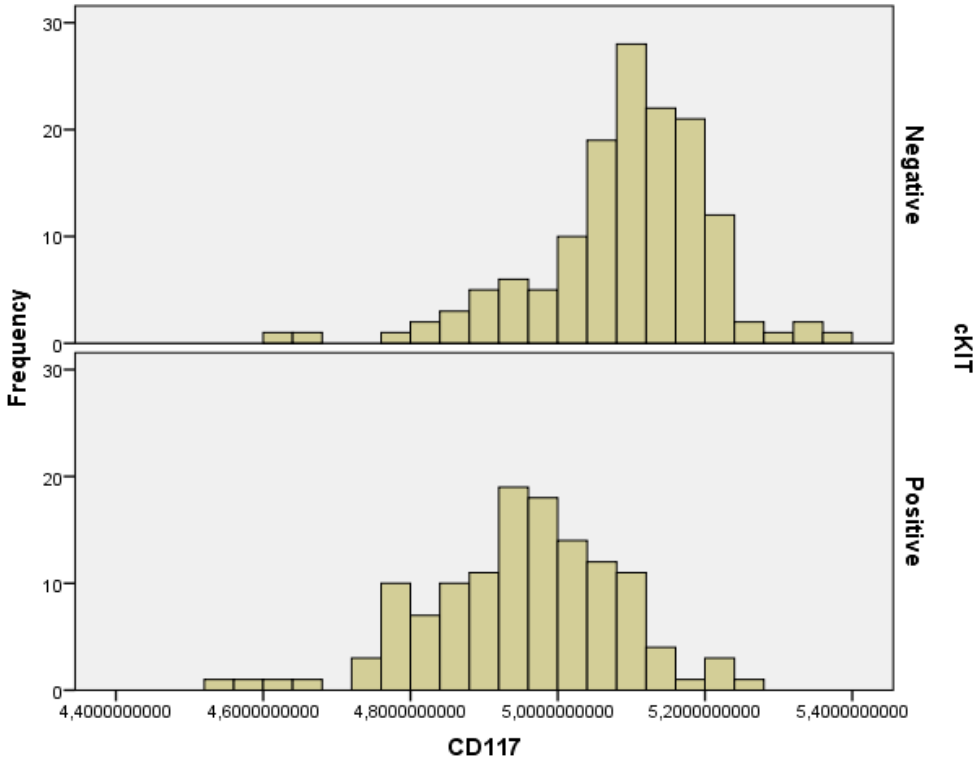
8. Close the report tab.
9. Check on the parameter bands channel whether some of the medians fall on the side line as it may happen on the CD71 channel and on the APC, CD56, CD123, PerCP-Cy5-5, CD22, CD34, HV500 and the CD13 channel. If so, correct for those medians by deleting the concerning side line. If a side line is deleted on a marker channel be sure to delete the side line of the regarding autofluorescence channel as it may cause a bias for later measurements.
10. Fetch the corrected medians as described from step 3 to 8.
11. When multiple corrections need to be made in one tube do not perform all of them at the same time, rather perform them one by one. Reinsert the deleted side lines from the first correction after each next correction as side line events will be deleted from the other channels of your tube. This same principle applies when a side line is removed from a backbone channel as for CD71.
12. Delete the table and right mouse click the same sheet, choose 'Add Component' > 'Statistic' again.
13. All changes are undone to obtain the gating file as saved in step 25 from 'Gating instructions'.
14. On the CD25 channel the positive cluster is marked as 'MC afwijkend' and the negative cluster is marked as 'MC normaal'. Make sure the CD45++ is marked from the backbone as representative as possible for its'true count, or if not possible from the backbone from all other channels individually.
15. Be sure to collect CD45++ from all channels and $CD25^{+/-}$ from tube 2 consequently as correction for the total count will be performed in later calculations!
16. The 'Open Report' tab is clicked and the current table on the third sheet is deleted.
17. A new statistics component is added.
18. All parameters are unchecked and the SSC-A, FSC-A and FSC-H of 'MC normaal', 'MC afwijkend' and 'CD45++' populations are checked.
19. Under 'Stats' tab 'Events', 'Total %', 'Median', 'First Quartile (Q1)' and 'Third Quartile (Q3)' are checked. Click on 'OK'.
20. Copy tab and export to excel.
21. Close the report tab.

Annex 2:

CD117



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10
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Positive
cKIT
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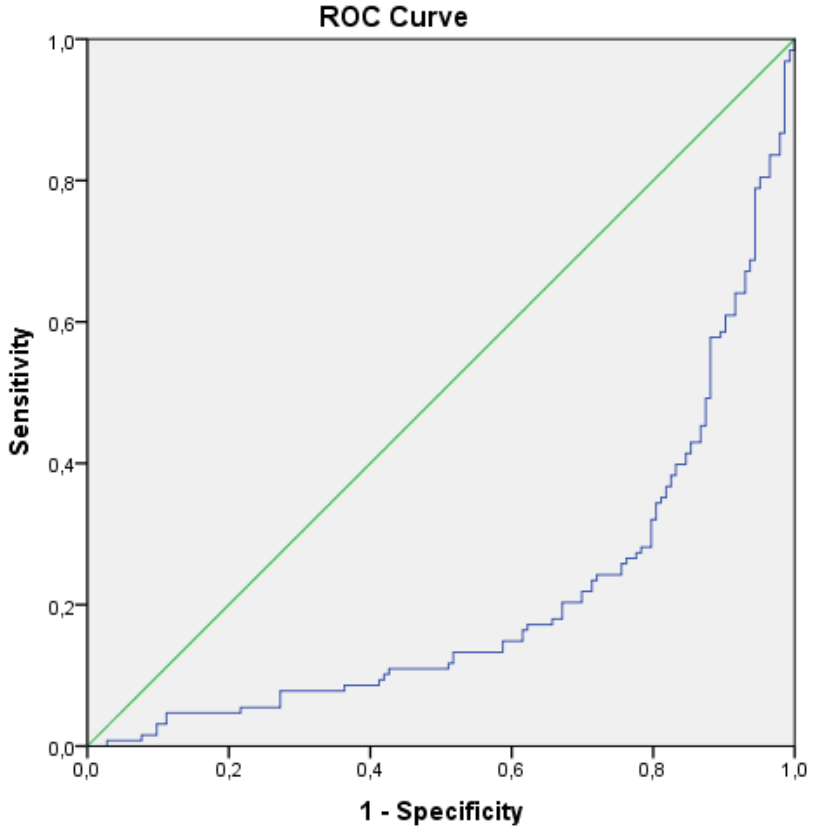
5,0000000000
5,2000000000
5,4000000000
CD117


ROC Curve
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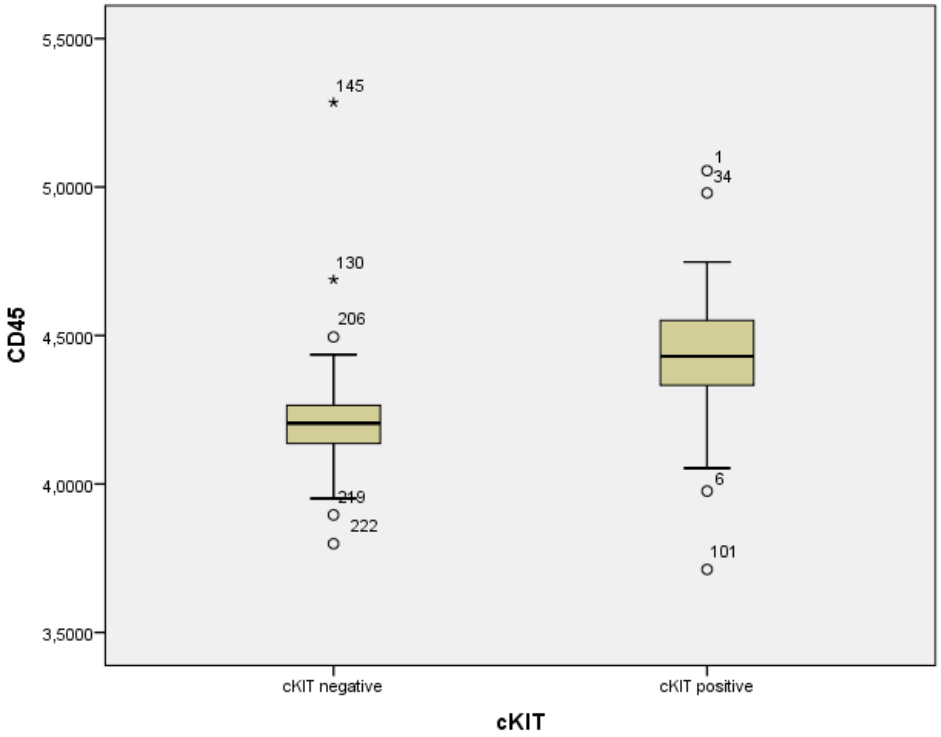
0,4
0,6
0,8
1,0

CD45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4,5000
4,0000
3,5000
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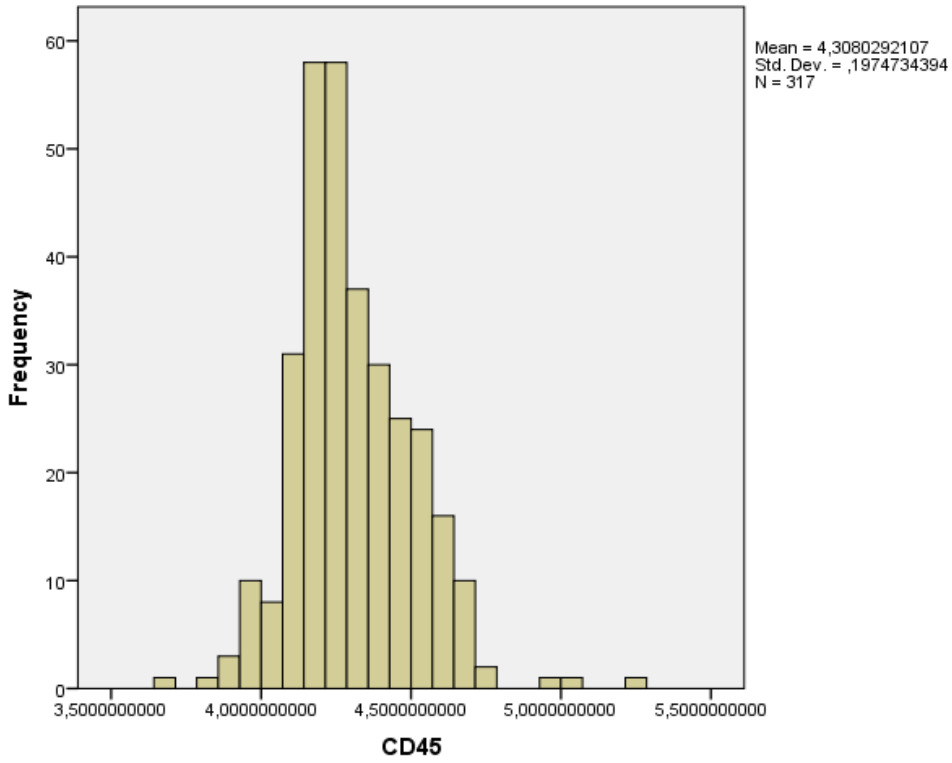
206
219
222
1
34
6
101
cKIT negative
cKIT positive
cKIT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Std. Dev. = ,1974734394
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30
20
10
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Frequency
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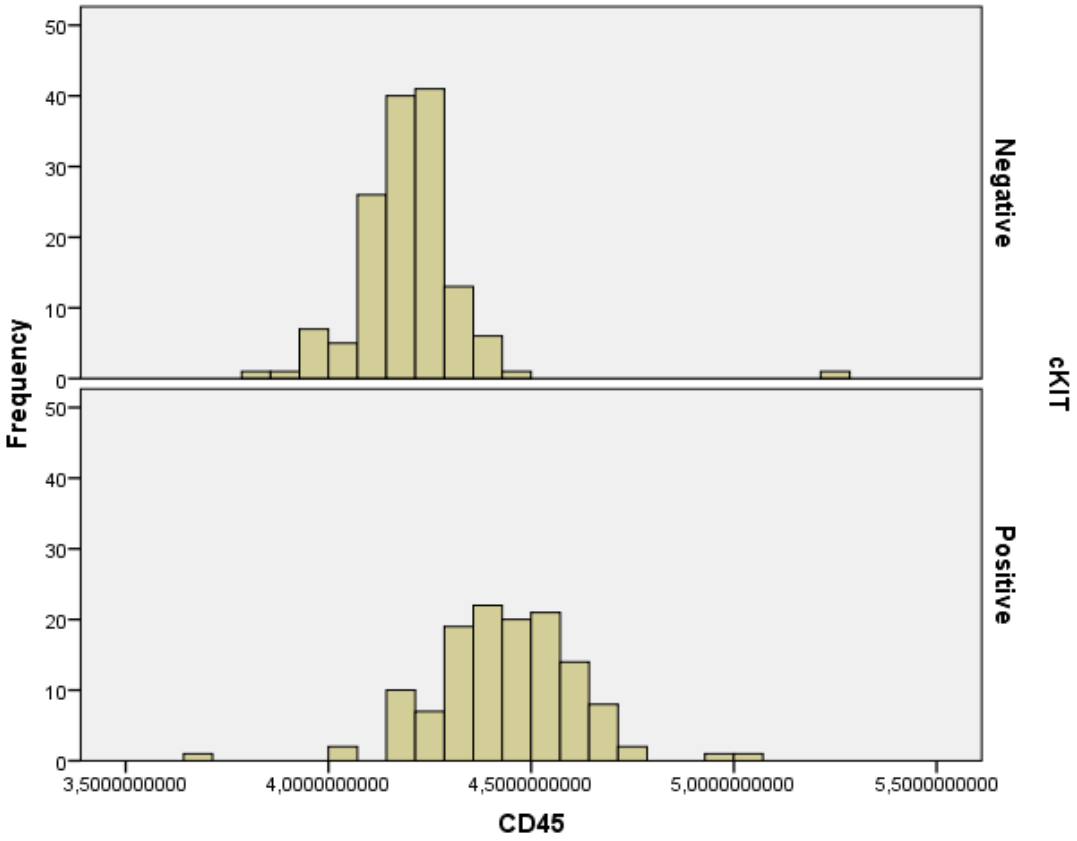
CD45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cKIT
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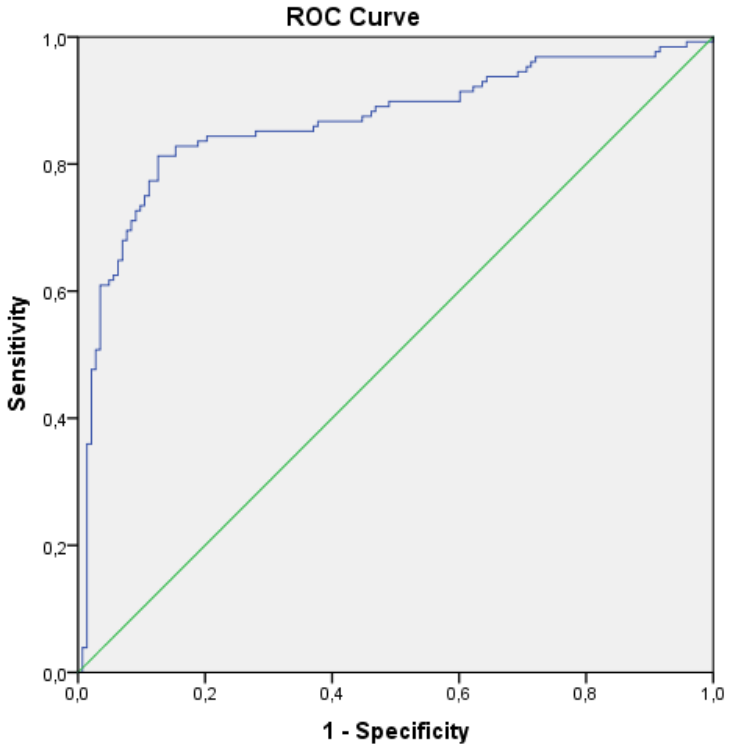
CD45


ROC Curve
Sensitivity
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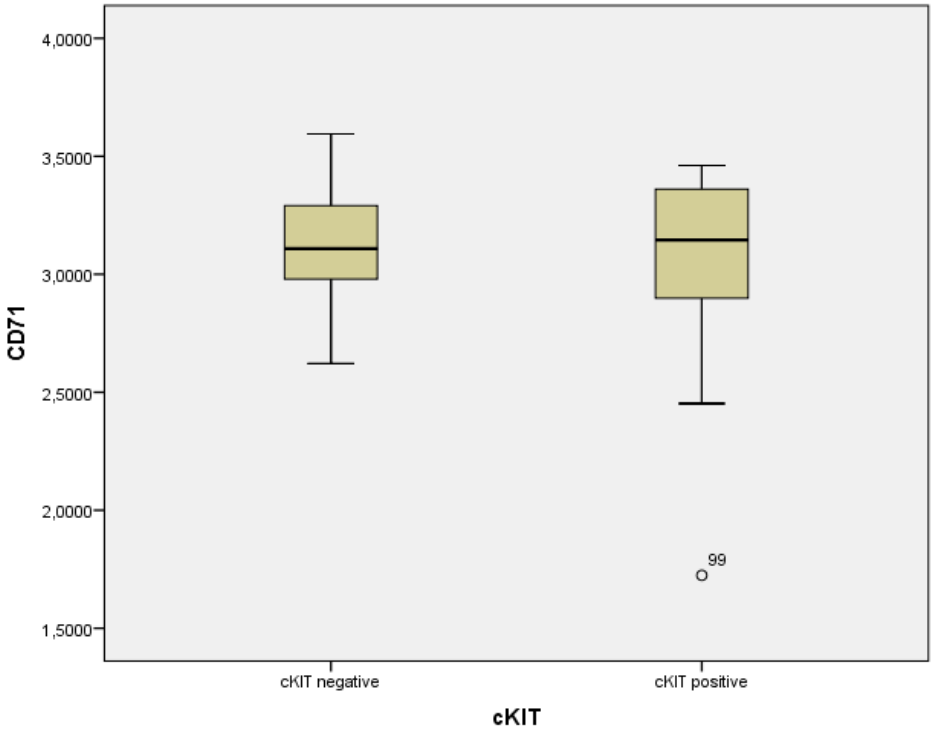
0,4
0,2
0,0
1 - Specificity

CD71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2,0000
1,5000
CD71
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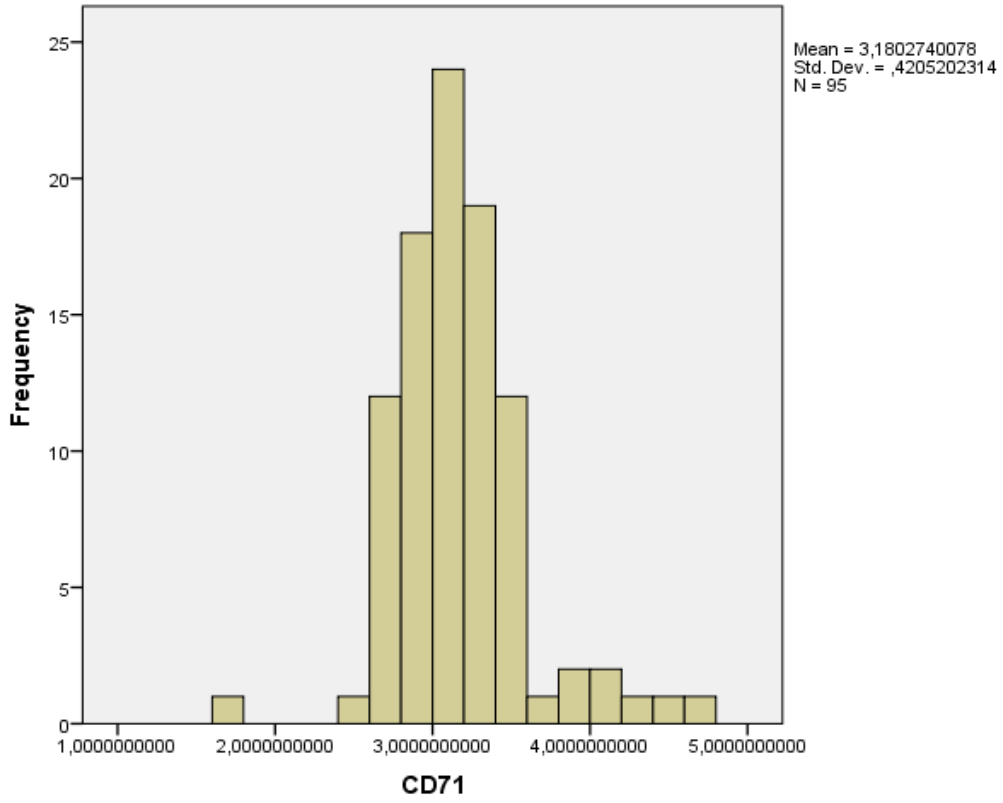
cKIT negative
cKIT positive
cKIT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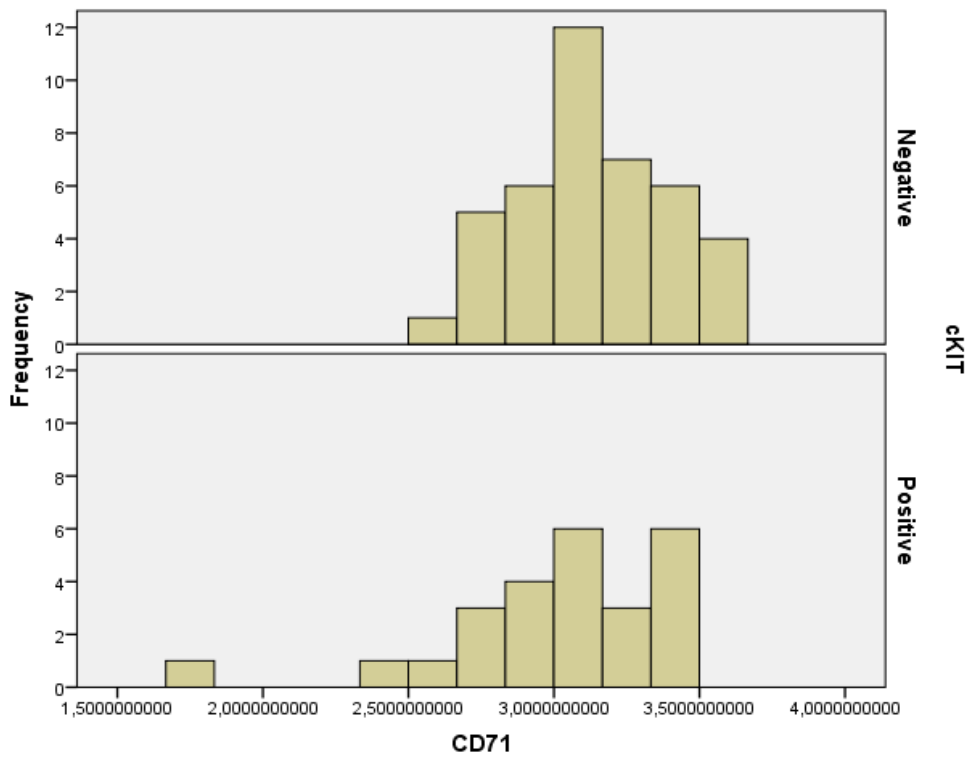
CD71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Positive
cKIT
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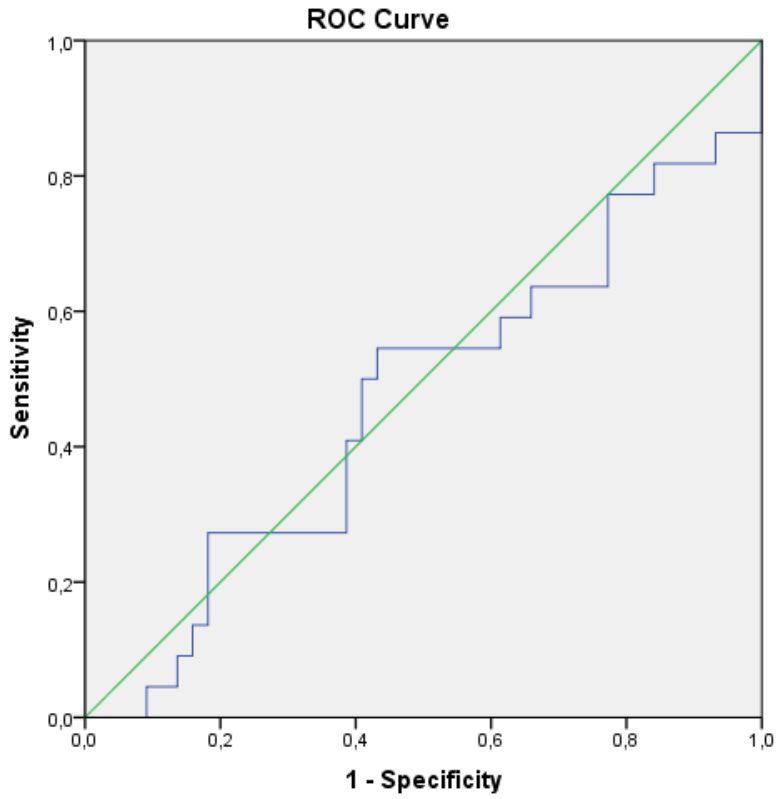
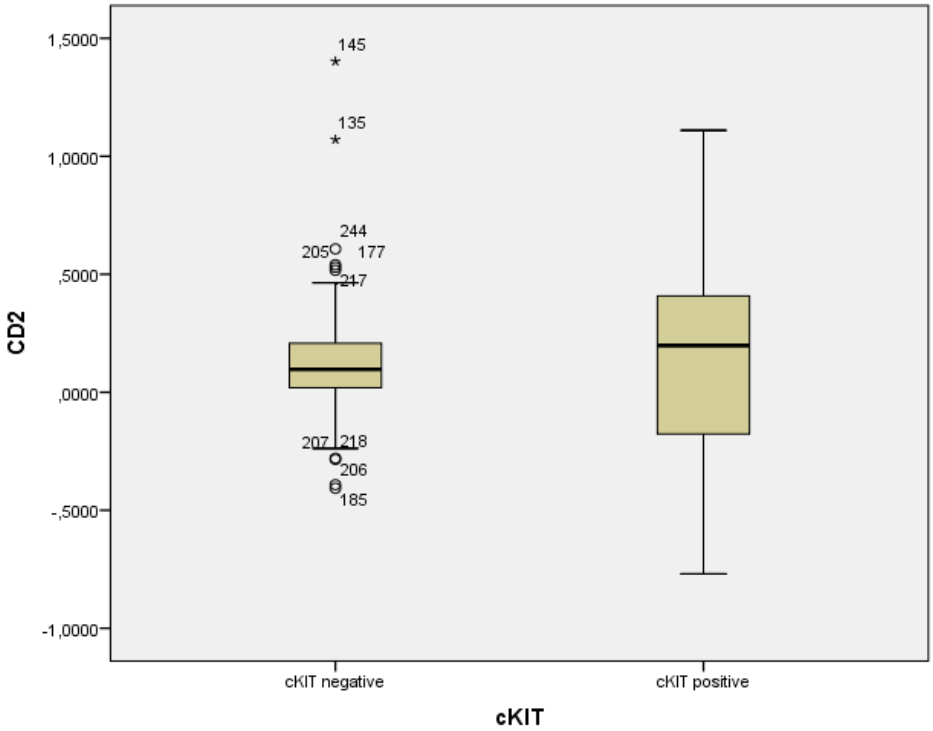

ROC Curve
Sensitivity
1 - Specificity

CD2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-1,0000
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207
218
206
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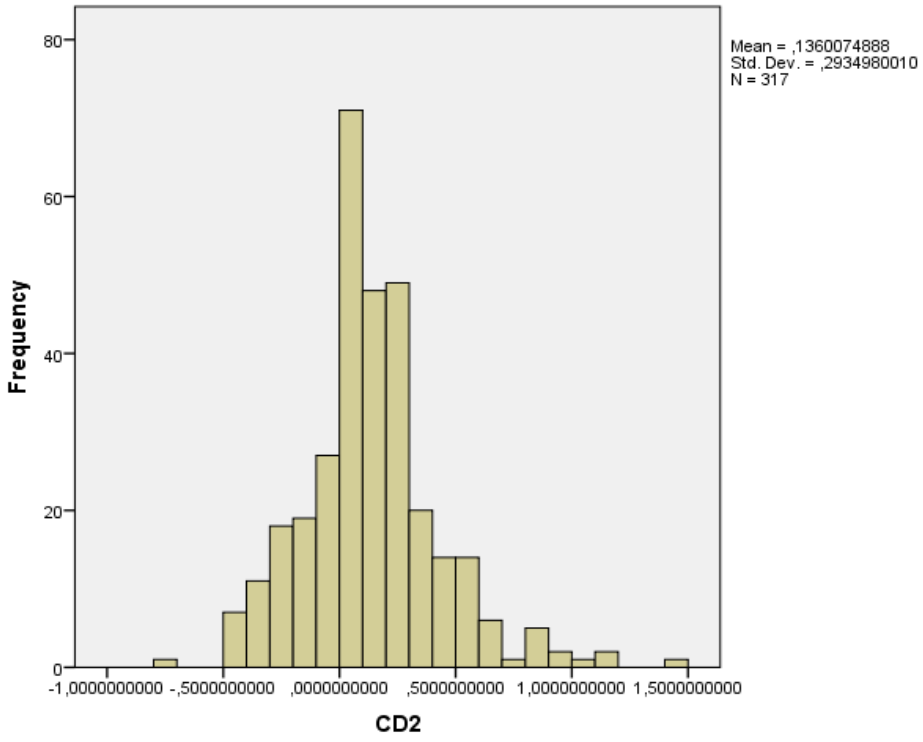
cKIT negative
cKIT positive
cKIT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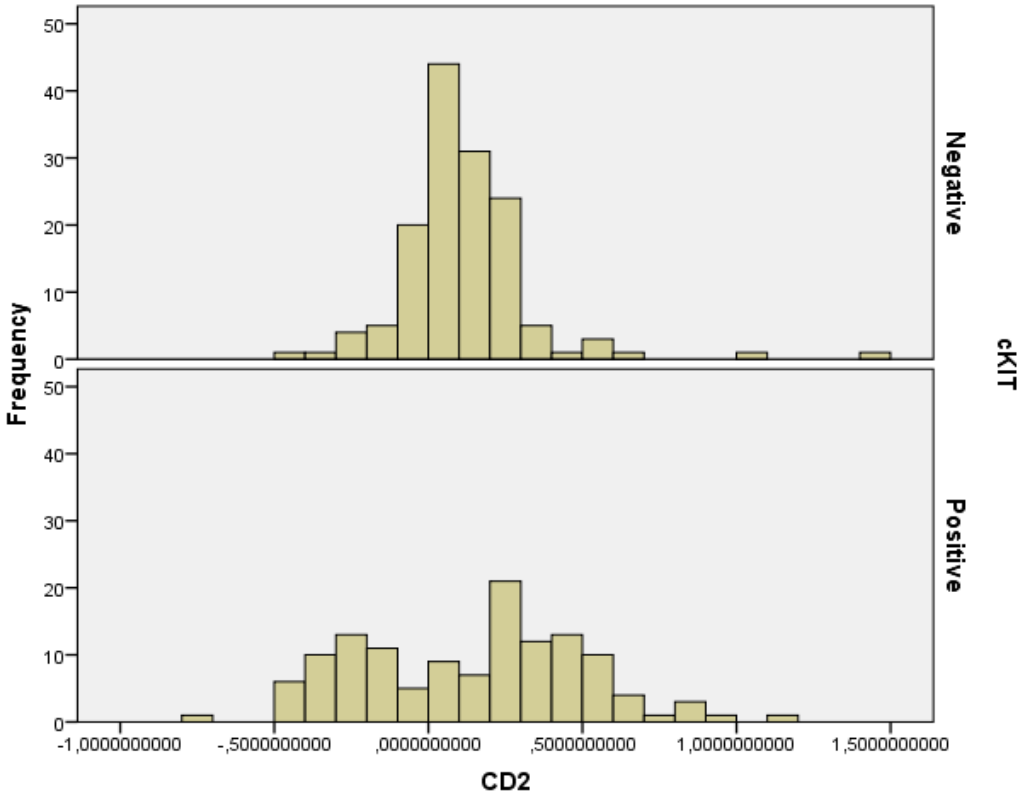
CD2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Positive
cKIT
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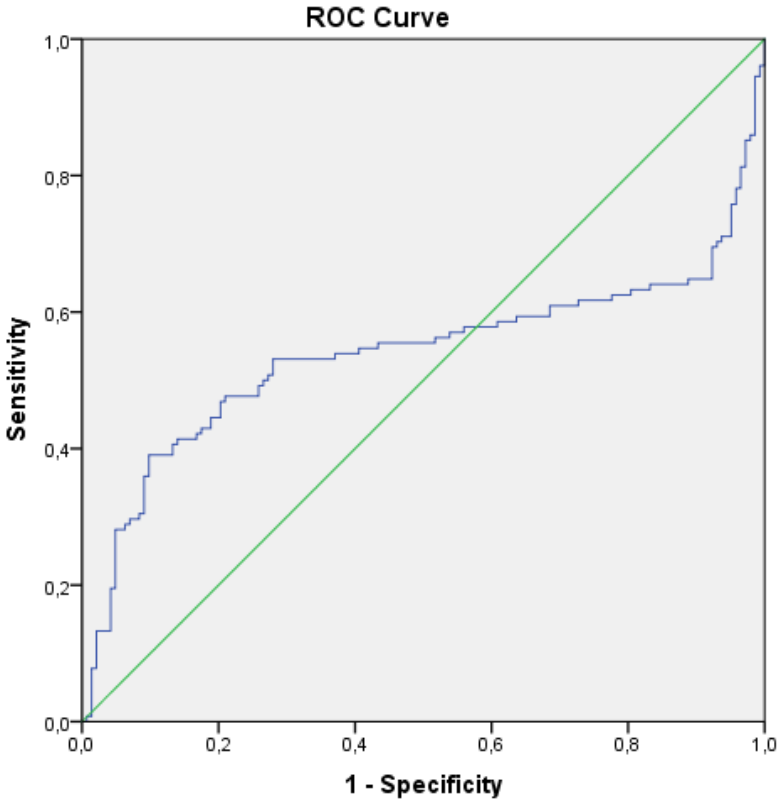
CD2


ROC Curve
Sensitivity
1 - Specificity
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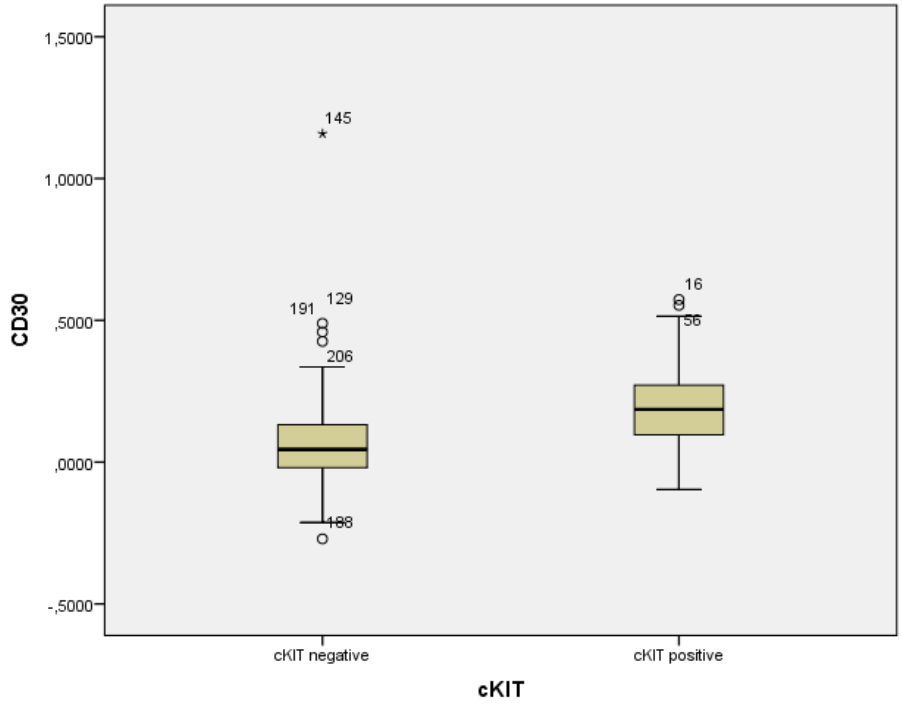
0,8
1,0

CD30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CD30
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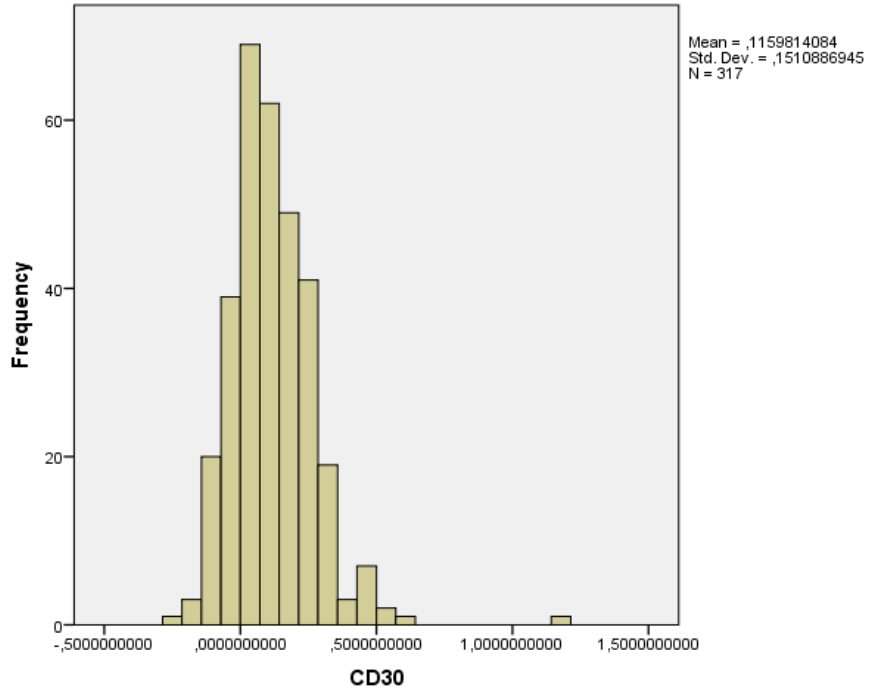
191
129
206
188
16
56
cKIT negative
cKIT positive
cKIT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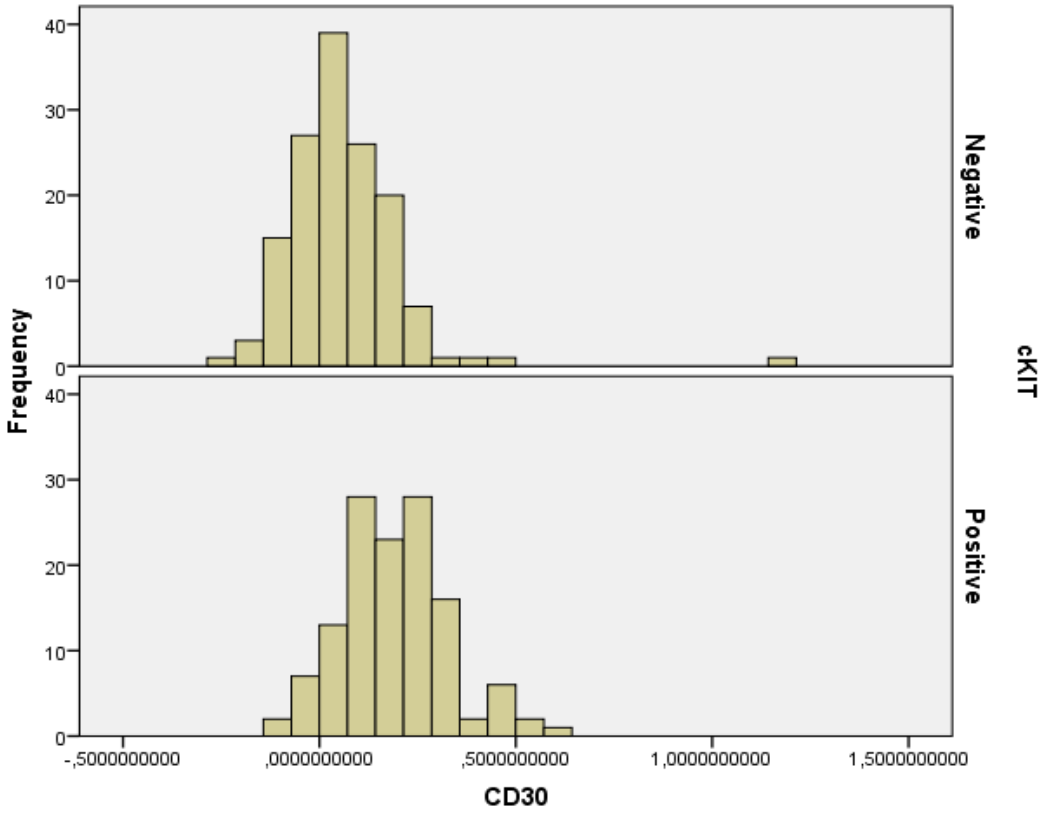
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cKIT
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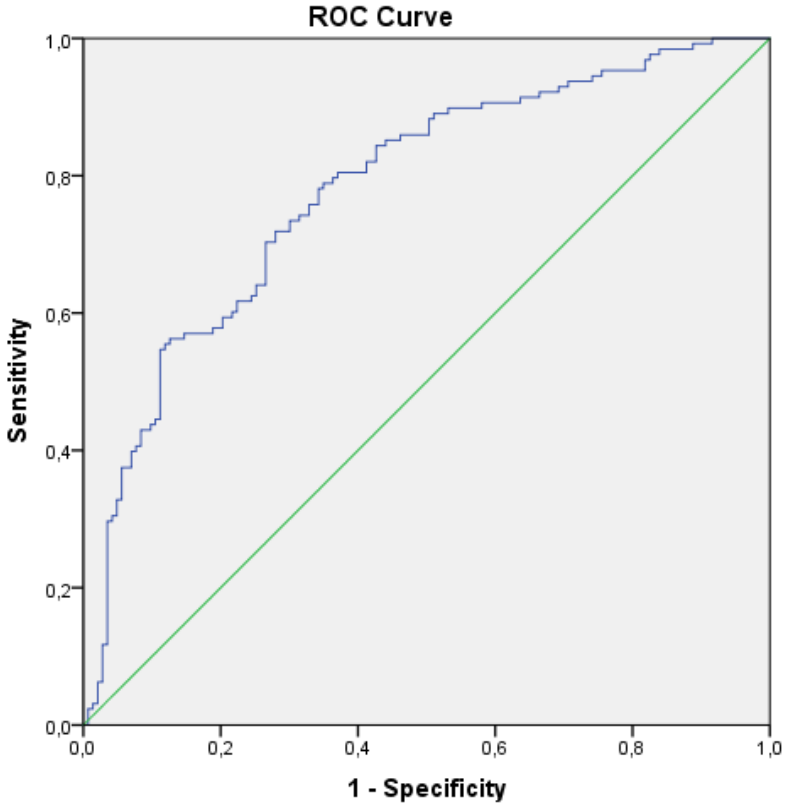
CD30


ROC Curve
Sensitivity
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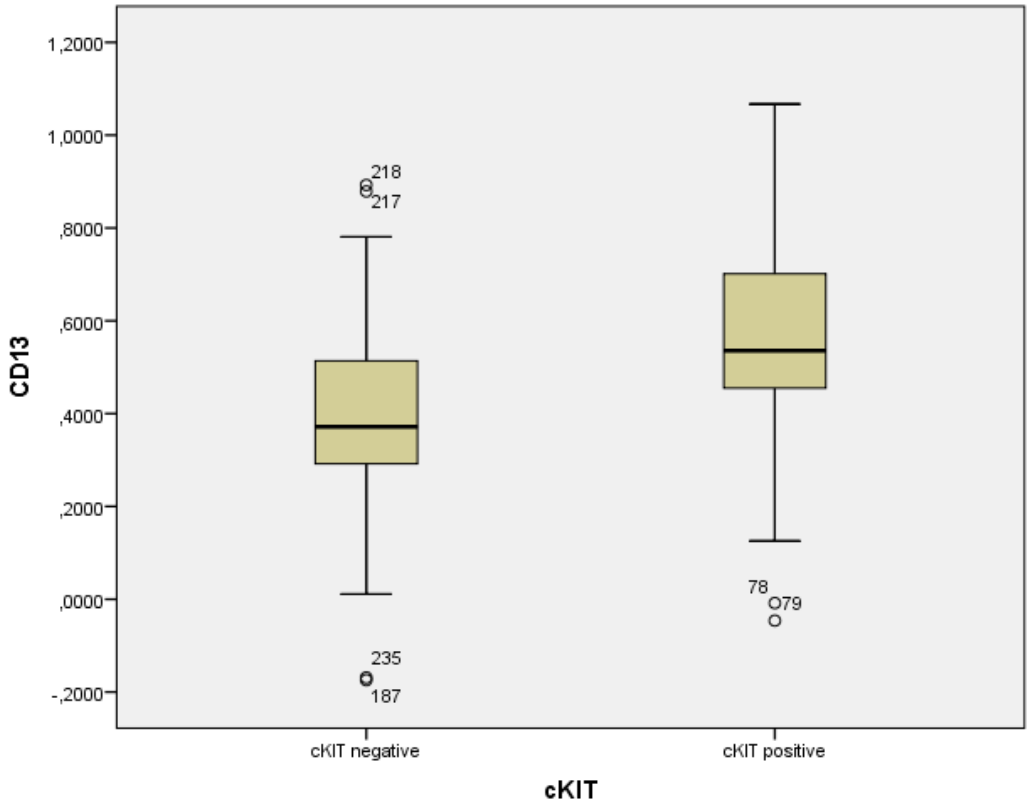
0,4
0,2
0,0
1 - Specificity

CD13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235
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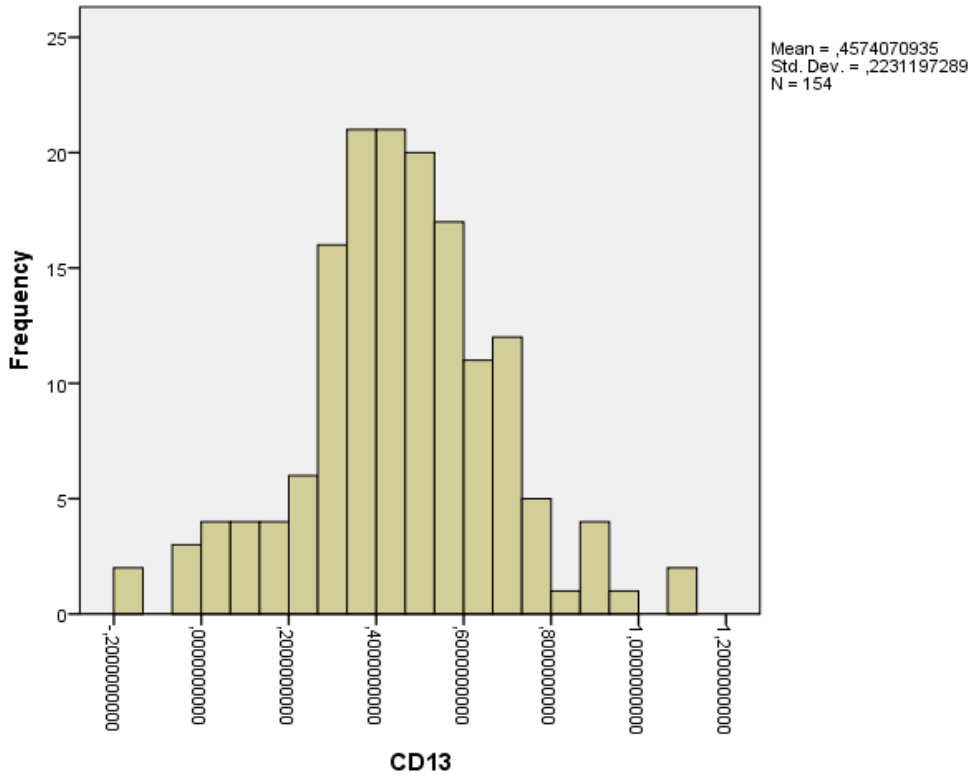
78
79
cKIT negative
cKIT positive
cKIT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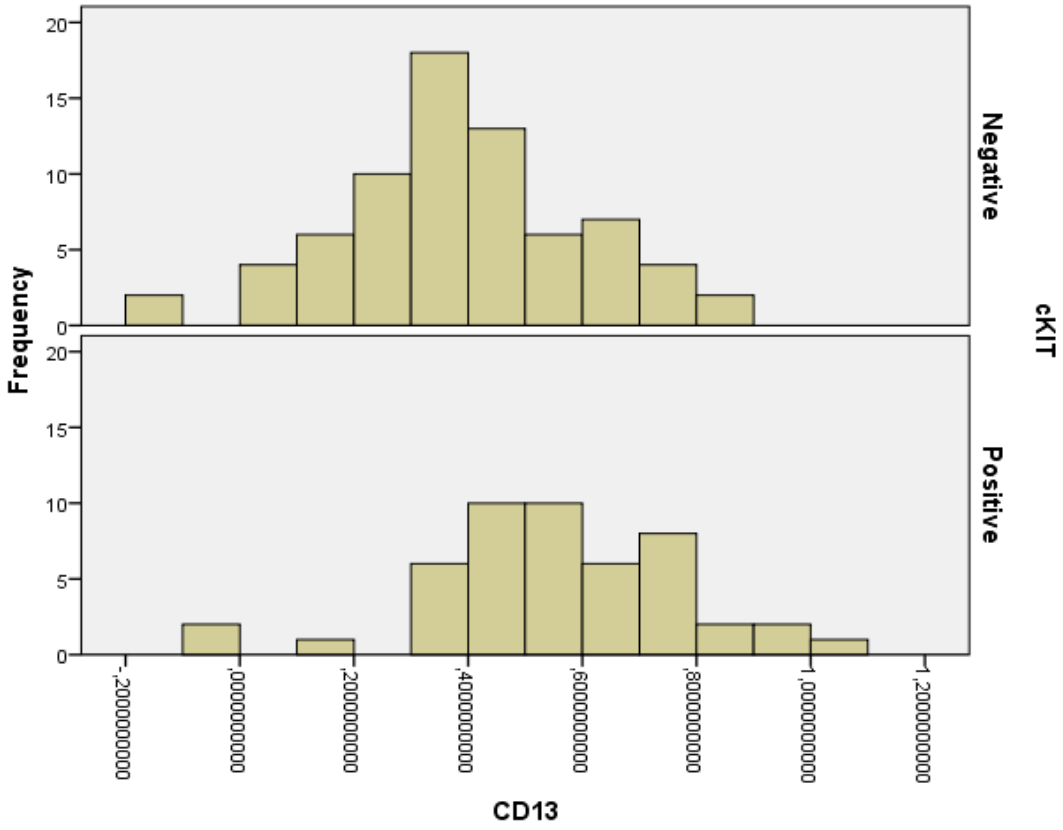
CD13


Frequency
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10
5
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Negative
Positive
cKIT
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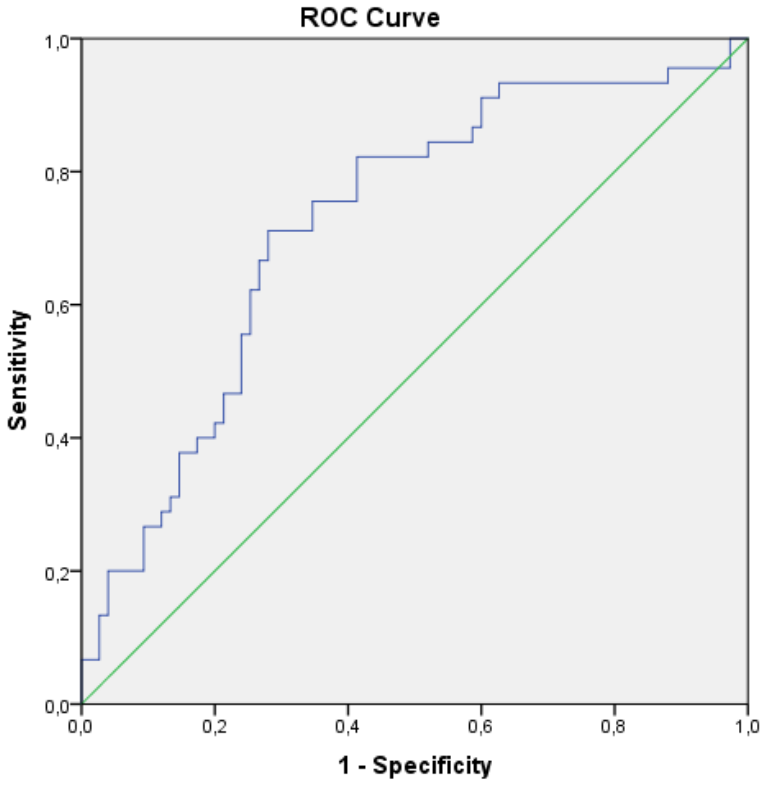
CD13


ROC Curve
Sensitivity
1 - Specificity
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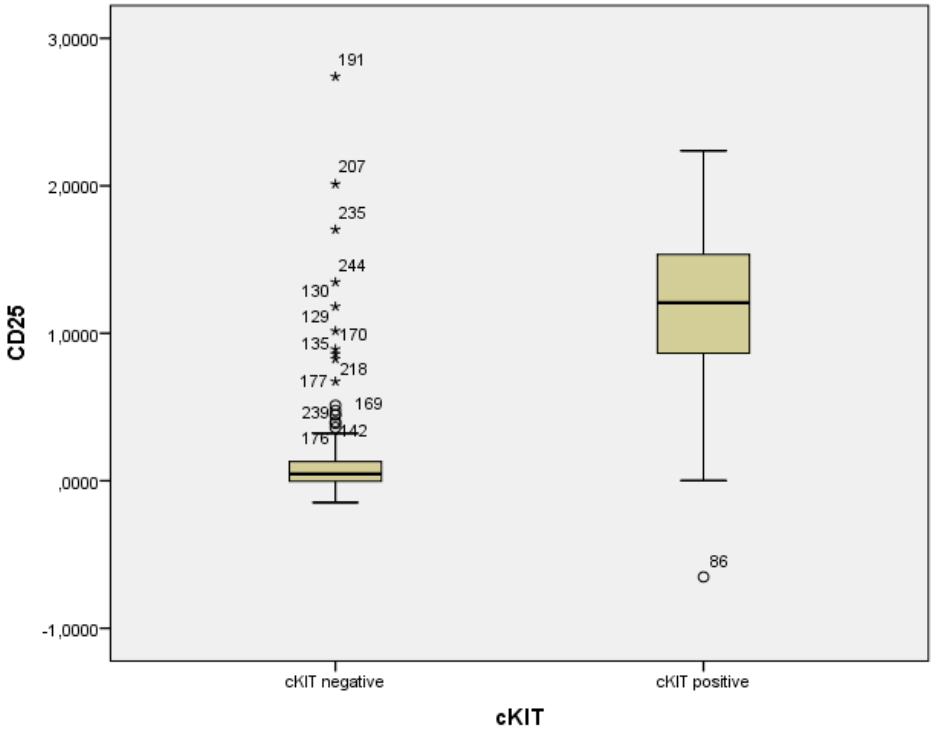
0,2
0,4
0,6
0,8
1,0

CD25


3,0000
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,0000
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CD25
191
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235
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129
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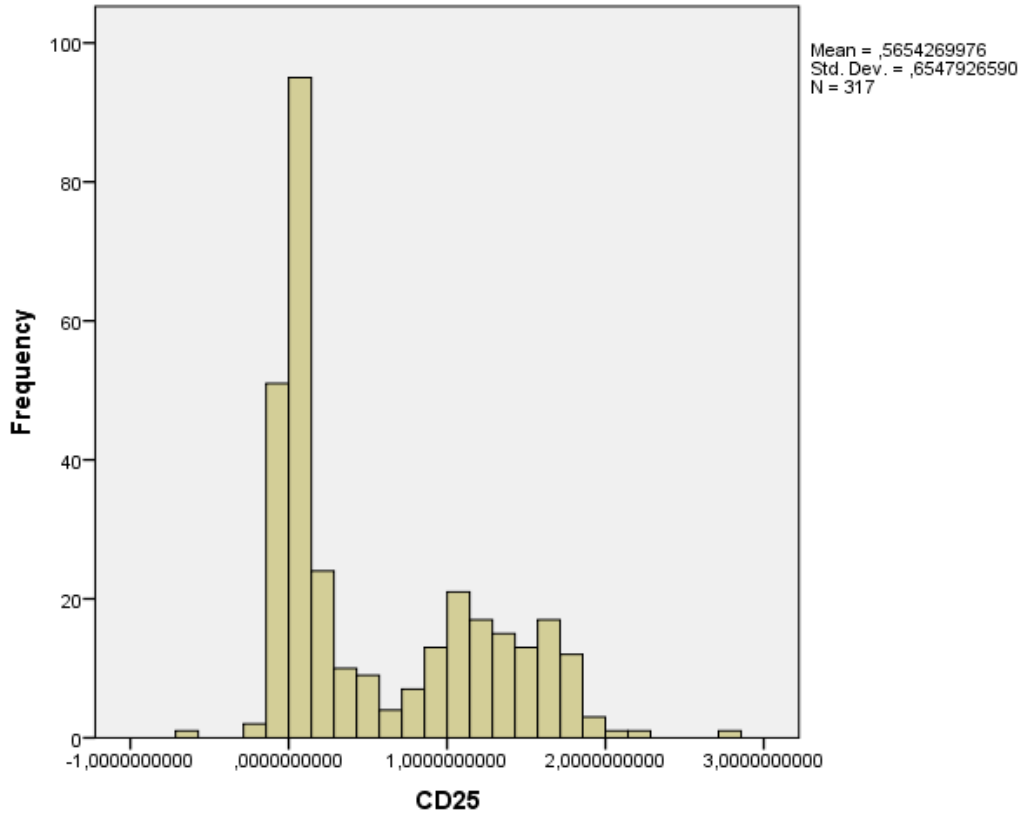
135
218
177
169
239
442
176
86
cKIT negative
cKIT positive
cKIT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0
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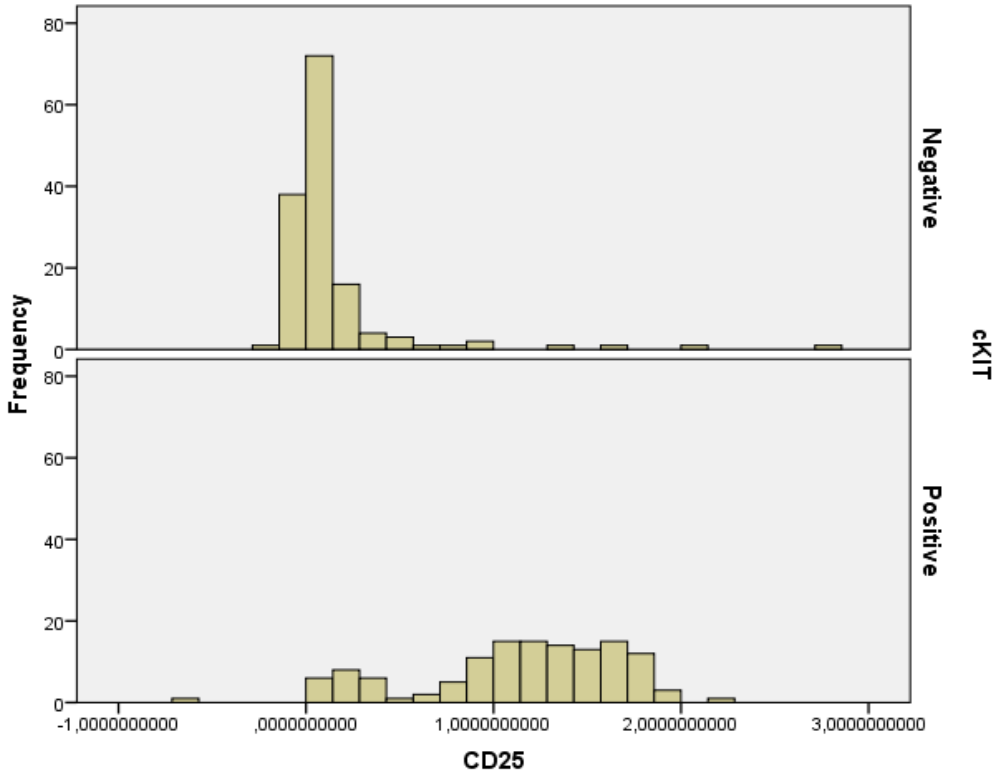
3,0000000000
CD25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20
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Positive
cKIT
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1,0000000000
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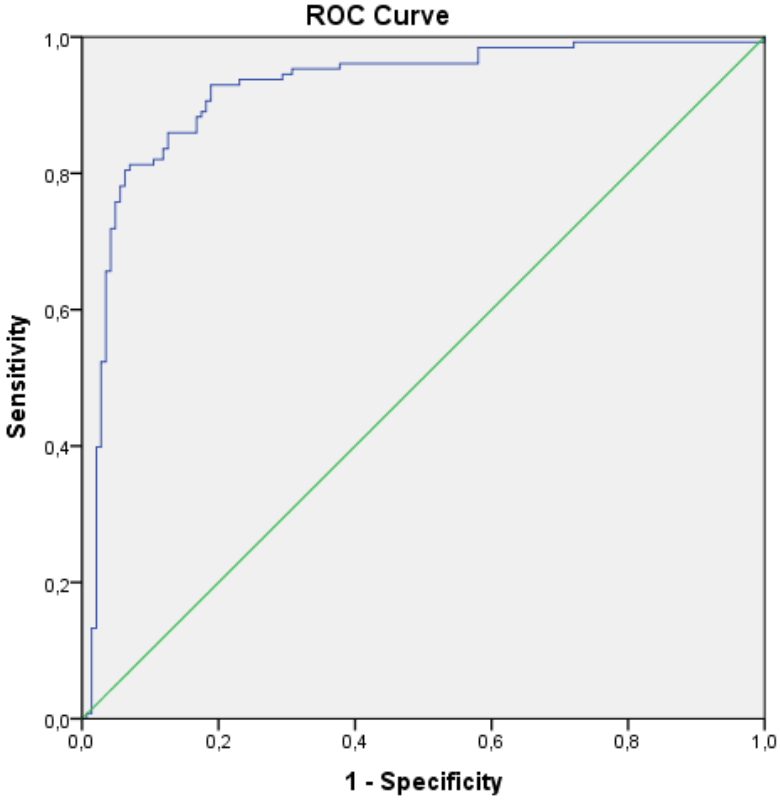
3,0000000000
CD25


ROC Curve
Sensitivity
1 - Specificity
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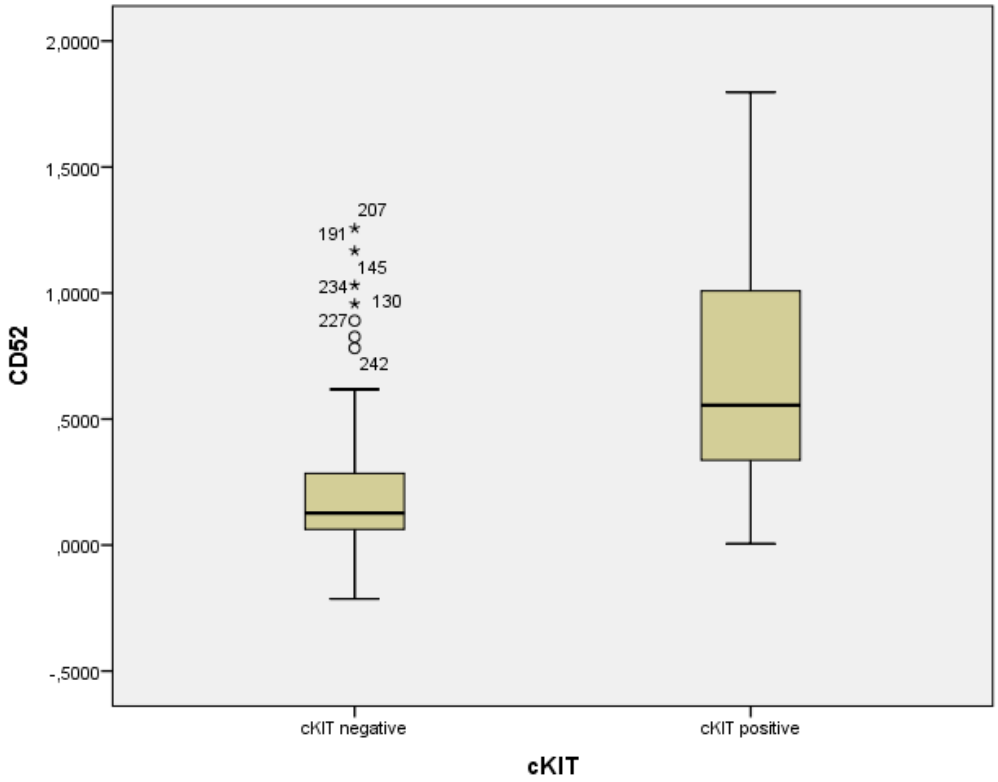
0,8
1,0

CD52


2,0000
1,5000
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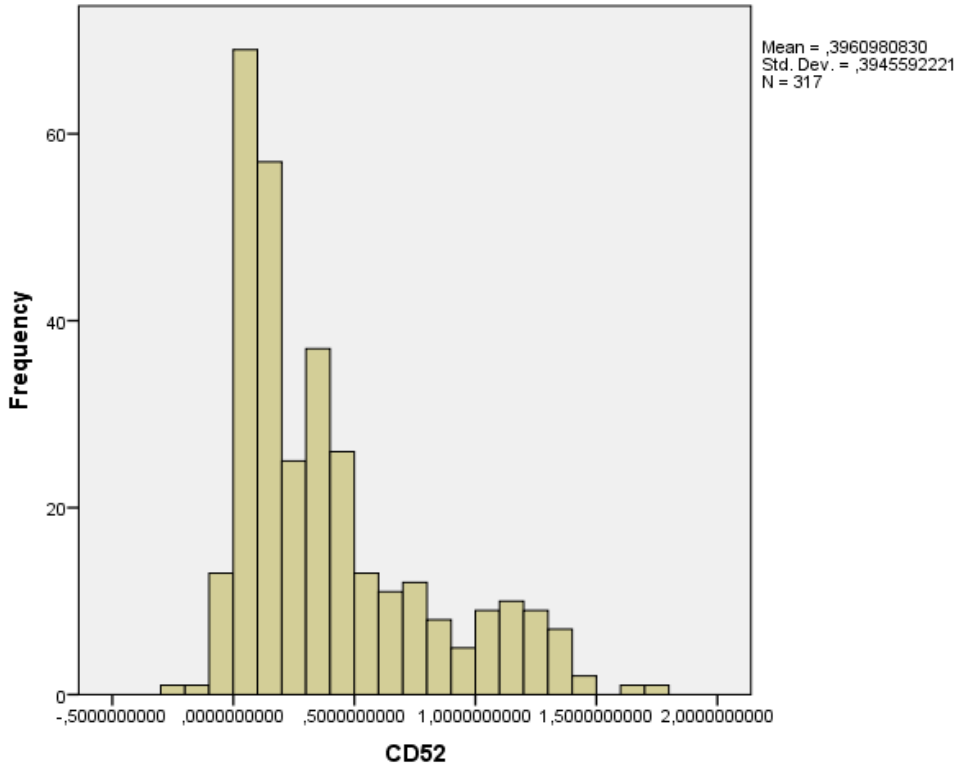
CD52
207
191
145
234
130
227
242
cKIT negative
cKIT positive
cKIT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Frequency
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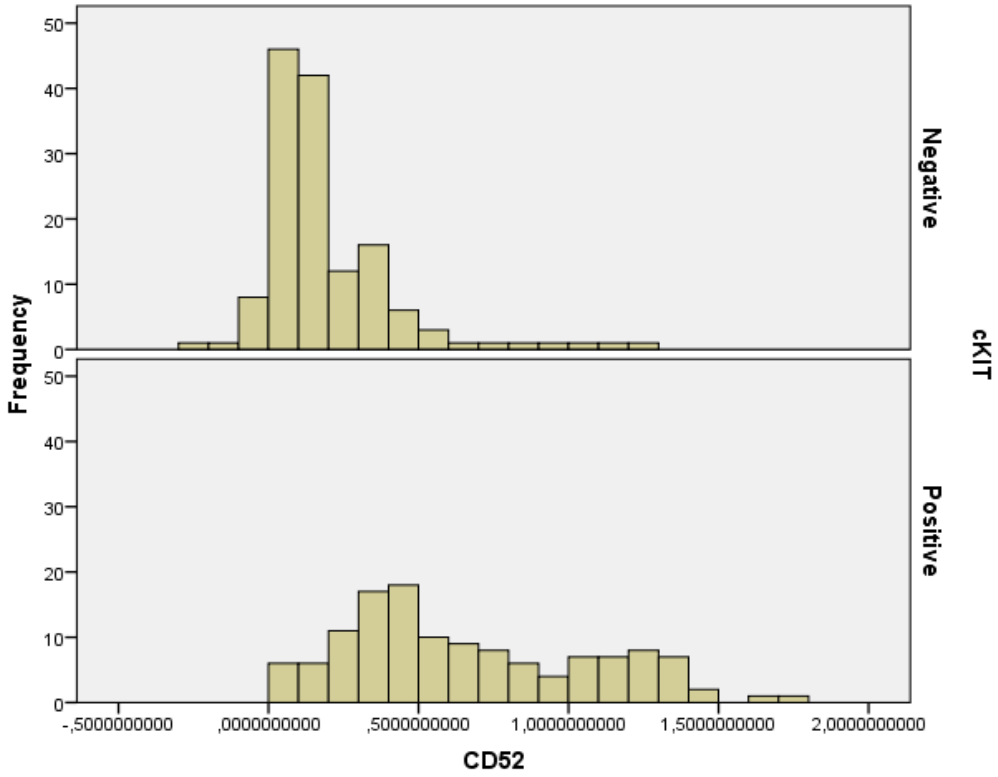
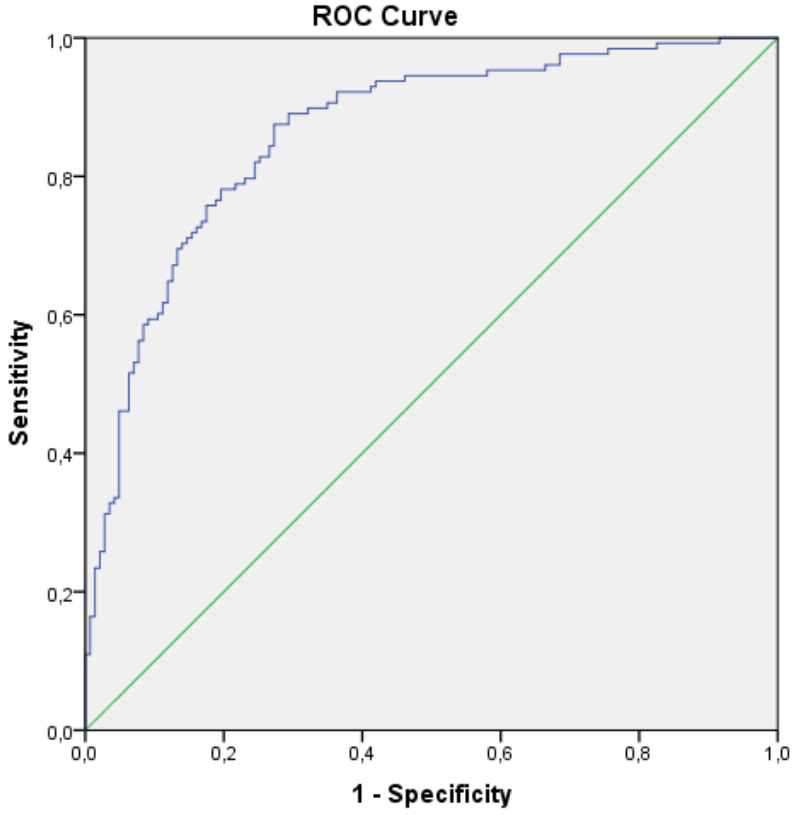
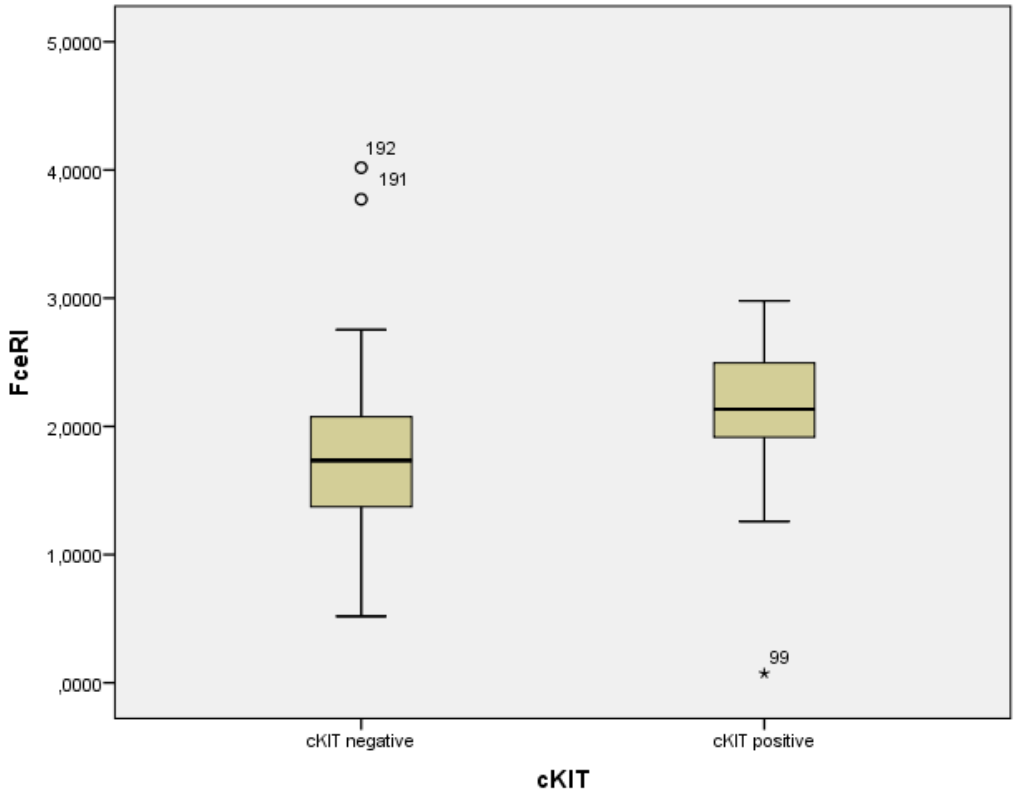
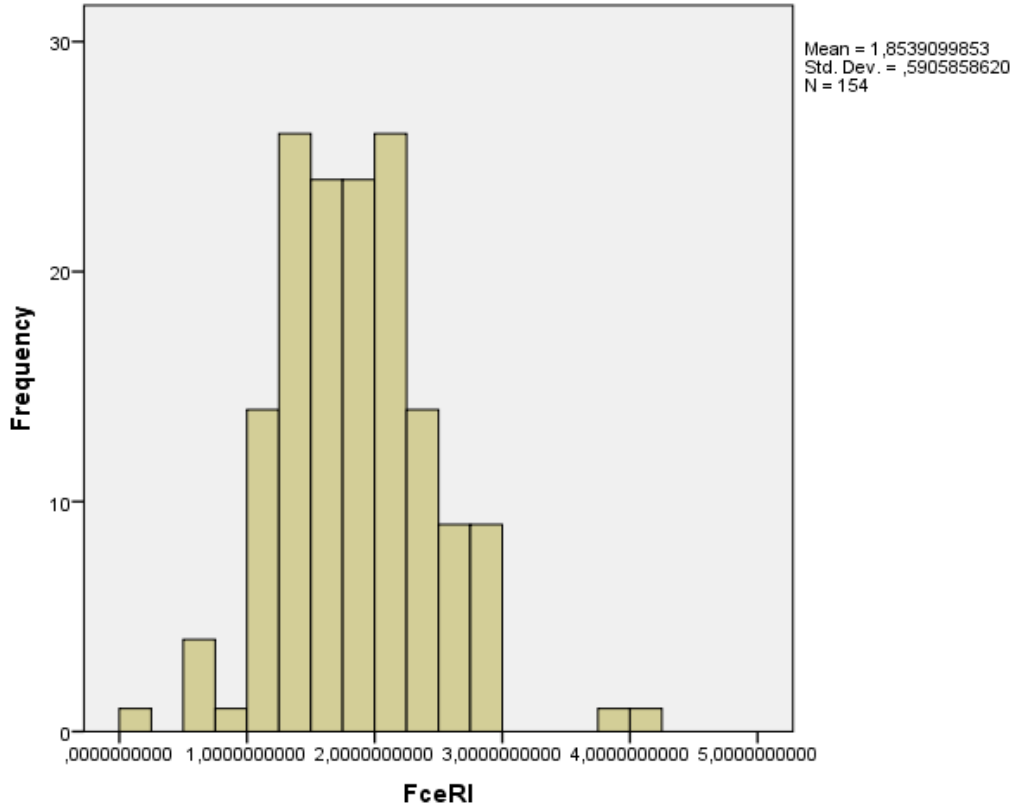
1,5000000000
2,0000000000
CD52

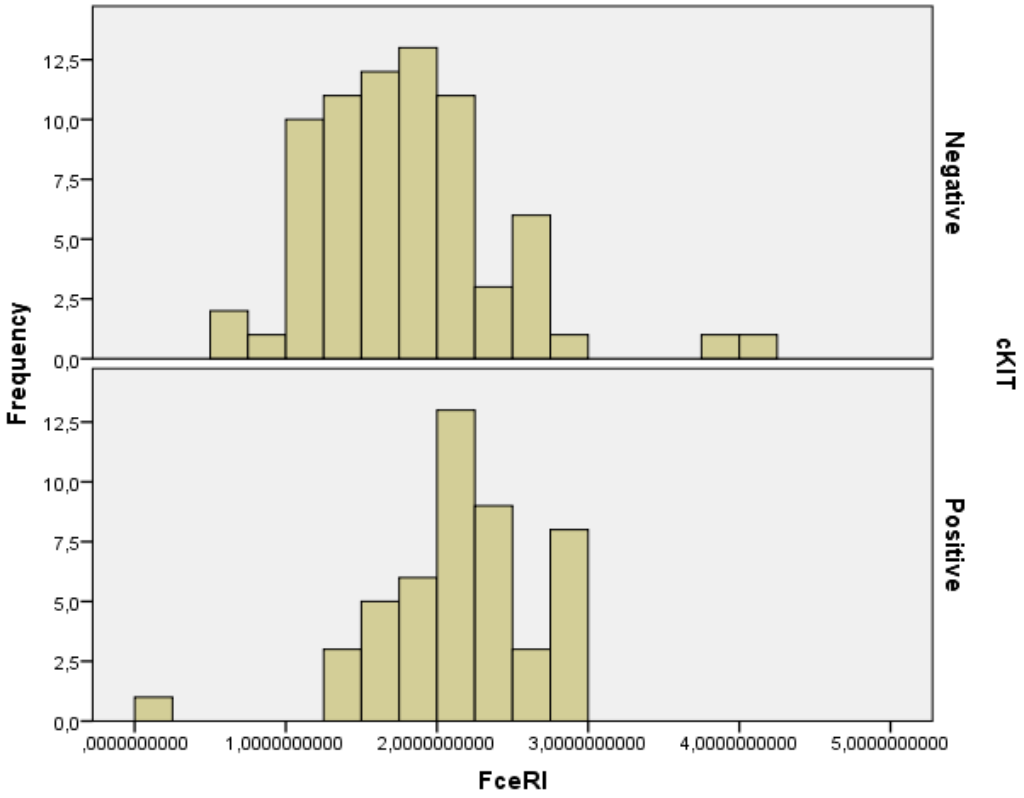

Frequency
50
40
30
20
10
0
Negative
Positive
cKIT
-,5000000000
,0000000000
,5000000000
1,0000000000
1,5000000000
2,0000000000
CD52

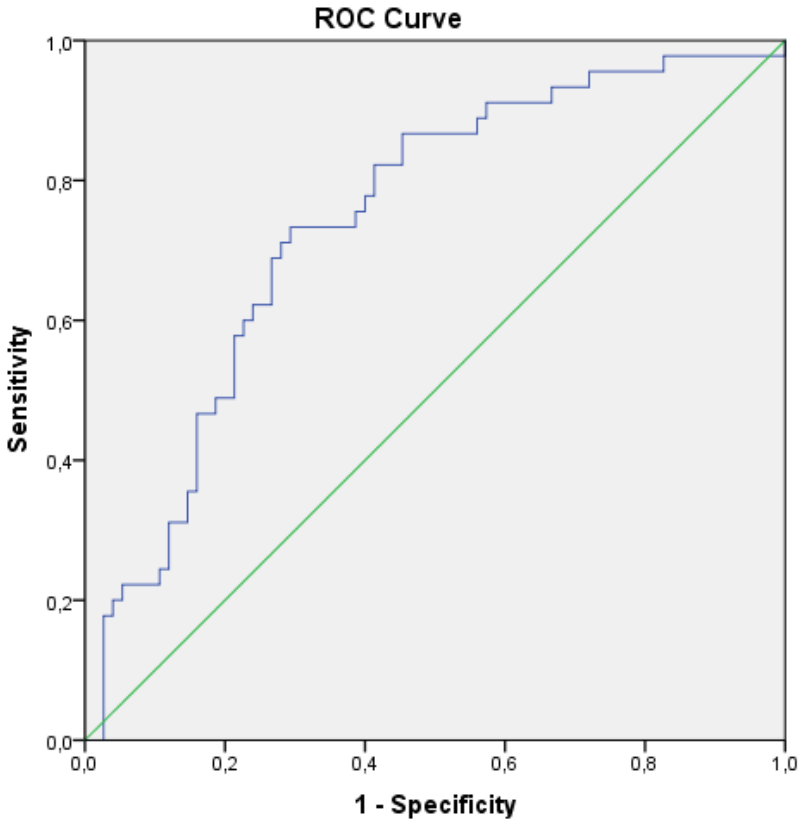

ROC Curve
Sensitivity
1,0
0,8
0,6
0,4
0,2
0,0
1 - Specificity

FceRI

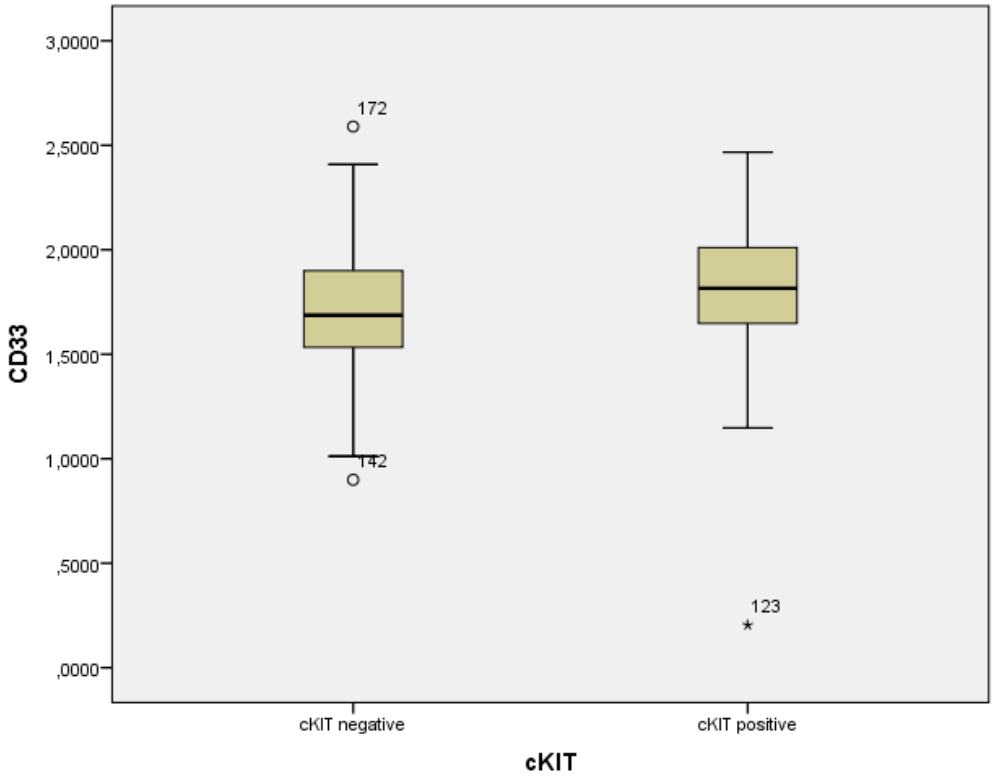

5,0000
4,0000
3,0000
2,0000
1,0000
,0000
FceRI
192
191
99
cKIT negative
cKIT positive
cKIT

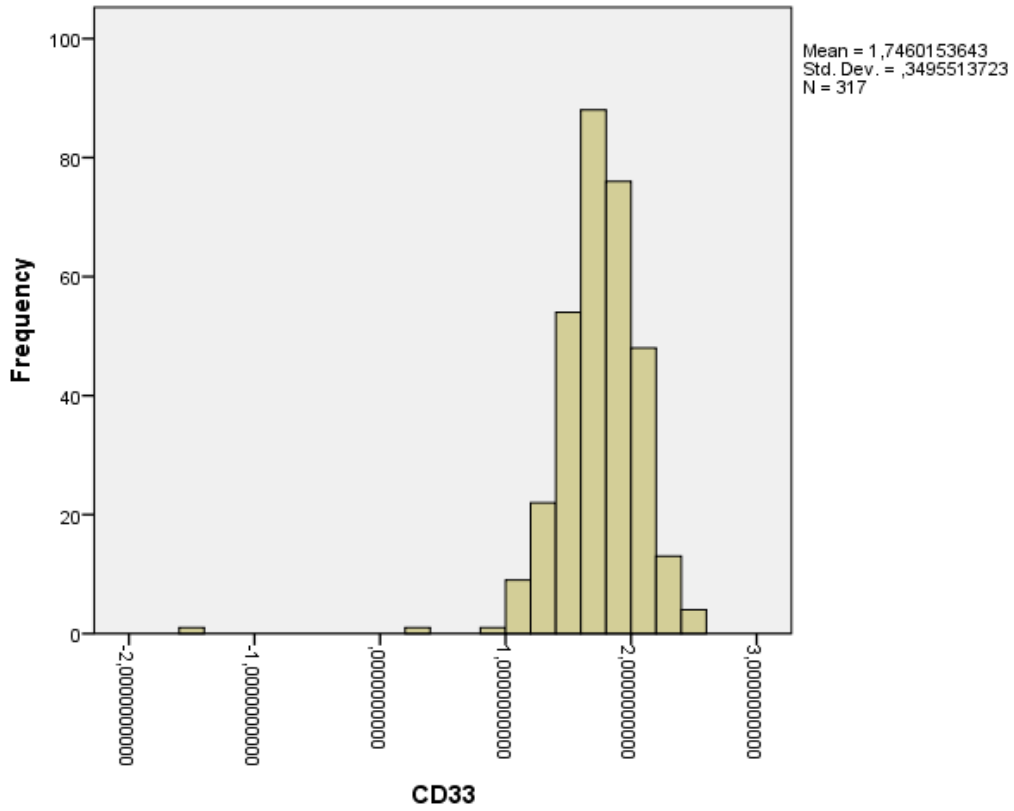

30
20
10
0
Frequency
Mean = 1,8539099853
Std. Dev. = ,5905858620
N = 154
,0000000000
1,0000000000
2,0000000000
3,0000000000
4,0000000000
5,0000000000
FceRI

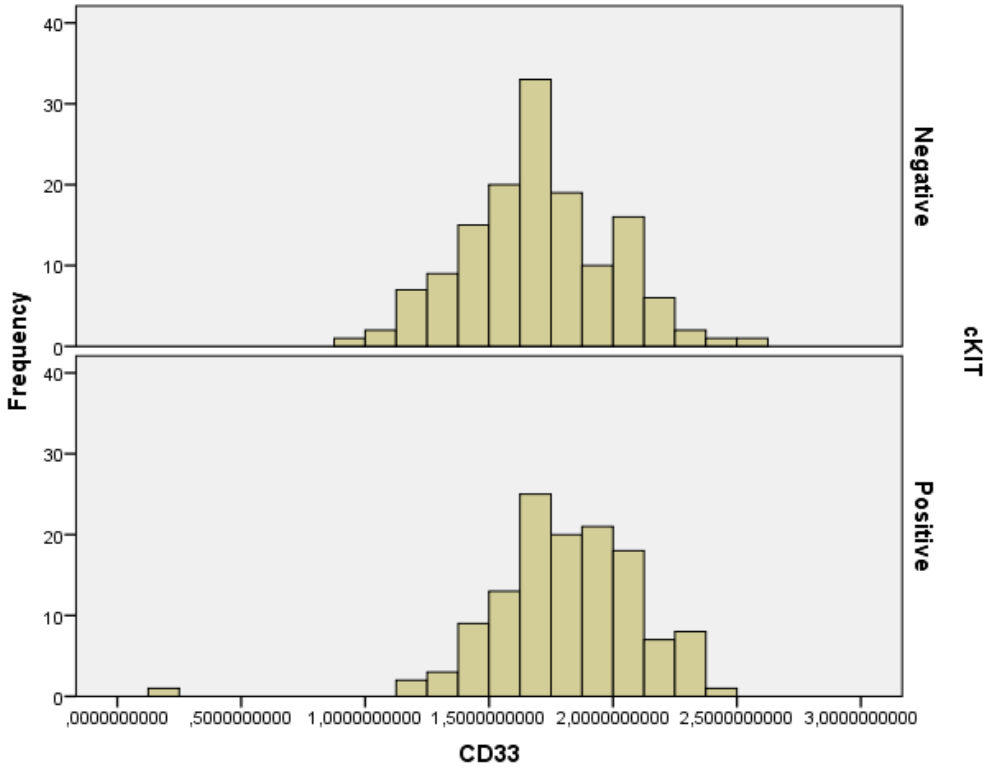

Frequency
12,5
10,0
7,5
5,0
2,5
0,0
Negative
Positive
cKIT
,0000000000
1,0000000000
2,0000000000
3,0000000000
4,0000000000
5,0000000000
FceRI

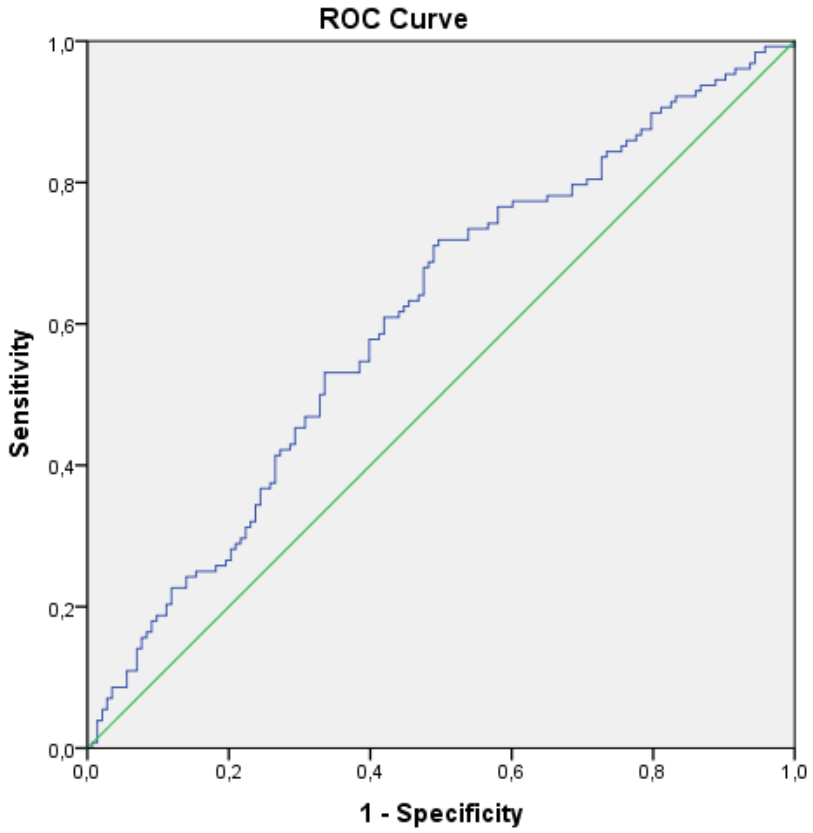

ROC Curve
Sensitivity
1,0
0,8
0,6
0,4
0,2
0,0
0,0
0,2
0,4
0,6
0,8
1,0
1 - Specificity

CD33

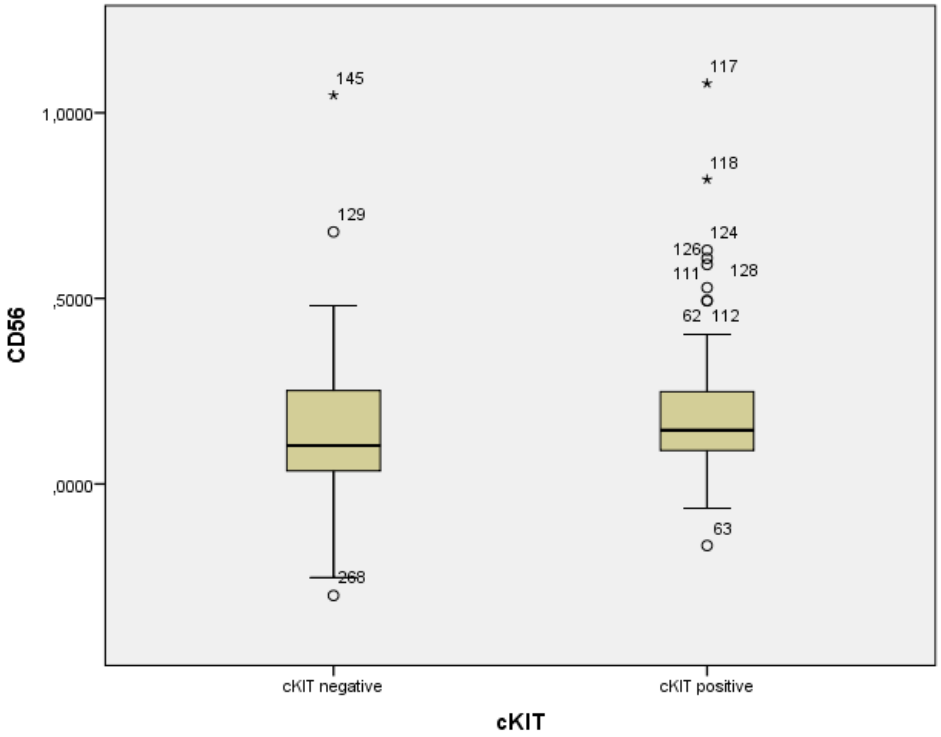

3,0000
2,5000
2,0000
1,5000
1,0000
,5000
,0000
CD33
172
142
123
cKIT negative
cKIT positive
cKIT

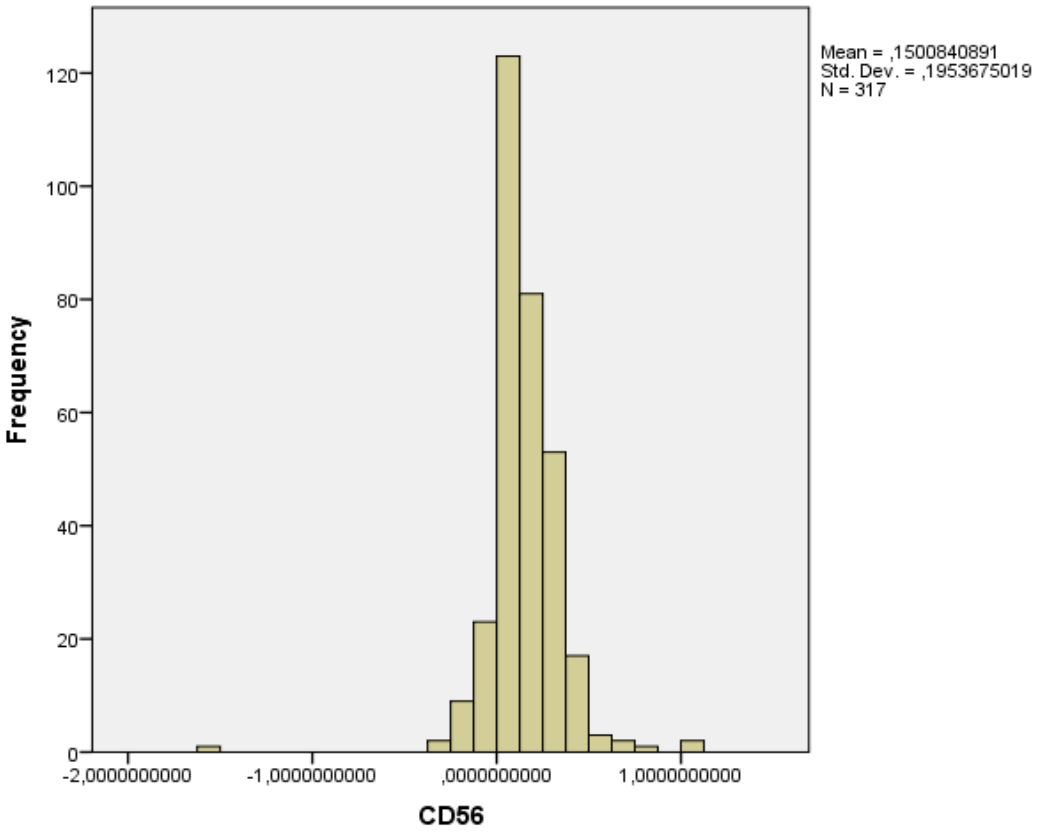

Mean = 1,7460153643
Std. Dev. = ,3495513723
N = 317
100
80
60
40
20
0
Frequency
-2,0000000000
-1,0000000000
,0000000000
1,0000000000
2,0000000000
3,0000000000
CD33

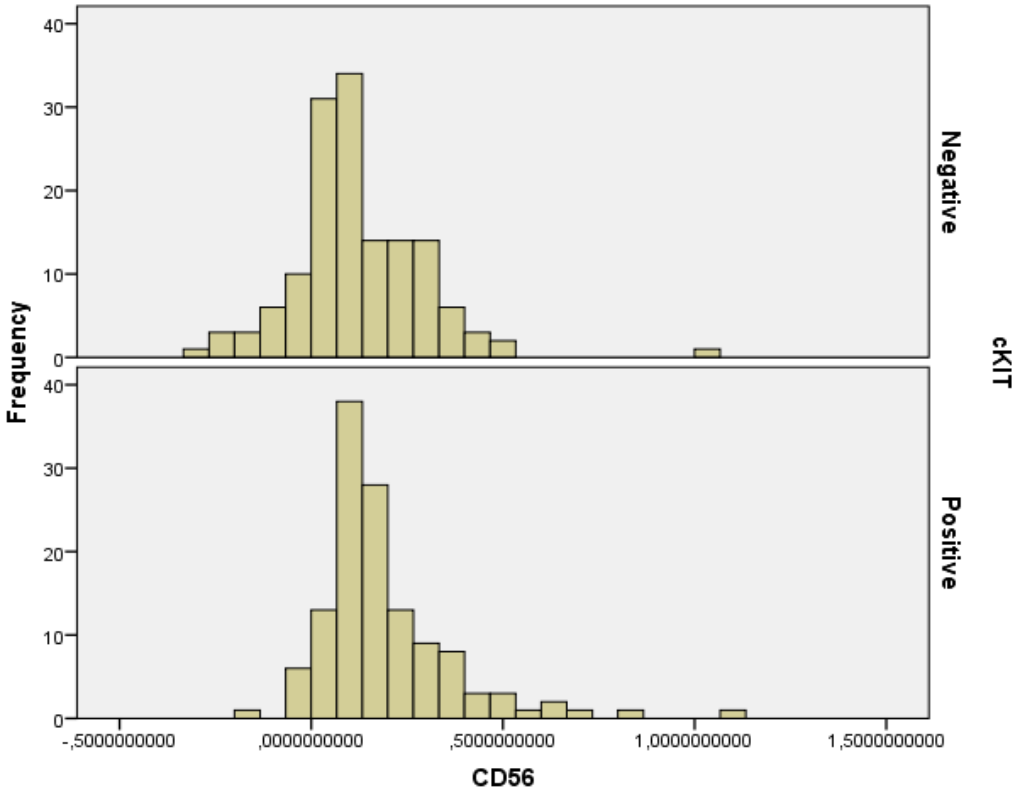

Frequency
40
30
20
10
0
Negative
Positive
cKIT
,0000000000
,5000000000
1,0000000000
1,5000000000
2,0000000000
2,5000000000
3,0000000000
CD33

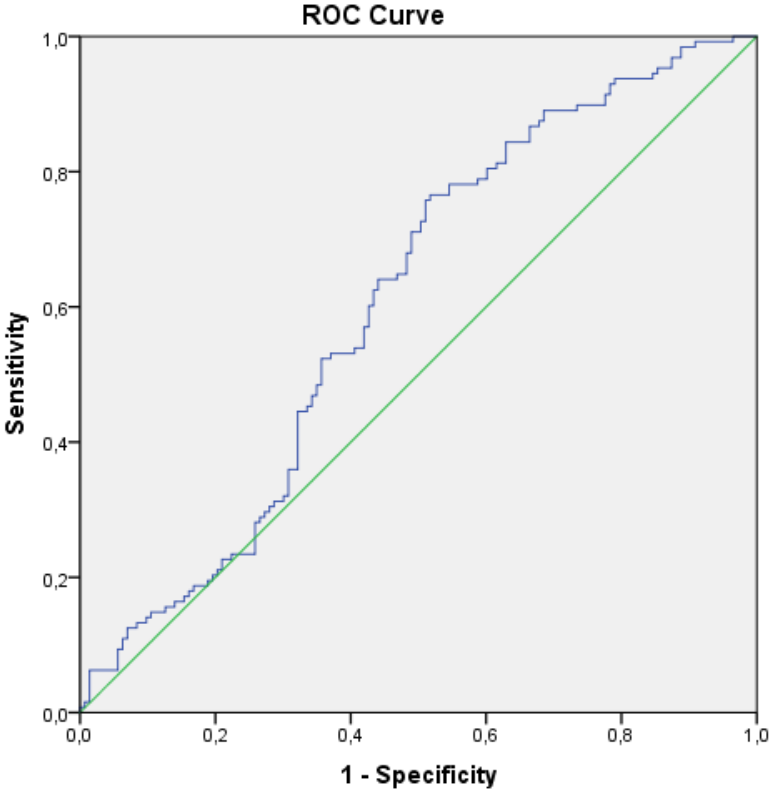

ROC Curve
Sensitivity
1 - Specificity
0,0
0,2
0,4
0,6
0,8
1,0

CD56

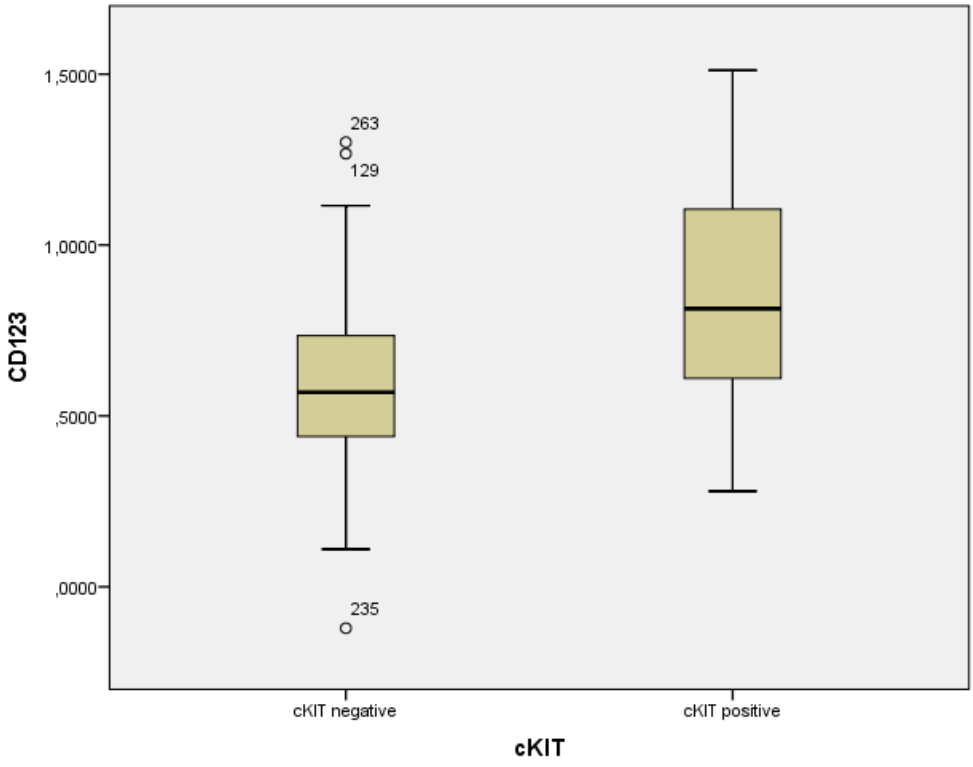

145
117
118
129
124
126
111
128
62
112
63
268
1,0000
,5000
,0000
CD56
cKIT negative
cKIT positive
cKIT

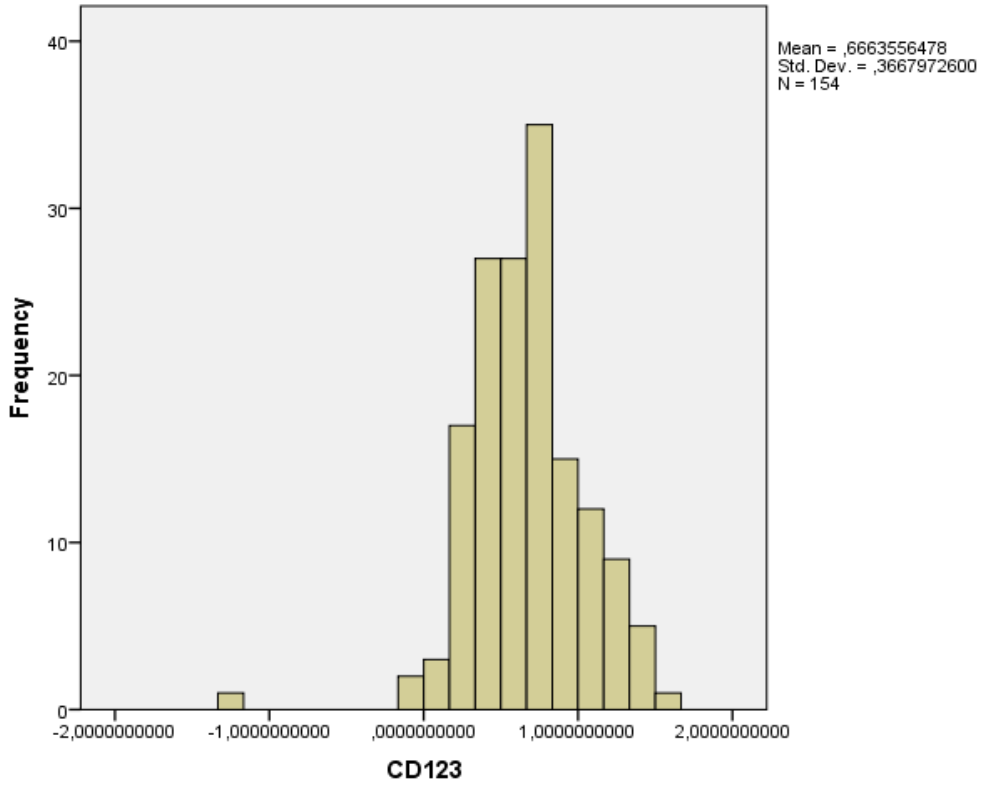

Mean = ,1500840891
Std. Dev. = ,1953675019
N = 317
120
100
80
60
40
20
0
Frequency
-2,0000000000
-1,0000000000
,0000000000
1,0000000000
CD56

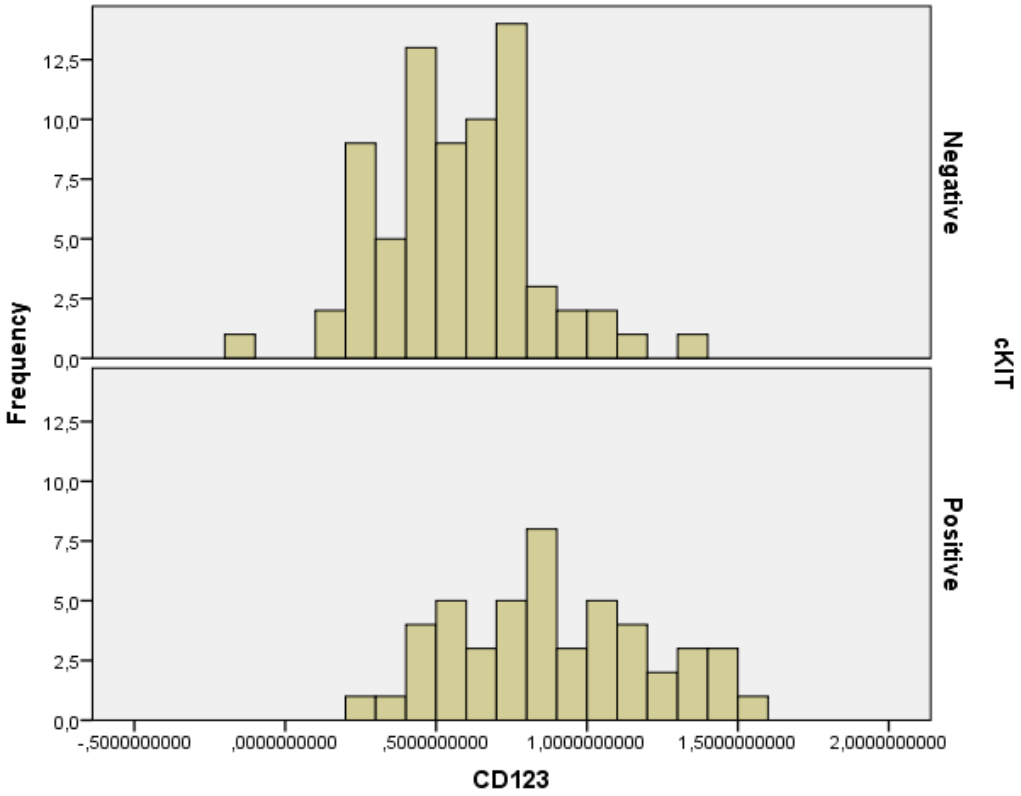

Frequency
40
30
20
10
0
Negative
Positive
cKIT
-,5000000000
,0000000000
,5000000000
1,0000000000
1,5000000000
CD56

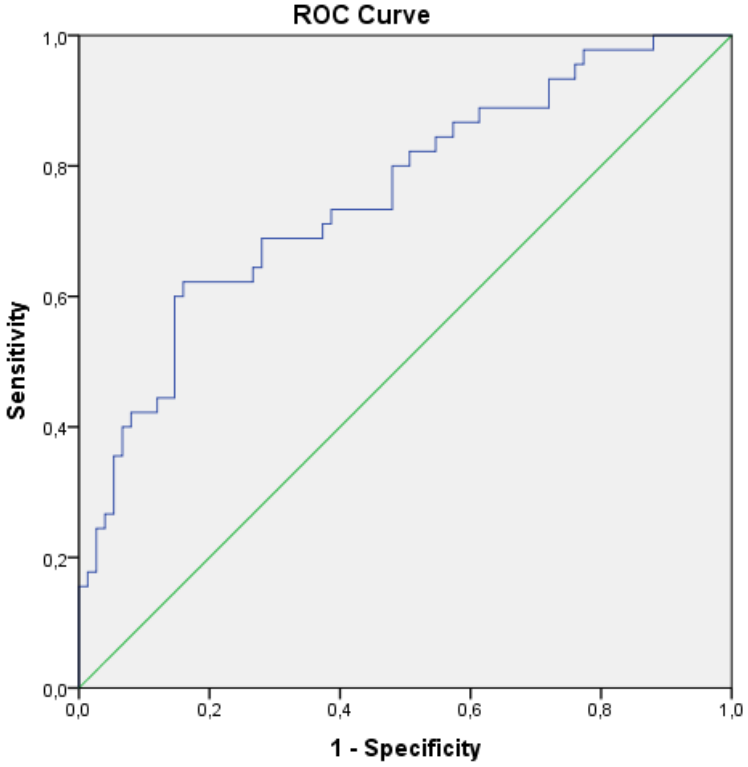

ROC Curve
Sensitivity
1,0
0,8
0,6
0,4
0,2
0,0
1 - Specificity

CD123

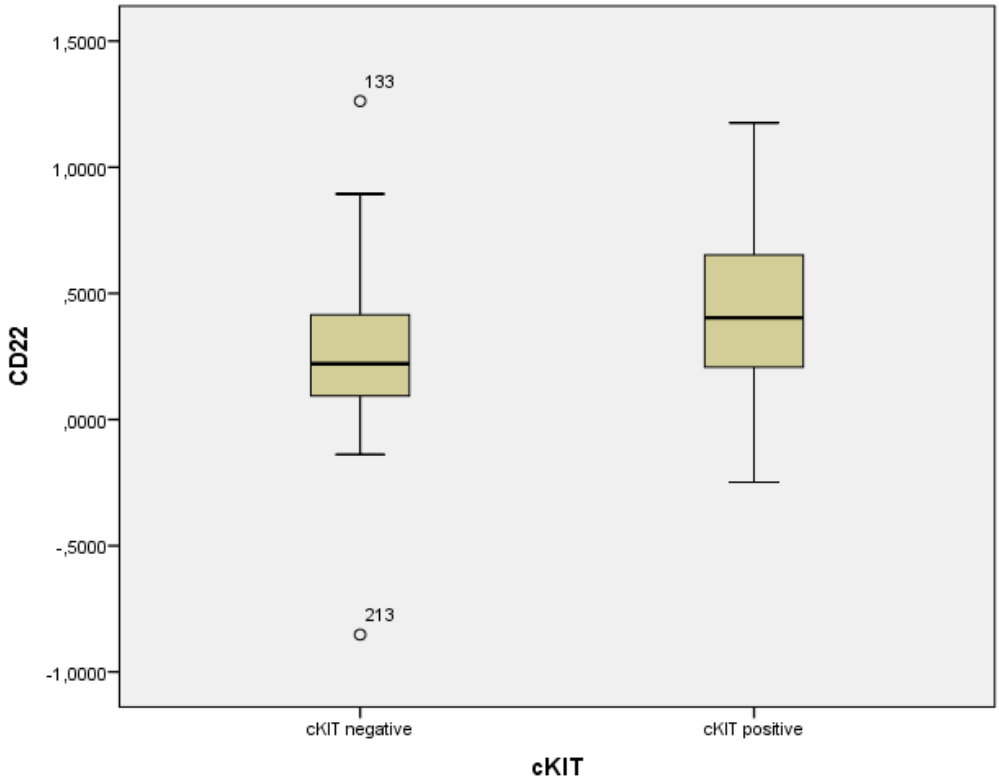

1,5000
1,0000
,5000
,0000
CD123
263
129
235
cKIT negative
cKIT positive
cKIT

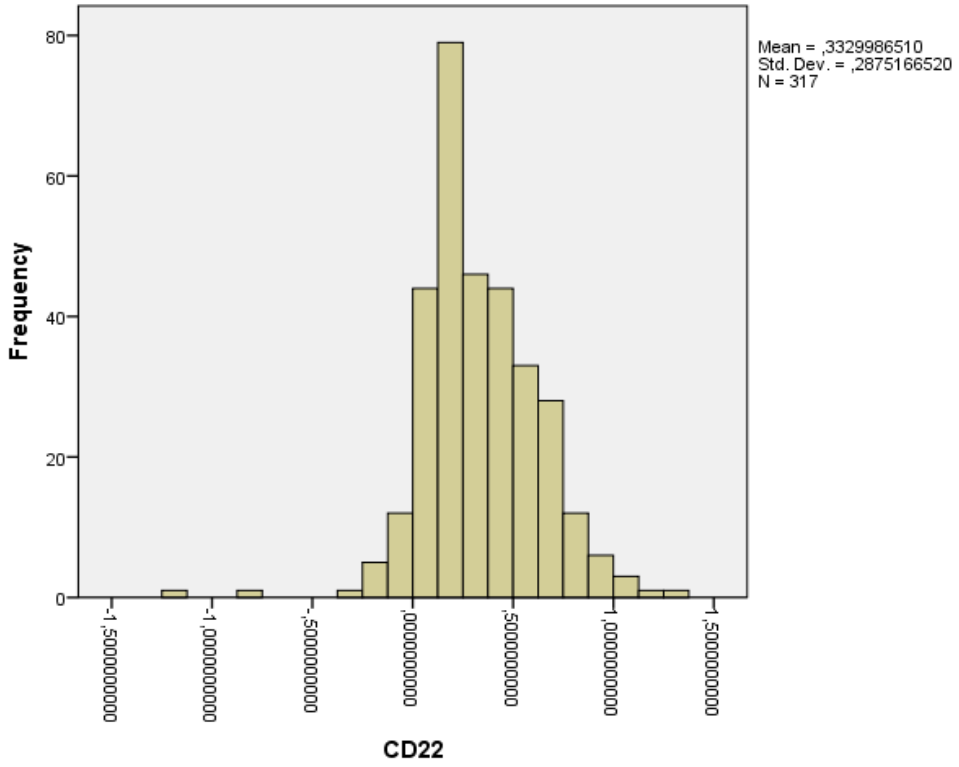

Mean = ,6663556478
Std. Dev. = ,3667972600
N = 154
40
30
20
10
0
Frequency
-2,0000000000
-1,0000000000
,0000000000
1,0000000000
2,0000000000
CD123

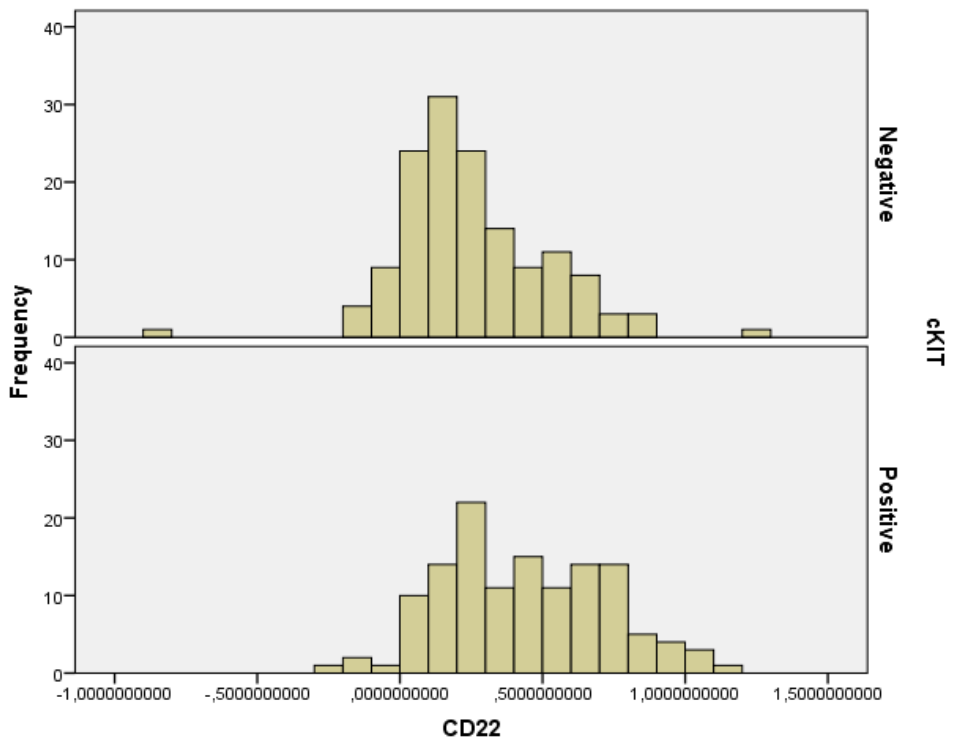

Frequency
12,5
10,0
7,5
5,0
2,5
0,0
Negative
Positive
cKIT
-,5000000000
,0000000000
,5000000000
1,0000000000
1,5000000000
2,0000000000
CD123

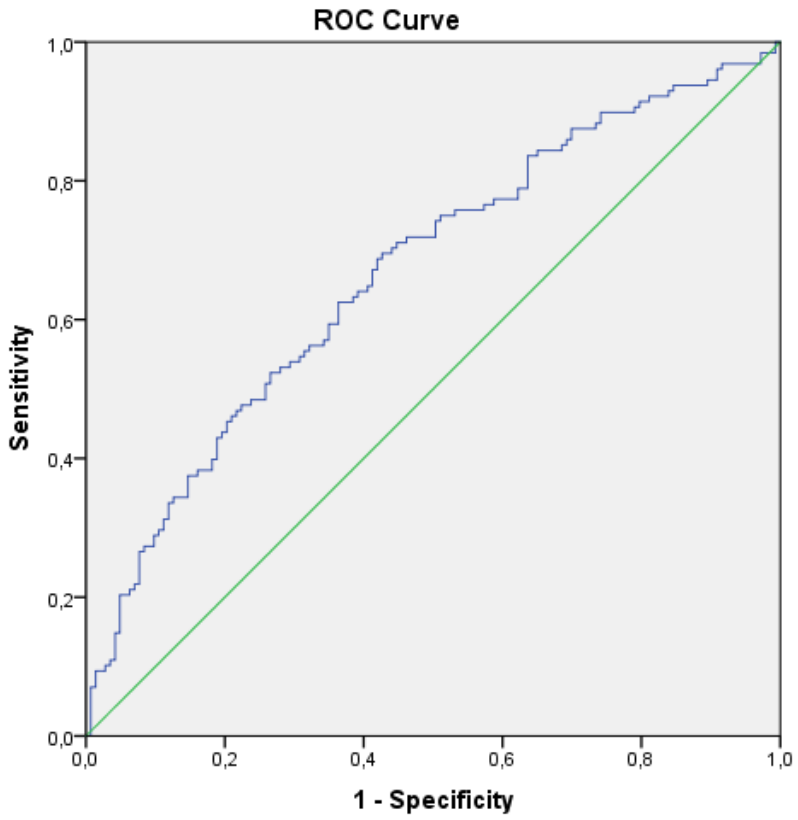

ROC Curve
Sensitivity
1,0
0,8
0,6
0,4
0,2
0,0
0,0
0,2
0,4
0,6
0,8
1,0
1 - Specificity

CD22

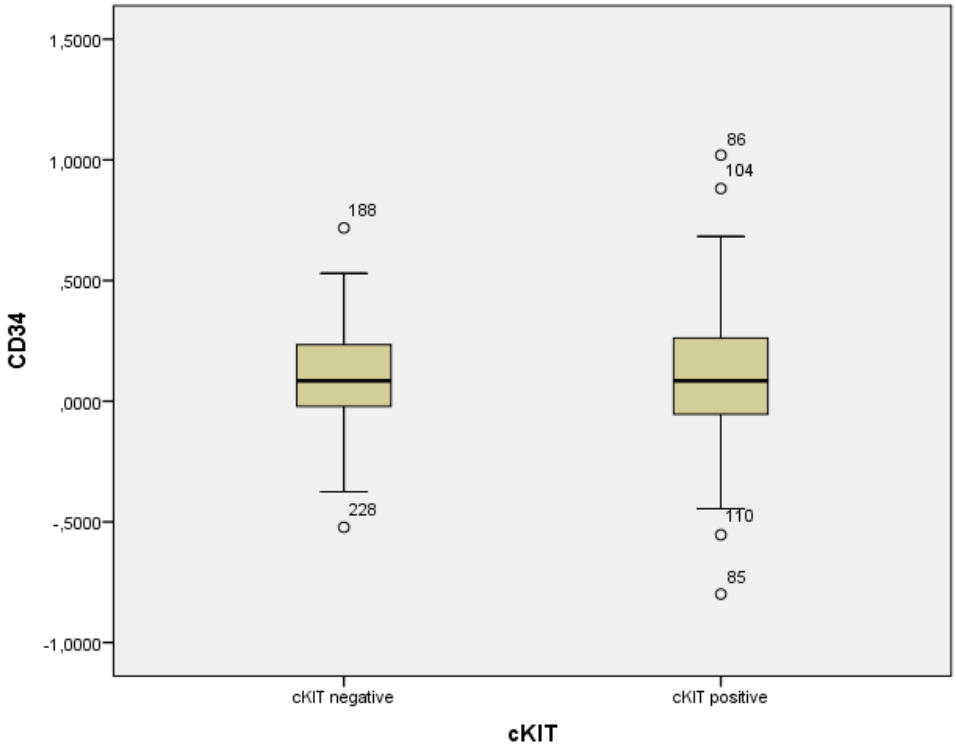

1,5000
1,0000
,5000
,0000
-,5000
-1,0000
133
213
CD22
cKIT negative
cKIT positive
cKIT

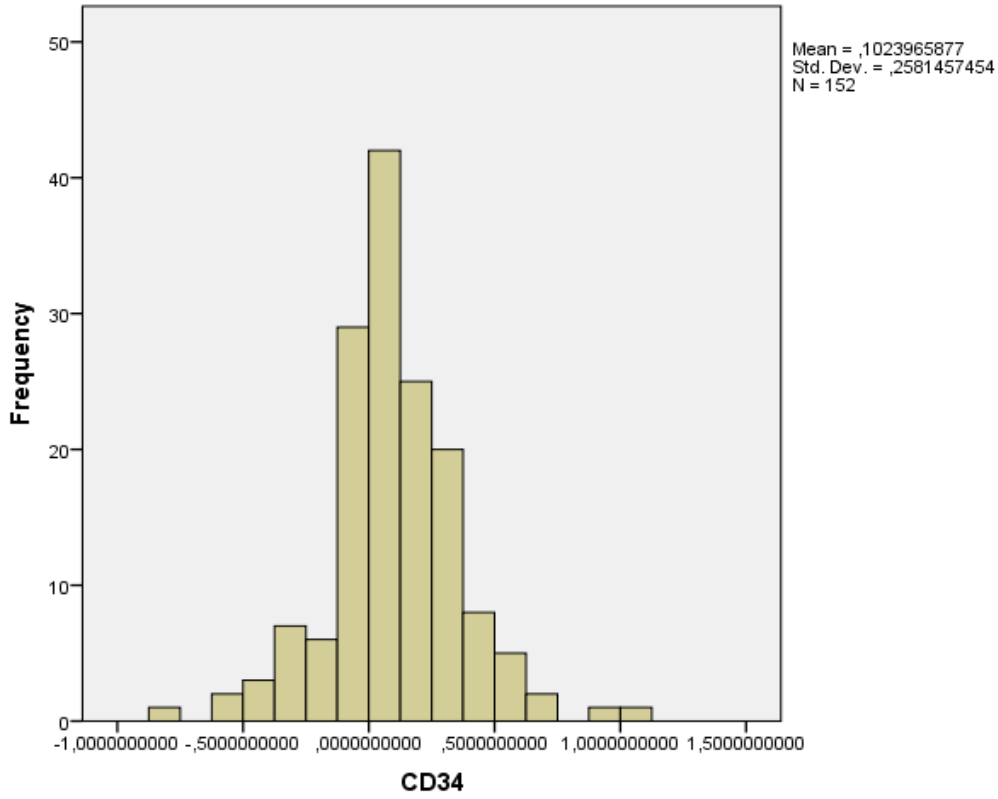

Mean = ,3329986510
Std. Dev. = ,2875166520
N = 317
80
60
40
20
0
Frequency
-1,5000000000
-1,0000000000
-,5000000000
,0000000000
,5000000000
1,0000000000
1,5000000000
CD22

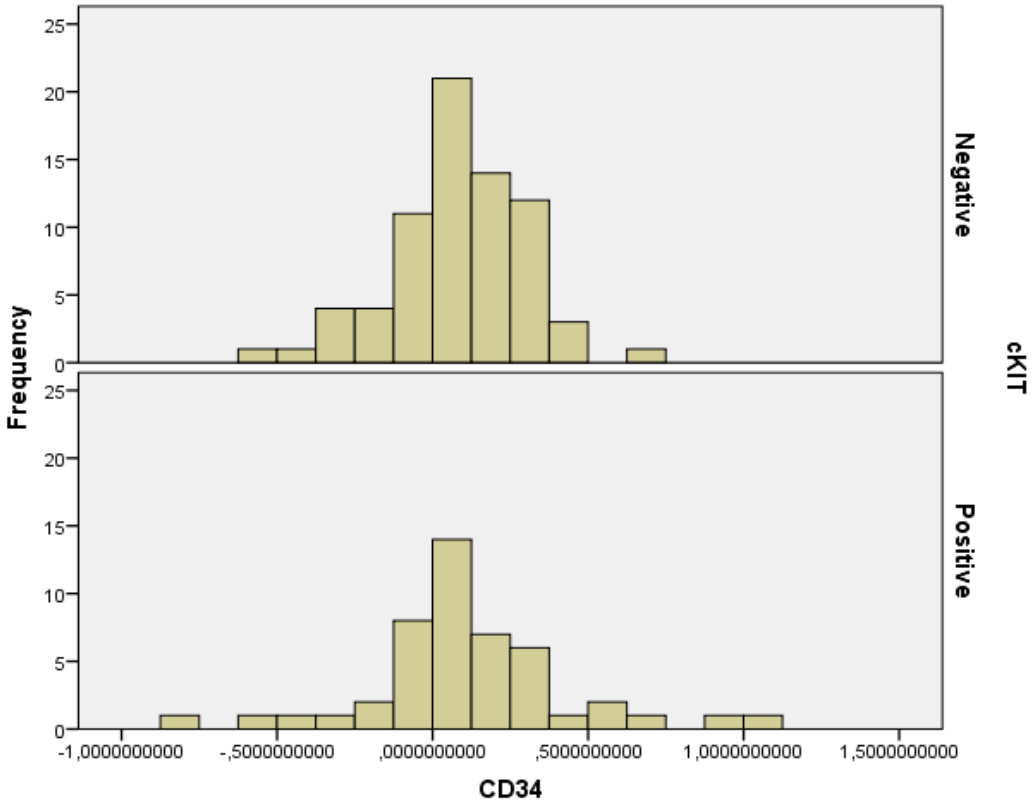

Frequency
40
30
20
10
0
Negative
Positive
cKIT
-1,0000000000
-,5000000000
,0000000000
,5000000000
1,0000000000
1,5000000000
CD22

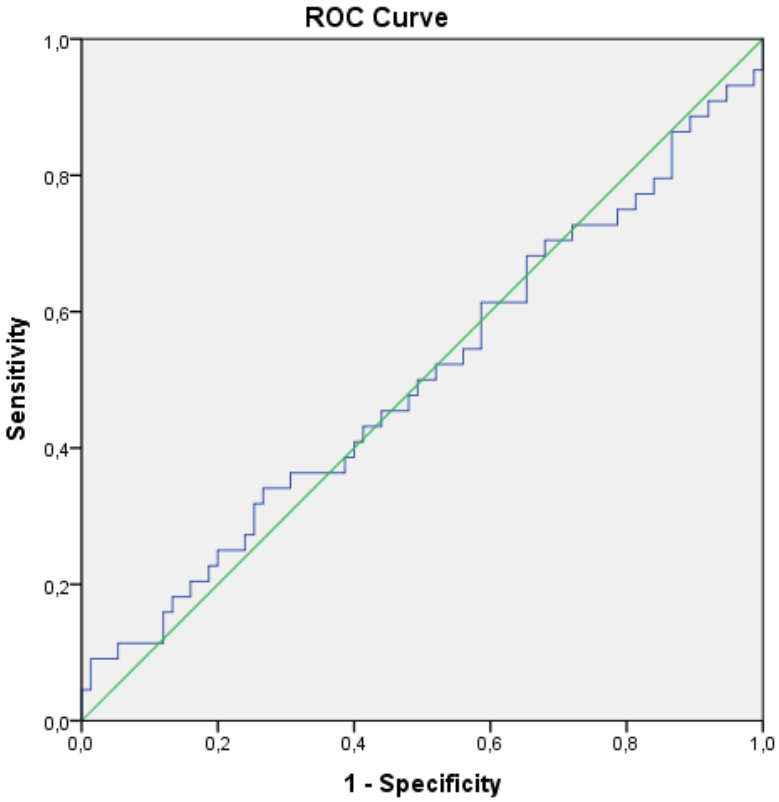

ROC Curve
Sensitivity
1,0
0,8
0,6
0,4
0,2
0,0
1 - Specificity

CD34

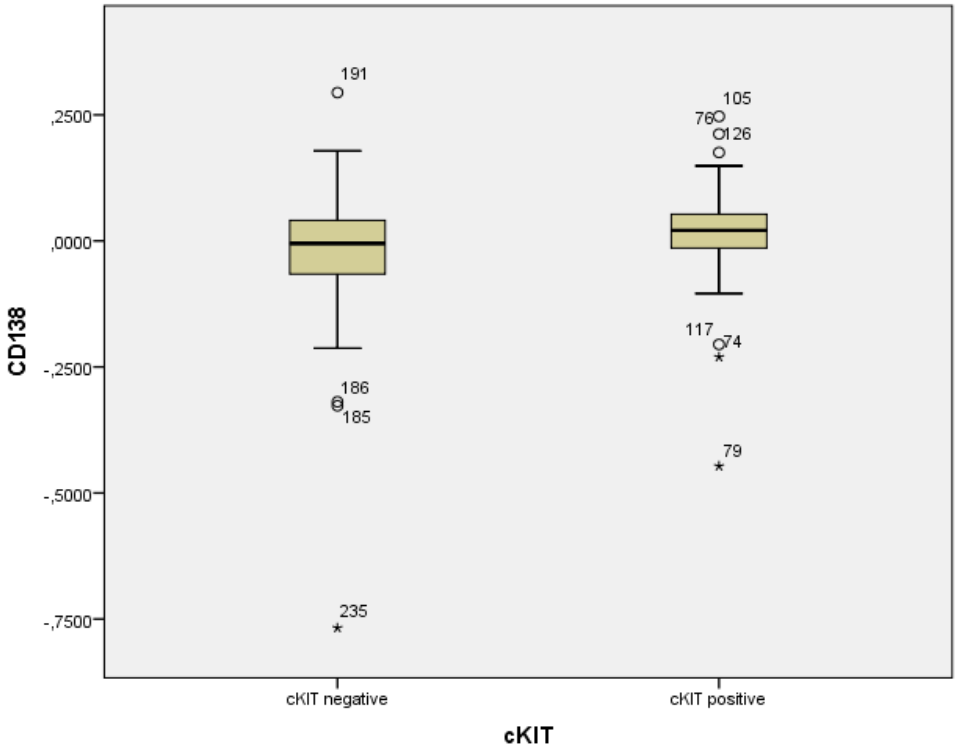

1,5000
1,0000
,5000
,0000
-,5000
-1,0000
CD34
188
228
86
104
110
85
cKIT negative
cKIT positive
cKIT

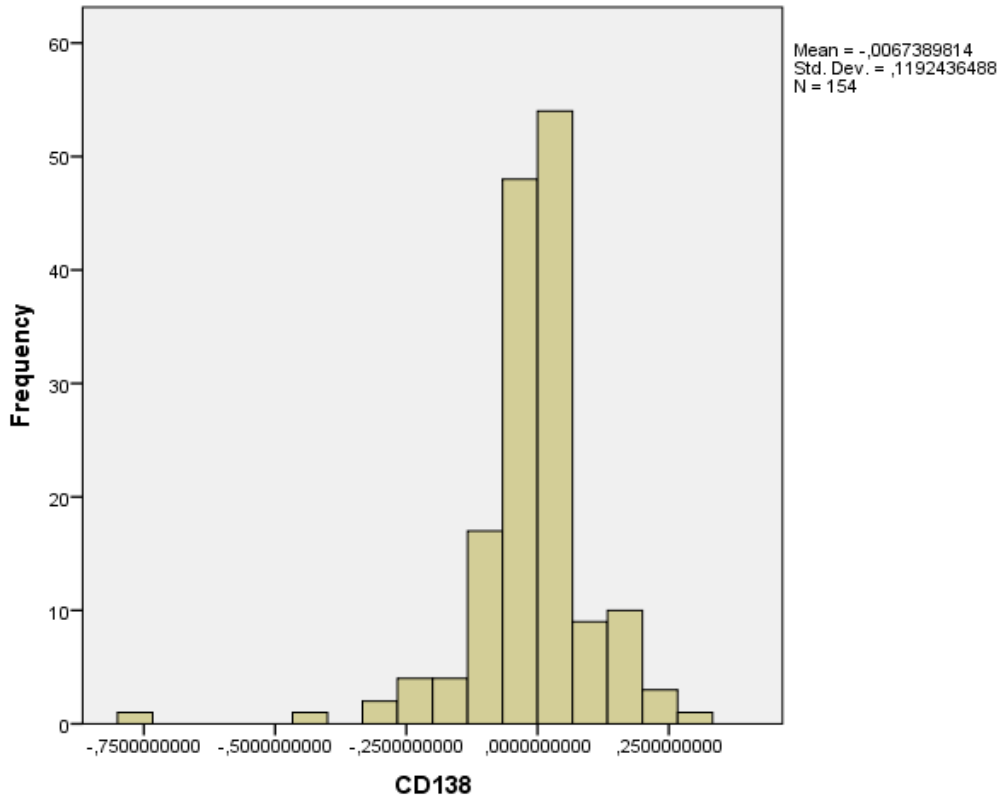

Mean = ,1023965877
Std. Dev. = ,2581457454
N = 152
50
40
30
20
10
0
Frequency
-1,0000000000
-,5000000000
,0000000000
,5000000000
1,0000000000
1,5000000000
CD34

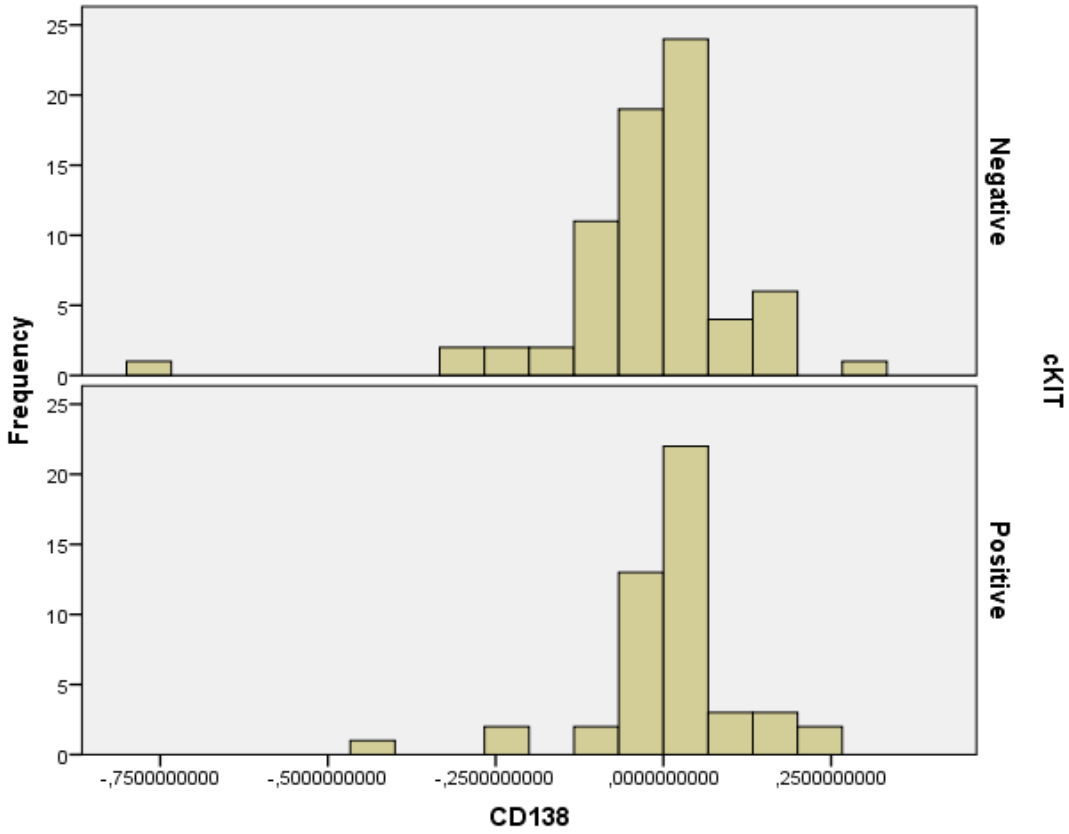

Frequency
25
20
15
10
5
0
Negative
Positive
cKIT
-1,0000000000
-,5000000000
,0000000000
,5000000000
1,0000000000
1,5000000000
CD34

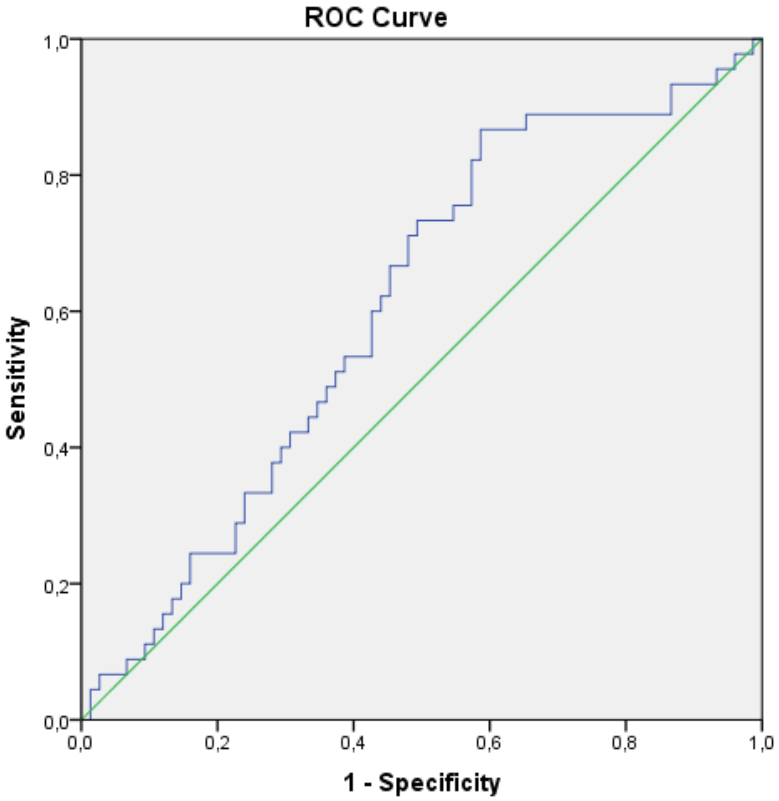

ROC Curve
Sensitivity
1 - Specificity
0,0
0,2
0,4
0,6
0,8
1,0

CD138


191
105
76
126
117
74
186
185
79
235
,2500
,0000
-,2500
-,5000
-,7500
CD138
cKIT negative
cKIT positive
cKIT


Mean = -,0067389814
Std. Dev. = ,1192436488
N = 154
60
50
40
30
20
10
0
Frequency
-,7500000000
-,5000000000
-,2500000000
,0000000000
,2500000000
CD138


Frequency
25
20
15
10
5
0
Negative
Positive
cKIT
-,7500000000
-,5000000000
-,2500000000
,0000000000
,2500000000
CD138


ROC Curve
Sensitivity
1 - Specificity
0,0
0,2
0,4
0,6
0,8
1,0