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(54) Titre : MOYENS ET PROCEDES DE STADIFICATION, TYPAGE ET TRAITEMENT D'UNE MALADIE CANCEREUSE
(54) Title: MEANS AND METHODS FOR STAGING, TYPING AND TREATING A CANCEROUS DISEASE

(57) **Abrégé/Abstract:**

The present invention relates to methods for diagnosing, staging and treating cancer, in particular melanoma. In particular, the present invention provides methods for determining the stage/type of a cancerous disease, comprising detecting somatic alterations of the DNA of one or more disseminated cancer cells (DCCs), obtained after homing to a distant organ, such as lymph node; and determining the somatic evolution of the DCC(s) based on the detected somatic alterations, wherein the somatic evolution is indicative of the stage/type of the cancerous disease.

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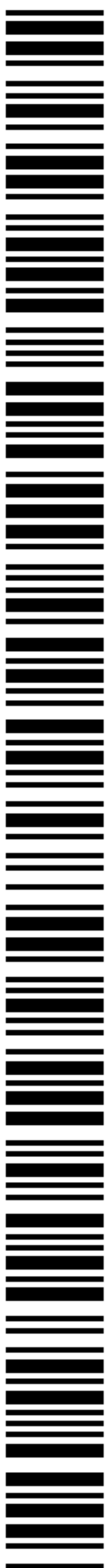


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(54) Title: MEANS AND METHODS FOR STAGING, TYPING AND TREATING A CANCEROUS DISEASE

(57) Abstract: The present invention relates to methods for diagnosing, staging and treating cancer, in particular melanoma. In particular, the present invention provides methods for determining the stage/type of a cancerous disease, comprising detecting somatic alterations of the DNA of one or more disseminated cancer cells (DCCs), obtained after homing to a distant organ, such as lymph node; and determining the somatic evolution of the DCC(s) based on the detected somatic alterations, wherein the somatic evolution is indicative of the stage/type of the cancerous disease.

Means and methods for staging, typing and treating a cancerous disease

The present invention relates to methods for diagnosing, staging and treating cancer. In particular, the present invention provides methods for determining the stage/type of a cancerous disease, comprising detecting somatic alterations of the DNA of one or more disseminated cancer cells (DCCs), obtained after homing to a distant organ, such as a lymph node; and determining the somatic evolution of the DCC(s) based on the detected somatic alterations, wherein the somatic evolution is indicative of the stage/type of the cancerous disease.

Cancer staging/typing is the process of determining the extent to which a cancer has developed by spreading. Contemporary practice is to assign a number from I-IV to a cancer, with I being an isolated cancer and IV being a cancer which has spread to the limit of what the assessment measures. Usually, stage IV indicates distant spread of the cancer. The stage generally takes into account the size of a tumor, whether it has invaded adjacent organs, how many regional (nearby) lymph nodes it has spread to (if any), and whether it has appeared in more distant locations (metastasized).

The determined stage of a cancer is generally used to find a suitable strategy for therapy of the cancer, e.g. surgical therapy or drug therapy. However, therapy, in particular drug therapy, is often unsuccessful due to, *inter alia*, resistance.

In this regard, our inability to cure cancer in many cases is directly linked to the selection of therapy-resistant variants. Insights into cancer drug resistance have been gained by studying advanced metastatic patients, where the total number of cancer cells correlates with the likelihood of resistance; see Holohan *et al.* (2013) Nature reviews Cancer 13, 714. Consequently, minimal systemic cancer after ‘curative’ surgery (i.e. the (neo-)adjuvant therapy setting) should be much easier to treat. The time-window after primary surgery and before manifestation of metastasis, i.e. during clinically undetectable minimal residual disease (MRD), defined by disseminated cancer cells (DCCs) and/or circulating tumor cells (CTCs)

that are left behind after primary tumor (PT) surgery, may be particularly suitable to prevent lethal metastasis (Aguirre-Ghiso *et al.* (2013) *Nature Med.* 19(3):276-7; Polzer and Klein (2013) *Nature Med.* 19(3):274-5). However, only circumstantial knowledge is available about MRD and consequently (neo-)adjuvant therapies, i.e. the administration of systemic drugs before or after surgery in non-metastatic cancer, improve outcome in only about 20% of patients (Cole *et al.* (2001) *Lancet* 358:277-286; Gianni *et al.* (2011) *Lancet Oncol.* 12:236-244). Moreover, when targeted therapies with documented efficacy in patients with manifested metastasis were applied in the adjuvant therapy setting, success was disappointingly low (Polzer and Klein (2013) *Nature Med.* 19(3):274-5). This clinical situation indicates that our current understanding of early systemic cancer is insufficient to successfully prevent metastasis.

First direct evidence for a characteristic biology of early-disseminated cancer cells and MRD came from the analysis of DCCs isolated from bone marrow of breast cancer patients before and after manifestation of metastasis. Genetic data indicated that DCCs from the two stages of metastatic spread differ (Klein *et al.* (2002) *Lancet* 360:683-689) Schmidt-Kittler *et al.* (2003) *PNAS* 100:7737-7742) and it was suggested that they might have disseminated early and evolved in parallel to the primary tumor (Klein (2009), *Nat Rev Cancer* 9:302-312). Subsequently, studies in transgenic mouse models (Eyles *et al.* (2010) *J Clin Invest.* 120:2030-2039; Husemann *et al.* (2008) *Cancer Cell* 13:58-68; Rhim *et al.* (2012) *Cell* 148:349-361) and in patients with pre-malignant lesions or in-situ carcinomas (Banys *et al.* (2012) *Breast Cancer Res Treat.* 131:801-808; Husemann *et al.* (2008) *Cancer Cell* 13:58-68; Sanger *et al.* (2011) *Int J Cancer* 129:2522-2526) corroborated this concept. Evidence for both the early and late dissemination models is available but inconclusive. Cancer of unknown primary (CUP), i.e. metastasis without primary tumor, comprises about 5% of cancer patients and proves that large advanced primary tumors are not needed for metastasis. Similarly, epidemiological data support dissemination of cancer cells and initiation of metastasis years before diagnosis (Engel *et al.* (2003) *Eur J Cancer* 39:1794-1806). Genetic data such as comparative sequencing studies of matched primary tumors and metastasis provide examples for both scenarios (Klein (2013) *Nature* 501:365-372). However, sequencing studies may be misleading or inconclusive as iatrogenic selection over several lines of systemic therapies may shift cancer populations significantly. In summary, clinical and patient-derived *ex vivo* data seem to support early dissemination and ectopic progression, whereas most experimental models assume and use late-arising, fully malignant metastatic

cells. In this regard, many reports use patient material derived from blood samples whereas samples derived from lymph nodes are considered a less preferred source, as for example described in WO 02/37113. Preference for plasma or blood samples is also disclosed in EP 2 503 009, which relates to a method for diagnosing tumors based on copy number of the ErbB2 gene and its expression profile. Experiments are done using exclusively serum or plasma samples of patients. As current clinical practice is based on a late-dissemination and intratumoral progression model (as opposed to a parallel progression model), consideration of a parallel progression model would fundamentally imply the need for novel diagnostic tools, for improved and more accurate determination of the stage/type of cancer and improved cancer therapy, in particular adjuvant therapy.

Thus, the technical problem underlying the present invention is the provision of accurate methods for the early determination of the stage/type of a cancerous disease and corresponding improved means and methods for treating a cancerous disease.

The solution is provided by the embodiments as defined herein below and as characterized in the claims.

The invention, accordingly, relates to a method for staging and/or typing of a cancerous disease, said method comprising the following steps:

- (a) detecting somatic alterations of the DNA of one or more disseminated cancer cell(s) DCC(s) obtained from one or more lymph node(s); and
- (b) determining the somatic evolution of the DCC(s) based on the detected somatic alteration(s) in step (a),

wherein the somatic evolution of the DCC(s) is indicative of the stage/type of the cancerous disease.

In a particularly preferred embodiment, the cancerous disease to be staged/typed is melanoma. Thus, the invention relates to a method for staging and/or typing of a cancerous disease, said method comprising the following steps:

- (a) detecting somatic alterations of the DNA of one or more disseminated cancer cell(s) DCC(s) obtained from one or more lymph node(s); and

- (b) determining the somatic evolution of the DCC(s) based on the detected somatic alteration(s) in step (a),
wherein the somatic evolution of the DCC(s) is indicative of the stage/type of the cancerous disease, wherein the cancerous disease is melanoma.

The illustrative appended Examples demonstrate that DCCs acquire alterations that are critical for metastatic progression within lymph nodes. This indicates that parallel progression of the primary tumor and DCCs takes place (as opposed by intratumoral progression of DCCs). Parallel progression of the primary tumor and DCCs means that analysis of the primary cancer cannot be used to evaluate whether DCCs progress to metastases. Parallel progression of the primary tumor and DCCs also means that progression of DCCs to metastasis cannot be prevented by resection of the primary cancer. Furthermore, parallel progression means that analysis of the primary cancer cannot be used to determine whether the metastases respond to a certain therapy.

In context of the present invention it has surprisingly been found that before progression to metastases DCCs show a metastasis signature (i.e. particular genetic alterations indicating that the DCCs will progress to metastases). According to the present invention this metastasis signature can advantageously be used to evaluate whether the DCCs will form metastases. In addition, this metastases signature can be used in order to evaluate whether the metastases will respond to a certain therapy.

Thus, the present invention relates to a method for identifying the metastasis signature of one or more DCC(s) (i.e. for staging and/or typing of a cancerous disease) said method comprising the following steps:

- (a) detecting somatic alterations of the DNA of one or more DCC(s) obtained from one or more lymph node(s); and
(b) determining the metastasis signature (i.e. the somatic evolution) of the DCC(s) based on the detected somatic alteration(s) in step (a).

In this method the metastases signature of the DCC(s) is indicative of the stage/type of the cancerous disease.

In a particularly preferred embodiment, the cancerous disease is melanoma.

According to the present invention the stage/type of a cancerous disease can be used to evaluate whether the cancerous disease (preferably a melanoma) responds to a certain therapy. Therefore, the method of the present invention is useful to determine whether a certain therapy should be initiated, continued or discontinued.

Thus, another embodiment of the present invention relates to a method for treating a cancerous disease, said method comprising the following steps:

- (a) detecting somatic alterations in the DNA of one or more DCC(s) obtained from one or more lymph node(s) of a subject;
- (b) determining the somatic evolution of the DCC(s) based on the detected somatic alteration(s) in step (a); and
- (c) determining the stage/type of the cancerous disease based on the somatic evolution of the DCC(s) determined in step (b),

wherein the stage/type of the cancerous disease is used to initiate, continue or discontinue therapy of said cancerous disease.

In a particularly preferred embodiment, the cancerous disease is melanoma.

The invention also relates to a pharmaceutical composition for use in treating a cancerous disease in a subject, wherein treatment is initiated, continued or discontinued based on the stage/type of said cancerous disease, wherein said stage/type of said cancerous disease is determined by:

- (a) detecting somatic alterations in the DNA of one or more DCC(s) obtained from one or more lymph node(s) of a subject;
- (b) determining the somatic evolution of the DCC(s) based on the detected somatic alteration(s) in step (a); and
- (c) determining the stage/type of the cancerous disease based on the somatic evolution of the DCC(s) determined in step (b).

In a particularly preferred embodiment, the cancerous disease is melanoma.

Said pharmaceutical composition may be any type of medicament for the treatment of a cancerous disease, including composition comprising a chemotherapeutic drug or a composition comprising a drug for immunotherapy.

One embodiment of the present invention relates to the methods or pharmaceutical composition of the invention, further comprising determination of the DCC density (DCCD), wherein the DCCD is the number of DCCs per million cells in the lymph node used to obtain the DCCs, wherein the DCCD is indicative of the stage/type of the cancerous disease. The appended Examples show that DCCs from lymph nodes with a DCCD of > 100 are able to grow to a colony and to develop to tumors in a xenograft experiment, in particular for melanoma. Therefore, one aspect of the present invention relates to the herein provided methods, or the herein provided pharmaceutical composition, wherein a DCCD of > 100 is indicative for the development of metastases, in particular in the case of melanoma.

In addition, it has been surprisingly found that immunological changes (i.e. signs of T cell exhaustion indicating a blunted T cell response and DCCs progressing to metastases) are locally observed and depend on the local tumor cell load. This information can be used to determine whether therapies targeting the microenvironment will be effective, in particular therapy targeting cells of the immune system. This is because the tissue origin of the primary tumor and target site of dissemination and metastasis differ. Thus, analysis of the microenvironment (e.g. immune cells) of the primary tumor may be less suited to evaluate whether the microenvironment at the target site of dissemination and metastasis is affected or whether a certain therapy targeting the microenvironment of DCC(s) and metastases will be effective. Rather, as is shown herein, the microenvironment in lymph nodes is indicative of the stage/type of the cancerous disease outside the primary tumor. Thus, it has been surprisingly found that, as is further demonstrated in the appended Examples, a DCCD of > 2000 , in particular in the case of melanoma, is indicative of a yet further advanced stage/type of the cancerous disease, in particular melanoma. Specifically, the appended Examples show that the number of antigen-experienced CD8 T cells increases with increasing DCCD, which is indicative of T cell activation related to tumor cell growth and metastases formation. In particular, PD-1 high expressing CD8 T cells are significantly enriched in lymph nodes with a DCCD at about 2000, i.e. at a value 20x higher than the DCCD > 100 which is indicative of metastasis development. PD-1 high CD8 T cells are considered as terminally exhausted T cells. These cells co-express Tim-3, another marker of exhausted T cells and have an impaired ability for cytokine production. Exhausted T cells lose effector functions in a hierarchical manner: IL-2 production, high proliferative capacity and cytolytic activity are lost first, followed by impaired cytokine production. PD-1 expressing CD8 T cells isolated from tumor tissue of metastatic melanoma patients were shown to be tumor-reactive. An increase in

MDSCs (myeloid derived suppressor cells) was concomitant with the increase with PD-1 high expressing CD8 T cells. MDSCs are immunosuppressive immune cells and cancer tissues with high infiltration of MDSCs are associated with poor patient prognosis and resistance to therapies. Also, the percentage of MDSC is increased in patients with a DCCD at about 2000, whereas the percentage of cytolytic CD56dim NK cells is decreased in lymph nodes with a high DCCD. CD56dim NK cells possess high cytolytic function and therefore can kill tumor cells. All together the appended Examples demonstrate that changes in the local immune microenvironment in the target organ of dissemination are associated with metastatic outgrowth, for which the DCCD gives an indicative measurement. The DCCD alone or in combination with information about changes in the immune microenvironment is indicative of the stage/type of the cancerous disease and/or is used to initiate, continue or discontinue therapy of said cancerous disease. Thus, in one embodiment, the methods or pharmaceutical composition of the invention, further comprise determination of the DCC density (DCCD), wherein the DCCD is the number of DCCs per million cells in the lymph node used to obtain the DCCs, wherein the DCCD is indicative of the stage/type of the cancerous disease, wherein a DCCD of > 100 is indicative for the development of metastases and/or wherein a DCCD of > 2000 is indicative of the immune system being unable to inhibit metastatic growth. Thus, where the DCCD is > 2000 , in particular in melanoma, treatment may further comprise immune therapy specifically addressing immune cells and their ability to inhibit metastasis growth.

As indicated above, the appended Examples show that depending on the metastasis signature (i.e. the type of somatic alterations) patient-derived DCCs are able to form tumors in mice. In particular, all DCCs that were able to form tumors had either a *BRAF* mutation, a loss of chromosome 9p11-13, a loss of chromosome 9p21-24, a gain of chromosome 7q21-36, or a *NRAS* mutation. In this regard, deletions of 9p11-13 and/or 9p21-24 are observed in about 90% of cells carrying more than one somatic alteration. All together, in about 20% of the samples, a loss of 9p11 was observed in combination with a loss of 9p24. About 50% have a loss of 9p11 and/or 9p24 in combination with a further alteration (*BRAF*, *NRAS* or gain on chromosome 7q21). Only about 20% have a loss of 9p11 and/or 9p24 plus a gain on chromosome 7q21 together with a mutation of *BRAF* or *NRAS*.

Therefore, one aspect of the present invention relates to the methods of the invention, or the pharmaceutical composition of the invention, wherein the somatic alterations comprise at

least one of the somatic alterations selected from the group consisting of a *BRAF* mutation, a loss of chromosome 9p11-13, a loss of chromosome 9p21-24, a gain of chromosome 7q21-36, and a *NRAS* mutation. According to the present invention the somatic alterations may also comprise at least two, three, four or all of the somatic alterations selected from the group consisting of a *BRAF* mutation, a loss of chromosome 9p11-13, a loss of chromosome 9p21-24, a gain of chromosome 7q21-36, and a *NRAS* mutation. In the methods of the invention or in context of the pharmaceutical composition of the invention, a *BRAF* mutation, a loss of chromosome 9p11-13, a loss of chromosome 9p21-24, a gain of chromosome 7q21-36, and/or a *NRAS* mutation indicates that the DCC(s) will develop to metastases.

However, according to the present invention also the proliferation activity of DCC(s) may be used in order to determine whether these cells will develop to metastases. Thus, another embodiment of the present invention relates to the methods of the invention, or the pharmaceutical composition of the invention, wherein step (a) further comprises evaluating the proliferation of the DCC(s), wherein in step (b) an increased proliferation of the DCC(s) indicates that the DCC(s) will develop to metastases. The proliferation can be measured, e.g., by analyzing the amount of proliferation markers such as the proliferation marker Ki-67. The frequency of Ki-67 positive cells can be determined by assessing the percentage of cells that stain positive for the marker, e.g. by immunohistochemistry, immunofluorescence. This percentage provides an estimate of cells in the cell cycle, i.e. the percentage of proliferating cells.

As indicated above, the stage/type of a cancerous disease can be used to evaluate whether the cancerous disease (preferably a melanoma) responds to a certain therapy. In particular, the presence or absence of genetic alterations within DCCs also indicates whether a certain treatment is to be initiated, continued or discontinued. In particular, one embodiment of the present invention relates to the methods of the invention, or the pharmaceutical composition of the invention, wherein if the DCC(s) are found to carry a *BRAF* mutation, a treatment that is directed against cells carrying said mutation is to be initiated or continued. However, if the DCC(s) were found to not carry a *BRAF* mutation, a treatment that is directed against cells carrying said mutation might be harmful to the patient. Similarly, if the DCC(s) are found to carry a *NRAS* mutation, a treatment that is directed against cells carrying said mutation is to be initiated or continued. However, if the DCC(s) are found to not carry a *NRAS* mutation, a

treatment that is directed against cells carrying said mutation might be harmful to the patient. If PD-1 high expressing T cells, which are thought to not respond to PD-1 blockade (PMID: 25797516), i.e. regain functionality, are found in increasing numbers in lymph node(s) harboring DCC(s), no therapy response might be observed under therapeutically PD-1 or PD-L1 blockade or the therapy might be even harmful to the patient (i.e. autoimmunity without anti-cancer response).

As described below in more detail, in the methods and pharmaceutical composition of the present invention, the type of cancerous disease is not particularly limited as long as the disease involves the dissemination of cells from a primary disease site into lymph node(s) and/or other tissue, for example bone marrow. It is preferred that the cancerous disease is a melanoma.

Accordingly, the present invention relates to a method for staging and/or typing of a cancerous disease, a method for treating a cancerous disease and a pharmaceutical composition for use in treating a cancerous disease, in particular cancer. In this regard, it was surprisingly and unexpectedly found that the stage/type of a cancerous disease, in particular cancer, can be determined by detecting the somatic alterations, in particular genetic and/or epigenetic alterations, in the DNA of disseminated cancer cells (DCCs) obtained from a tissue sample of a subject, in particular obtained from one or more lymph node(s) of a subject, in particular draining and/or regional lymph node(s) and/or sentinel lymph node(s).

Also provided herein are methods for staging and/or typing of a cancerous disease by detecting the somatic alterations, in particular genetic and/or epigenetic alterations, in the DNA of circulating tumor cells (CTCs). Accordingly, the present invention provides a method for staging and/or typing of a cancerous disease, a method for treating a cancerous disease and a pharmaceutical composition for use in treating a cancerous disease, in particular cancer where the stage/type of a cancerous disease, in particular cancer, is determined by detecting the somatic alterations, in particular genetic and/or epigenetic alterations, in the DNA of one or more CTC(s) obtained from blood of a subject, in particular obtained from a blood sample obtained from a subject or obtained using an *in vivo* CTC-capturing-device. That is, the embodiments provided herein may also be applied to CTC(s), unless indicated otherwise, instead or in addition to DCC(s). The skilled person is readily aware how to adapt the teaching provided herein for DCC(s) to CTC(s) accordingly.

The inventors determined the time point of metastatic seeding of melanoma relative to the depth of dermal invasion and characterized ectopic molecular evolution in cancer, in particular melanoma; see *inter alia* Example 1 and Figure 1. More specifically, as also large melanomas may be non-invasive, the time point of metastatic seeding of melanoma relative to the depth of dermal invasion (not relative to the total size) of the melanoma was analyzed. Dissemination to lymph nodes preferentially occurred around 0.4 mm (95%CI 0.04-0.75 mm) before intradermal expansion. Disseminated cancer cells (DCCs) lacked typical driver changes before lymphatic colony formation regardless of primary tumor thickness; see *inter alia* Example 5 and Figures 2, 3 and 4. However, driver mutations in *BRAF* or *NRAS* and signature chromosomal rearrangements became significantly enriched in colony-forming DCCs and/or xenografts from DCCs and put patients at high risk for death. Thus, in cancerous diseases, in particular cancer, DCCs leave primary tumors early and evolve at different sites in parallel; see *inter alia* Example 6 and Figure 5. Accordingly, staging and/or typing of cancerous diseases, in particular cancer, can be improved, in particular a more accurate method of staging and/or typing of cancerous diseases can be provided, by using information from cells derived from primary tumors, in particular DCCs obtained from different sites where cells evolve, i.e. sites where DCCs migrate, like for example lymph nodes, or sites where cells, in particular CTCs derived from a primary tumor circulate, for example blood. In this regard, the inventors surprisingly found that the somatic evolution of said DCCs or CTCs, respectively, determined based on somatic, in particular genetic and/or epigenetic alterations detected in the DNA of said DCCs or CTCs, respectively, can be used to stage/type a cancerous disease, in particular cancer. These surprising and unexpected findings led to the present invention, in particular more accurate means and methods for staging and/or typing of cancerous diseases, which are based on a novel model of cancerous diseases, in particular cancer.

As outlined further above, the stage of a cancerous disease is usually determined using, for example, the TNM staging system. The TNM Classification of Malignant Tumours (TNM) is a cancer staging notation system that gives codes to describe the stage of a patient's cancer, wherein T describes the size of the original (primary) tumor and whether it has invaded nearby tissue, N describes nearby (regional) lymph nodes that are involved, and M describes distant metastasis (spread of cancer from one part of the body to another). With particular regard to the tumour, i.e. parameter T, there are several stages: Tx: tumor cannot be evaluated,

Tis: carcinoma in situ, T0: no signs of tumor, and T1, T2, T3, T4: size and/or extension of the primary tumor. As it was found by the present inventors, the above stages of cancerous diseases, in particular cancer, for example melanoma, are in many cases insufficient to determine a suitable strategy for therapy of a cancerous disease. Therefore, a new model was found, which uses the somatic evolution of cells, in particular DCCs obtained from one or more lymph node(s) of a subject, in particular draining and/or regional lymph node(s) and/or sentinel lymph node(s), but also, if available, DCCs from other organs than lymph nodes, such as bone marrow or liquor, informing about the molecular state of brain DCCs. The somatic evolution of cells may also be determined using CTCs obtained from blood of a subject. Accordingly, in contrast to classical staging/typing of cancerous diseases, which mainly depends on phenotypic assessment of a primary tumor, the novel and inventive methods of the present invention use the somatic evolution of cells, in particular DCCs and/or CTCs, as indicator of the stage/type of a cancerous disease, in particular cancer.

DCCs are cells that disseminated from a primary tumor site before surgery and spread lymphatically or hematogeneously. They may be detected using epithelial cell markers or melanocytic markers in case of melanoma, which are not found on the surface of lymphatic cells or cells found in bone marrow. For example, EpCAM and/or cytokeratins are cell markers that may be targeted in the detection of DCCs (Klein (2009), Nat Rev Cancer 9:302-312), in particular in the case of carcinomas. The targeting cell markers can be expressed and/or located intracellularly and/or at the surface of the cell. Preferably, DCCs from melanoma may be detected by using gp100 and/or MCSP (melanoma-associated chondroitin sulfate proteoglycan) and/or Melan A (MART-1), and/or CD146 as targeting cell markers. DCCs can be detected and isolated for example from bone marrow aspirates or lymph nodes. After disaggregating and/or processing into single cell suspension, the DCCD can be determined by immunocytology. In particular, the single cell suspension may be stained with antibodies against the said targeting cell markers such as EpCAM (Guzvic *et al.* (2014) Cancer Res. 74:7383-7394), cytokeratin (Schardt *et al.* (2005) Cancer Cell 8:227-239, gp100 or MCSP (Ulmer *et al.* (2014) PLoS Med. 11:e1001604. The number of cells positive for the targeting cell marker per million of cells (preferably lymph node cells) that were stained defines the DCC-density (DCCD).

CTCs are cells that are also derived from a primary tumor site before surgery, but that circulate in the blood system. After primary tumor surgery they may be derived from latent or manifest metastatic colonies. They may be detected and isolated using EpCAM as targeting

cell marker, but are not limited to this targeting cell marker. Enrichment and detection markers are similar to those used for detection in other mesenchymal organs (bone marrow, lymph nodes) and include for example EpCAM and Cytokeratins for epithelial markers and the mentioned melanocytic markers in melanoma. CTCs may be obtained from blood (e.g. from leukapheresis products) (Fischer *et al.* (2013) PNAS 110:16580-16585) using methods well-known in the art, in particular FDA-cleared Cell Search® system (Veridex LLC), DEPArray™ technology or flowcytometry, but CTC-isolation is not limited to these systems (Polzer *et al.* (2014) EMBO Mol Med. 6:1371-138) and may comprise a variety of novel devices such as CellSieve or Parsortix. In vivo CTC-capturing-devices may also be used. Examples for such devices are Gilupi, CellCollector (Gorges *et al.* (2015) ClinCancer Res. 1416.2015, Epub ahead of print), cancer cell binding nanoparticles Galanzha *et al.* (2011) Cytometry 79:814-824) or medical wires (Saucedo-Zeni *et al.* (2012) Int J Oncol. 41:1241-1250), but are not limited to these. Such devices or particles are implanted or infused into a subject/patient to capture and enrich CTCs in the body of the subject/patient. The CTCs can then be retrieved from the CTC-capturing-device or -particles ex vivo or *in vivo*.

In this regard, the term “somatic evolution” as used herein relates to the accumulation of alterations in cells, in particular cancerous disease cells, in particular DCCs, during a lifetime, and the effects of those alterations on the fitness of those cells. Somatic evolution of cancer cells is due to spontaneous or induced genetic/epigenetic alterations and subsequent natural selection during clonal expansion, which selects cells according to fitness, proliferation rate, apoptosis rate and the like. In addition, cancer therapy acts as a form of artificial selection, killing sensitive cancer cells, but leaving behind resistant cells. Often the tumor will regrow from those resistant cells, the patient will relapse, and the therapy that had been previously used will no longer be effective. Thus, somatic evolution is an ongoing process during the development of cancerous diseases and/or therapy of a cancerous disease. In this regard, there are multiple levels of genetic heterogeneity associated with somatic evolution in cancerous diseases. In the methods of the present invention, somatic evolution is determined based on somatic alterations, including epigenetic alterations and/or genetic alterations. Therefore, based on the number and/or nature of somatic alterations, including epigenetic alterations and/or genetic alterations, the somatic evolution of a cell, in particular a DCC, can be determined.

The term “somatic alterations” as used herein includes any alteration of nucleic acids, in particular DNA, comprised in a cell, in particular a DCC. Alteration means a deviation from a

reference state like for example a state at a given point of time in a reference sample obtained from a subject, for example in a sample obtained from a subject comprising cells derived from other tissue than the sample to be analyzed in the methods of the present invention and/or comprising stem cells of the subject, or a state generally taken as reference with regard to a particular segment of DNA, for example a DNA sequence taken from a sequence database or a known pattern of epigenetic alterations such as a methylation pattern. Accordingly, a somatic alteration may be detected by comparing an observed state with a reference state. Any observed deviation may then be categorized as somatic alteration. In a second phase, said alteration may be categorized as real somatic alteration or an error introduced by the employed detection method, for example sequencing method. In this regard, the present invention discloses means and methods particularly useful for error-free detection of somatic alterations. Such methods are preferred to be used in the methods of the present invention. Somatic alterations within the meaning of the present invention include epigenetic and/or genetic alterations. Genetic alterations include alterations on the level of the sequence of the DNA, i.e. the succession of nucleotides comprised in DNA, and alterations of the relative abundance of segments of the DNA comprised in the sample to be analyzed. Accordingly, genetic alterations within the meaning of the present invention may include, *inter alia*, single nucleotide variations (SNV), sequence mutations, Microsatellite shifts and instability, Loss of heterozygosity (LOH), copy number alterations, karyotypic variations including chromosome structural aberrations and/or aneuploidy. Epigenetic alterations include chemical alterations of DNA that are not on the level of the DNA sequence and alterations of external factors involved in DNA structure formation, processing and the like. Accordingly, epigenetic alterations within the meaning of the present invention may include, *inter alia*, methylation, in particular methylation of CG pairs, deficiencies of DNA repair proteins, and/or alterations in histone architecture or structure like methylation, acetylation, sumoylation, activating or inactivating histone marks and/or alterations in chromatin architecture like for example in eu- or hetero-chromatin and the like.

In one aspect of the invention, somatic alterations, in particular genetic alterations, comprise one or more driver mutation(s) and/or passenger mutation(s). The term “driver mutation” refers to mutations that give a selective advantage to a clone in its microenvironment, through either increasing its survival or reproduction. Driver mutations tend to cause clonal expansions. Accordingly, the somatic alterations, in particular genetic alterations, determined in the methods of the present invention may comprise one or more driver mutation(s). In this

regard, one common feature of cellular, in particular DCC progression is the expansion of a clone with a somatic alteration, in particular a genetic and/or epigenetic alteration. This is generally due to the expanding clone having a competitive advantage (either a reproductive or survival advantage) over other cells in the respective tissue. Since expanding clones often have more than one somatic alteration, in particular genetic and/or epigenetic alteration, in their genomes, it is often not clear which of those alterations cause a reproductive or survival advantage and which other alterations are simply hitchhikers or passenger mutations on the clonal expansion. However, several driver mutations are known in the art. For example, driver mutations may be, *inter alia*, mutations in known oncogenes. Oncogenes are genes known to be associated with the development of a cancerous disease, in particular cancer. Thus, driver mutation(s) within the meaning of the present invention include, but are not limited to, mutations in known oncogenes. For examples, the genes coding for the neuroblastoma RAS viral oncogene homolog (NRAS) or BRAF are known oncogenes. Further known oncogenes are reported in databases such as the COSMIC database by Forbes *et al.* (2014) Nucleic Acid Res. 43, D805-811, or in reports by Vogelstein *et al.* (2013) Science 339(6127):1546-58, Akagi *et al.* (2004) Nucleic Acid Res. 32, D523-7 or Huret *et al.* (2000) Nucleic Acid Res. 28(1):349-51. Such databases are constantly updated for oncogenes and known driver mutations. Accordingly, any of the therein reported driver mutations may be detected in the methods of the present invention. In this regard, the methods used in the present invention for detecting the somatic alterations, in particular genetic and/or epigenetic alterations, in oncogenes are suitable for simultaneous detection of somatic alterations in multiple oncogenes. Particular driver mutations that may be detected in the methods of the present invention comprise driver mutations in the gene coding for NRAS comprising, *inter alia*, the exon 3 mutation c181C>A. Accordingly, the present invention relates to a method for staging and/or typing of a cancerous disease, the method comprising the steps of detecting somatic alterations of the DNA of one or more DCC(s) obtained from a tissue sample, in particular one or more lymph node(s); and determining the somatic evolution of the DCC(s) based on the detected somatic alteration(s) in the DNA of the one or more DCC(s), wherein the somatic evolution of the DCC(s) is indicative of the stage/type of the cancerous disease, wherein detection of somatic alterations comprises detection of the nucleotide at position 2986 of SEQ ID NO:1, wherein a cytosine at position 2986 of SEQ ID NO:1 is indicative of a non-diseased stage/type and an adenine at position 2986 of SEQ ID NO:1 is indicative of a diseased stage/type. Furthermore, a guanine at position 2987 of SEQ ID NO:1 or a thymine at position 2987 of SEQ ID NO:1 is indicative of a diseased stage. The present invention furthermore

relates to a method for treating a cancerous disease, the method comprising the steps of detecting somatic alterations in the DNA of one or more DCC(s) obtained from a tissue sample, in particular one or more lymph node(s) of a subject; determining the somatic evolution of the DCC(s) based on the detected somatic alteration(s) in the DNA of the one or more DCC(s); and determining the stage/type of the cancerous disease based on the somatic evolution of the cancerous disease determined based on the detected somatic alterations in the DNA of the one or more DCC(s), wherein the stage/type of the cancerous disease is used to initiate, continue or discontinue therapy of said cancerous disease, wherein detection of somatic alterations comprises detection of the nucleotide at position 2986 of SEQ ID NO:1, wherein a cytosine at position 2986 of SEQ ID NO:1 is used to discontinue therapy and an adenine at position 2986 of SEQ ID NO:1 is used to initiate or continue therapy of the cancerous disease. Furthermore, a guanine at position 2987 of SEQ ID NO:1 or a thymine at position 2987 of SEQ ID NO:1 is used to initiate or continue therapy of the cancerous disease.

The invention furthermore relates to a pharmaceutical composition for use in treating a cancerous disease in a subject, wherein treatment is initiated, continued or discontinued based on the stage/type of the cancerous disease, wherein the stage/type of the cancerous disease is determined by detecting somatic alterations in the DNA of one or more DCC(s) obtained from a tissue sample, in particular one or more lymph node(s) of a subject; determining the somatic evolution of the DCC(s) based on the detected somatic alteration(s) in the DNA of the one or more DCC(s); and determining the stage/type of the cancerous disease based on the somatic evolution of the cancerous disease determined based on the detected somatic alterations in the DNA of the one or more DCC(s), wherein detection of somatic alterations comprises detection of the nucleotide at position 2986 of SEQ ID NO:1, wherein a cytosine at position 2986 of SEQ ID NO:1 is used to discontinue treatment and an adenine at position 2986 of SEQ ID NO:1 is used to continue or initiate treatment. Furthermore, a guanine at position 2987 of SEQ ID NO:1 or a thymine at position 2987 of SEQ ID NO:1 is used to continue or initiate treatment of the cancerous disease. The detection of somatic alterations may comprise the detection of genetic and/or epigenetic alterations of one or more oncogenes.

As outlined above, the detection of somatic alterations, including genetic and/or epigenetic alterations, may comprise the detection of somatic alterations in known oncogenes. As described above for NRAS, known driver mutations may also be detected in BRAF, another

known oncogene encoding a protein involved in directing cell growth. As such, several genetic alterations, in particular (driver) mutations, are described in the art to cause a constitutively active form of BRAF, which is associated with the development of cancer. In view of its role in disease, several BRAF inhibitors directed to the native and mutated form have been described; see *e.g.* Wan *et al.* (2004) Cell 116 (6): 855-67; Tsai *et al.* (2008) PNAS 105 (8): 3041-6; and Bollag *et al.* (2010) Nature 467 (7315) : 596-9. For example, sorafenib is a small molecular kinase inhibitor drug approved for the treatment of primary kidney cancer (advanced renal cell carcinoma), advanced primary liver cancer (hepatocellular carcinoma), and radioactive iodine resistant advanced thyroid carcinoma. Sorafenib inhibits, *inter alia*, wild-type and mutant BRAF. A further exemplary BRAF inhibitor is vemurafenib, which is a selective inhibitor of the V600E mutated BRAF protein responsible for an aggressive form of melanoma. It has also been shown to be an effective inhibitor of V600K mutated BRAF protein. In melanoma patients having wild-type BRAF, vemurafenib promotes tumor growth; see Hatzivassiliou *et al.* (2010) Nature 464 (7287): 431-5 or Halaban *et al.* (2010) Pigment Cell Melanoma Res. 23(2): 190-200. However, classical melanoma diagnosis does not comprise determination of the mutational state of patients with regard to the BRAF oncogene. Moreover, as it has been surprisingly found by the present inventors, the herein described new model of parallel evolution of cells in the primary tumor and DCC(s) indicates that detection of the mutational state in the primary tumor is insufficient for determination of the best suitable therapy of melanoma patients. Accordingly, the herein provided more accurate methods of cancer staging/typing may comprise a step of detecting the mutational state of the BRAF oncogene. Therefore, the present invention relates to a method for staging and/or typing of a cancerous disease, the method comprising the steps of detecting somatic alterations of the DNA of one or more DCC(s) obtained from a tissue sample, in particular one or more lymph node(s); and determining the somatic evolution of the DCC(s) based on the detected somatic alteration(s) in the DNA of the one or more DCC(s), wherein the somatic evolution of the DCC(s) is indicative of the stage/type of the cancerous disease, wherein detection of somatic alterations comprises detection of the nucleotide triplet at positions 171428 to 171430 of SEQ ID NO:2, wherein a nucleotide triplet guanine at position 171428, tyrosine at position 171429 and guanine at position 171430 of SEQ ID NO:2 is indicative of a non-diseased stage/type and a nucleotide triplet guanine at position 171428, adenine at position 171429 and guanine at position 171430 of SEQ ID NO:2 is indicative of a diseased stage/type. Furthermore, a nucleotide triplet adenine at position 171428, adenine at position 171429 and guanine at position 171430 of SEQ ID NO:2 and a nucleotide triplet adenine at

position 171428, guanine at position 171429 and guanine at position 171430 of SEQ ID NO:2 is indicative of a diseased stage/type. The present invention furthermore relates to a method for treating a cancerous disease, the method comprising the steps of detecting somatic alterations in the DNA of one or more DCC(s) obtained from a tissue sample, in particular one or more lymph node(s); determining the somatic evolution of the DCC(s) based on the detected somatic alteration(s) in the DNA of the one or more DCC(s); and determining the stage/type of the cancerous disease based on the somatic evolution of the cancerous disease determined based on the detected somatic alterations in the DNA of the one or more DCC(s), wherein the stage/type of the cancerous disease is used to initiate, continue or discontinue therapy of said cancerous disease, wherein detection of somatic alterations comprises detection of the nucleotide triplet at positions 171428 to 171430 of SEQ ID NO:2, wherein a nucleotide triplet guanine at position 171428, tyrosine at position 171429 and guanine at position 171430 of SEQ ID NO:2 is used to discontinue treatment and a nucleotide triplet guanine at position 171428, adenine at position 171429 and guanine at position 171430 of SEQ ID NO:2 is used to continue or initiate treatment. Furthermore, a nucleotide triplet adenine at position 171428, adenine at position 171429 and guanine at position 171430 of SEQ ID NO:2 and a nucleotide triplet adenine at position 171428, guanine at position 171429 and guanine at position 171430 of SEQ ID NO:2 is used to initiate or continue treatment. It is preferred that treatment comprises the use of an inhibitor of BRAF. Accordingly, treatment preferably comprises the use of sorafenib or vemurafenib. Moreover, it is preferred that the cancerous disease is melanoma. The invention furthermore relates to a pharmaceutical composition for use in treating a cancerous disease in a subject, wherein treatment is initiated, continued or discontinued based on the stage/type of the cancerous disease, wherein the stage/type of the cancerous disease is determined by detecting somatic alterations in the DNA of one or more DCC(s) obtained from a tissue sample, in particular one or more lymph node(s) of a subject; determining the somatic evolution of the DCC(s) based on the detected somatic alteration(s) in the DNA of the one or more DCC(s); and determining the stage/type of the cancerous disease based on the somatic evolution of the cancerous disease determined based on the detected somatic alterations in the DNA of the one or more DCC(s), wherein detection of somatic alterations comprises detection of the nucleotide triplet at positions 171428 to 171430 of SEQ ID NO:2, wherein a nucleotide triplet guanine at position 171428, tyrosine at position 171429 and guanine at position 171430 of SEQ ID NO:2 is used to discontinue treatment and a nucleotide triplet guanine at position 171428, adenine at position 171429 and guanine at position 171430 of SEQ ID NO:2 is used to continue or initiate

treatment. Furthermore, a nucleotide triplet adenine at position 171428, adenine at position 171429 and guanine at position 171430 of SEQ ID NO:2 and a nucleotide triplet adenine at position 171428, guanine at position 171429 and guanine at position 171430 of SEQ ID NO:2 is used to initiate or continue treatment. It is preferred that the pharmaceutical composition comprises an inhibitor of BRAF. Accordingly, it is preferred that the pharmaceutical composition comprises sorafenib or vemurafenib. Moreover, it is preferred that the cancerous disease is liver cancer, kidney cancer or melanoma. It is more preferred that the cancerous disease is melanoma. Accordingly, the present invention relates to, *inter alia*, a pharmaceutical composition comprising a BRAF inhibitor, preferably sorafenib or vemurafenib, for use in treating a cancerous disease in a subject, wherein treatment is initiated, continued or discontinued based on the stage/type of the cancerous disease, wherein the stage/type of the cancerous disease is determined by detecting somatic alterations in the DNA of one or more DCC(s) obtained from a tissue sample, in particular one or more lymph node(s) of a subject; determining the somatic evolution of the DCC(s) based on the detected somatic alteration(s) in the DNA of the one or more DCC(s); and determining the stage/type of the cancerous disease based on the somatic evolution of the cancerous disease determined based on the detected somatic alterations in the DNA of the one or more DCC(s), wherein detection of somatic alterations comprises detection of the nucleotide triplet at positions 171428 to 171430 of SEQ ID NO:2, wherein a nucleotide triplet guanine at position 171428, tyrosine at position 171429 and guanine at position 171430 of SEQ ID NO:2 is used to discontinue treatment and a nucleotide triplet guanine at position 171428, adenine at position 171429 and guanine at position 171430 of SEQ ID NO:2 is used to continue or initiate treatment. Furthermore, a nucleotide triplet adenine at position 171428, adenine at position 171429 and guanine at position 171430 of SEQ ID NO:2 and a nucleotide triplet adenine at position 171428, guanine at position 171429 and guanine at position 171430 of SEQ ID NO:2 is used to initiate or continue treatment. The cancerous disease is preferably liver cancer, kidney cancer or melanoma. It is more preferred that the cancerous disease is melanoma.

In accordance with the above, the invention provides means and methods of treating a cancerous disease, in particular cancer. Accordingly, the present invention, *inter alia*, relates to a method of treating a cancerous disease, in particular cancer, comprising obtaining and analyzing a sample from a patient suspected of having or having a cancerous disease, in particular cancer; wherein analyzing comprises sequencing of the DNA of DCC(s) comprised in the sample; detecting the presence or absence of somatic alterations, in particular genetic

and/or epigenetic alterations, in the DNA, wherein the presence of somatic alterations indicates that an effective amount of a pharmaceutical composition has to be administered to the patient. The invention furthermore relates to a method of diagnosing a cancerous disease, comprising obtaining and analyzing a sample from a patient suspected of having a cancerous disease, in particular cancer; wherein analyzing comprises sequencing of the DNA of DCC(s) comprised in the sample; detecting the presence or absence of somatic alterations, in particular genetic and/or epigenetic alterations, in the DNA, wherein the presence of somatic alterations indicates a patient has, or is likely to have, a cancerous disease and the absence of somatic alterations indicates the patient does not have, or is not likely to have, a cancerous disease.

Somatic alterations, in particular genetic alterations, determined in the methods of the present invention may also comprise one or more passenger mutations. A passenger mutation is a mutation that has generally no or only little effect on the fitness of a clone but may be associated with a clonal expansion because it occurs in the same genome with a driver mutation. In this regard, it is important to recognize that a passenger mutation may have no or only little effect on the fitness of a cell within a particular tissue while the same mutation may have drastic effect on the fitness of a cell within another tissue. However, within the meaning of the present invention, passenger mutations may be those mutations that are known to have a higher probability to be found in a genome where a driver mutation occurred or is likely to occur in the future.

Somatic alterations, in particular genetic or epigenetic alterations, determined in the methods of the present invention may also comprise one or more copy number alterations. Copy-number alterations are alterations of the DNA of a genome that result in the cell having an abnormal or, for certain genes, a normal variation in the number of copies of one or more sections of the DNA. A copy number alteration may correspond to parts of the genome that have been deleted (fewer than the normal number) or multiplied (more than the normal number).

Each alteration may correspond to the deletion or gain, in particular duplication, of a genomic region, which may range from about one kilobase (1,000 nucleotide bases) to several megabases (1,000,000 nucleotide bases) in size. Copy number alterations have been associated with susceptibility or resistance to disease. For example, gene copy number can be elevated in cells associated with cancer, e.g. in DCCs. Accordingly, copy number alterations

may also be determined in the methods of the present invention in order to determine the somatic evolution of a cell, in particular of a DCC. The somatic evolution may then be used to determine the stage/type of a cancerous disease, in particular cancer. Particular copy number alterations determined in the methods of the present invention comprise loss of chromosome 9p11-13, loss of chromosome 9p21-24 and/or gain of chromosome 7q21. Accordingly, the present invention relates to the methods or pharmaceutical compositions of the invention, wherein the somatic alterations of the DNA comprise at least one of the alterations selected from a loss of chromosome 9p11-13, a loss of chromosome 9p21-24 and gain of chromosome 7q21.

In addition or alternatively to the nature/type of somatic alterations, in particular genetic and/or epigenetic alterations, detected in the methods of the present invention, the number of somatic alterations, in particular genetic and/or epigenetic alterations, may also be determined in order to determine the somatic evolution of a cell, in particular a DCC. In this regard, it can generally be assumed that the higher the number of accumulated somatic alterations, the more advanced the somatic evolution of the cells, in particular the DCCs. While there are driver mutations, as described above, whose presence is indicative of an advanced stage/type of the cancerous disease, in particular the cancer, the overall number of accumulated somatic alterations may also achieve a threshold value, which is indicative of an advanced stage of the cancerous disease, in particular the cancer; see e.g. Shain *et al.* (2015) N Engl J Med 373; 20:1926.

The accuracy of the methods of the present invention may be further increased by using additional parameters to determine the overall somatic evolution of a cell population, in particular DCCs comprised in a lymph node of a subject or derived from other tissue (early) invaded by cancer cells, e.g. bone marrow or brain. In this regard, it was surprisingly found that at a specific cell density, cells, in particular DCCs, form cell-cell contacts leading to DCCs being organized in small nests of cells rather than isolated cells. Moreover, it was surprisingly found that in xenotransplantation experiments, only mice transplanted with cells, in particular DCCs, derived from lymph nodes of patients in which said cells, in particular DCCs, were present at a cell density above a specific threshold value gave rise to a tumor in transplanted mice; see Figure 5C. From these experiments, it was deduced that the overall cell number of DCCs observed in the respective tissue sample, e.g. a sample derived from a lymph node or a bone marrow sample, may serve as additional parameter to further increase accuracy of the methods of the present invention. Based on these experiments, the threshold

value indicative for a high probability that cells accumulated important driver mutations in their genome so that an advanced stage/type of the cancerous disease, in particular cancer, is determined to be in the range of about 50 or more, preferably 60, 70, 80, 90 or more, most preferably 100 cells per one million cells in the respective tissue sample. Accordingly, a DCCD between about 50 and about 100, preferably between about 60 and about 100, preferably between about 70 and about 100, preferably between about 80 and about 100 and most preferably between about 90 and about 100 is indicative of an advanced stage/type of a cancerous disease, in particular cancer. In accordance with the above, based on the number of cells found in the tissue sample obtained from a patient, the cell density can be calculated with respect to the overall number of cells in the obtained sample or with respect to another cell population found in the obtained sample. In this regard, it is preferred that the DCC cell population comprised in the tissue sample obtained from a patient is detected using a marker, e.g. EpCAM and/or cytokeratins. The DCC cell population comprised in a melanoma tissue sample obtained from a patient is preferably detected using gp100 as a marker. However, alternative DCC markers may also be used, which may cause alternative threshold values corresponding to an advanced stage/type of the cancerous disease, as the person skilled in the art will appreciate. Accordingly, it should be recognized that the determined DCCD in a tissue sample, in particular lymph node sample or bone marrow sample obtained from a patient, which is used as an indicator of the stage/type of a cancerous disease, in particular cancer, can vary based on the method used to determine the cell density. In this regard, it is preferred that gp100 is used as a marker to determine the number of DCCs comprised in the obtained sample, wherein DCCs are gp100 positive. Accordingly, it is preferred that the methods of the present invention, in addition to determination of somatic alterations, in particular genetic and/or epigenetic alterations, comprised in one or more DCCs obtained from one or more lymph nodes of a patient, comprise a step of determining the DCCD in the lymph node used to obtain the DCCs, in particular the lymph node obtained from a patient.

Where the stage/type of a cancerous disease is determined based on the somatic evolution of CTCs, the methods of the present invention may further comprise a step of determining the CTCD in order to improve accuracy. Accordingly, it is preferred that, in addition to determination of somatic alterations, in particular genetic and/or epigenetic alterations, comprised in one or more CTCs from a patient, in particular retrieved from blood samples or in vivo CTC-capturing-devices, comprise a step of determining the CTC density (CTCD). In this regard, the CTCD is the number of CTCs per one million cells in the blood sample

obtained from a patient, or in the case of an in vivo capturing-device by some parameter that relates the CTC number to the filtered blood volume (blood volume or filtering time as surrogate). At a specific threshold value that may be determined based on the used detection method of CTCs, for example comprising the use of MCSP or EpCAM as a marker, but not limited to this, an advanced stage/type of the cancerous disease may be determined.

In the methods of the present invention, the one or more cell(s), in particular the one or more DCC(s) is/are preferably obtained from one or more lymph node(s). Lymph nodes are oval-shaped organs of the lymphatic system, distributed widely throughout the body including the armpit and stomach and linked by lymphatic vessels. Lymph nodes are major sites of B, T, and other immune cells. Lymph nodes are important for the proper functioning of the immune system, acting as filters for foreign particles and cancer cells. Lymph nodes are known to have clinical significance. They become inflamed or enlarged in various infections and diseases, which range from throat infections to cancer. It is furthermore preferred that the one or more lymph node(s) is/are regional lymph node(s), wherein a regional lymph node is a lymph node close to a site being in a status of developing a disease, in particular a site susceptible to developing a cancerous disease, in particular cancer or a site that has developed a cancerous disease, in particular cancer. In particular, in the context of cancer, the regional lymph node is a lymph node anatomically close to a cancer site, for example a tumor. It is more preferred that the one or more lymph node used in the methods of the invention is/are (a) draining lymph node(s). Draining lymph nodes are lymph nodes to which cells migrate. Often, the draining lymph node(s) of a diseased site or a site susceptible of developing a disease is/are the first lymph node(s) invaded by cells migrating from said site. Such lymph node(s) is/are also called sentinel lymph node(s), i.e. the hypothetical first lymph node or group of nodes draining a cancerous disease, in particular a cancer. In case of established cancerous dissemination the sentinel lymph node/s is/are the target organs primarily reached by metastasizing cancer cells from the tumor. Thus, sentinel lymph nodes can be totally void of cancer because they were detected prior to dissemination. Accordingly, it is most preferred that the DCCs used in the methods of the present invention are derived from one or more sentinel lymph node(s) in order to determine whether a subject is susceptible to develop a cancerous disease, in particular cancer, or determine the stage/type of a cancerous disease, in particular cancer.

The person skilled in the art is well-aware of methods how to obtain lymph node(s) from a subject, in particular a human subject. As used herein, the term “sample obtained from one or

more lymph node(s)” refers to a sample of cells, tissue or fluid that is removed from a subject for the purpose of determining if the sample contains cancerous tissue, in particular DCC(s), and/or for determining the somatic evolution of said cells. In some embodiments, such a sample is obtained because a subject is suspected of having cancer due to an earlier diagnosis and/or a predisposition. The sample may then also be examined for the presence or absence of cancer, cancer stem cells, and/or cancer stem cell gene signature expression using methods known in the art. For example, the lymph node samples used in the methods of the present invention may be obtained by lymph node biopsy, for example needle biopsy or open biopsy as described in the art; see e.g. Chang KL et al. (2009) *Modern Surgical Pathology*. 2nd ed. Philadelphia, PA: Saunders Elsevier; 2009:chap 41.

As used herein, the term “subject” refers to any animal (e.g., a mammal), including, but not limited to, humans, non-human primates, rodents, and the like, which is to be the recipient of a particular treatment. Typically, the terms “subject” and “patient” are used interchangeably herein in reference to a human subject. Accordingly, it is preferred that the one or more DCC(s) are obtained from a human subject, more preferred a human patient, wherein the subject/patient may be suspected of having cancer or may be susceptible to a cancerous disease, in particular cancer.

As used herein, the term “subject suspected of having cancer” or “patient susceptible to a cancerous disease” refers to a subject/patient that presents one or more symptoms indicative of a cancer (e.g., a noticeable lump or mass) or is being screened for a cancer (e.g., during a routine physical). A subject suspected of having cancer or a patient susceptible to a cancerous disease, in particular cancer, can also have one or more risk factors. A “subject suspected of having cancer” or “patient susceptible to a cancerous disease” encompasses an individual who has received an initial diagnosis but for whom the stage of cancer is not known. The term further includes people who once had cancer (e.g., an individual in remission). The term also refers to a subject with one or more risk factors for developing a specific cancer. Risk factors include, but are not limited to, gender, age, genetic predisposition, environmental exposure, previous incidents of cancer, preexisting non-cancer diseases, and lifestyle.

The methods of the present invention are for staging and/or typing and/or predicting outcome and/or treating a cancerous disease, in particular cancer. In the methods of the present invention, the type of cancerous disease is not particularly limited as long as the disease involves the dissemination of cells from a primary disease site into lymph node(s) and/or

other tissue, for example bone marrow. Generally, dissemination relates to the process of migration by active migration or passive transport by body fluids from a primary disease site to a secondary site, for example a lymph node or bone marrow or blood. Accordingly, the methods of the present invention are for staging/typing of a cancerous disease, in particular a cancerous disease involving dissemination of cells, preferably involving lymphatic dissemination. Thus, the methods of the present invention are preferably for staging and/or typing and/or predicting outcome and/or treating of solid cancer. As used herein, a “solid cancer” refers to one or more cells which are growing or have grown in an uncontrolled manner to form cancer tissue. As used herein, the term “solid cancer” includes, but is not limited to “carcinomas”, “adenocarcinomas” and “sarcomas”. “Sarcomas” are cancers of the connective tissue, cartilage, bone, muscle, and the like. “Carcinomas” are cancers of epithelial (lining) cells. “Adenocarcinoma” refers to carcinoma derived from cells of glandular origin. The terms “cancer” and “tumor” are used interchangeably throughout the subject specification.

Solid cancers may arise in nearly any tissue of the body and the methods of the present invention may be used for staging and/or typing and/or predicting outcome and/or treating of any of these cancers. Exemplary “solid cancers” which may be staged and/or typed and/or whose outcome may be predicted and/or which may be treated in accordance with the present invention include acoustic neuroma, adenocystic carcinoma, adrenocortical cancer, alveolar soft-part sarcoma, anal cancer, angiosarcoma, basal cell carcinoma (bcc), bladder cancer, bone cancers, bowel cancer, brain stem glioma, breast cancer, CNS cancers, carcinoid cancers, cervical cancer, childhood brain cancers, childhood soft tissue sarcoma, chondrosarcoma, choriocarcinoma, colorectal cancers, dermatofibrosarcoma-protuberans, desmoplastic small round cell cancer, ductal carcinoma, endocrine cancers, endometrial cancer, ependymoma, esophageal cancer, Ewing's sarcoma, extra hepatic bile duct cancer, eye cancer, eye: melanoma, retinoblastoma, fallopian tube cancer, fibrosarcoma, gall bladder cancer, gastric cancer, gastrointestinal cancers, gastrointestinal carcinoid cancer, genitourinary cancers, germ cell cancers, gestational trophoblastic disease, glioma, gynecological cancers, head and neck cancer, hepatocellular cancer, hereditary breast cancer, human papillomavirus, hypopharynx cancer, intra-ocular melanoma, Kaposi's sarcoma, kidney cancer, laryngeal cancer, leiomyosarcoma, lip cancer, liposarcoma, liver cancer, lung cancer, male breast cancer, malignant rhabdoid cancer of kidney, medulloblastoma, melanoma, merkel cell cancer, mesothelioma, metastatic cancer, mouth cancer, multiple endocrine neoplasia, nasal cancer, nasopharyngeal cancer, neuroblastoma, neuroblastoma,

neurofibromatosis, non-melanoma skin cancer, non-small cell lung cancer (nsc), ocular cancers, oesophageal cancer, oral cavity cancer, oropharynx cancer, osteosarcoma, pancreas cancer, paranasal cancer, parathyroid cancer, parotid gland cancer, penile cancer, peripheral neuroectodermal cancers, pituitary cancer, prostate cancer, rare cancers and associated disorders, renal cell carcinoma, retinoblastoma, rhabdomyosarcoma, salivary gland cancer, sarcoma, skin cancer, small cell lung cancer (scl), small intestine cancer, soft tissue sarcoma, spinal cord cancers, squamous cell carcinoma (scc), stomach cancer, synovial sarcoma, testicular cancer, thymus cancer, thyroid cancer, transitional cell cancer (bladder), transitional cell cancer (renal-pelvis/-ureter), trophoblastic cancer, urethral cancer, urinary system cancer, uroplakins, uterine sarcoma, uterus cancer, vaginal cancer, vulva cancer, and Wilms' Cancer. It is preferred that the cancer to be staged and/or typed and/or whose outcome may be predicted and/or which may be treated is melanoma. Accordingly, in a preferred embodiment, the present invention relates to a method for staging and/or typing of melanoma, said method comprising the steps of detecting somatic alterations of the DNA of one or more DCC(s) obtained from one or more lymph node(s); and determining the somatic evolution of the DCC(s) based on the detected somatic alteration(s) in the DNA of the one or more DCC(s), wherein the somatic evolution of the DCC(s) is indicative of the stage/type of melanoma. In a further embodiment, the present invention relates to a method for treating melanoma, said method comprising the steps of detecting somatic alterations in the DNA of one or more DCC(s) obtained from lymph node(s) of a patient; determining the somatic evolution of the DCC(s) based on the detected somatic alteration(s) in the DNA of the one or more DCC(s); and determining the stage/type of melanoma of said patient based on the somatic evolution of melanoma determined based on the detected somatic alteration(s) in the DNA of the one or more DCC(s), wherein the stage/type of melanoma is used to initiate, continue or discontinue melanoma therapy. Furthermore, the present invention relates to a therapeutic intervention, preferably a pharmaceutical composition, for use in treating melanoma in a patient, wherein treatment is initiated, continued or discontinued based on the stage/type of melanoma, wherein said stage/type of melanoma is determined by detecting somatic alterations in the DNA of one or more DCC(s) obtained from lymph node(s) or liquor (preferably from lymph node(s), more preferably from a sentinel lymph node) of said patient; determining the somatic evolution of the DCC(s) based on the detected somatic alteration(s) in the DNA of the one or more DCC(s); and determining the stage/type of melanoma of said patient based on the somatic evolution of melanoma determined by detecting somatic alterations in the DNA of one or more DCC(s) obtained from lymph node(s) or liquor of said patient.

In a further preferred embodiment, the invention relates to a method for staging and/or typing of melanoma, said method comprising the steps of detecting somatic alterations of the DNA of one or more CTC(s) obtained from blood; and determining the somatic evolution of the CTC(s) based on the detected somatic alteration(s) in the DNA of the one or more CTC(s), wherein the somatic evolution of the CTC(s) is indicative of the stage/type of melanoma. As described herein above, for obtaining the CTCs from blood, in vivo CTC-capturing-devices may be used. In a further embodiment, the present invention relates to a method for treating melanoma, said method comprising the steps of detecting somatic alterations in the DNA of one or more CTC(s) obtained from blood of a patient; determining the somatic evolution of the CTC(s) based on the detected somatic alteration(s) in the DNA of the one or more CTC(s); and determining the stage/type of melanoma of said patient based on the somatic evolution of melanoma determined based on the detected somatic alteration(s) in the DNA of the one or more CTC(s), wherein the stage/type of melanoma is used to initiate, continue or discontinue melanoma therapy. Furthermore, the present invention relates to a pharmaceutical composition for use in treating melanoma in a patient, wherein treatment is initiated, continued or discontinued based on the stage/type of melanoma, wherein said stage/type of melanoma is determined by detecting somatic alterations in the DNA of one or more CTC(s) obtained from blood of said patient; determining the somatic evolution of the CTC(s) based on the detected somatic alteration(s) in the DNA of the one or more CTC(s); and determining the stage/type of melanoma of said patient based on the somatic evolution of melanoma determined by detecting somatic alterations in the DNA of one or more CTC(s) obtained from blood of said patient.

The methods of the present invention comprise a step of detecting somatic alterations of the DNA of one or more DCC(s) or CTC(s), respectively, obtained from a tissue sample, in particular a lymph node sample or bone marrow sample or blood. In this regard, the person skilled in the art is well-aware of methods suitable for detecting somatic alterations of DNA. As explained further above, somatic alterations may comprise epigenetic and/or genetic alterations.

With regard to epigenetic alterations, the person skilled in the art is well-aware of methods capable of detecting epigenetic alterations with regard to a reference sample or with regard to

a reference standard known in the art. As explained above, epigenetic alterations to be detected in the DNA of one or more DCC(s) may include methylation, in particular methylation of CpG(s), deficiencies of DNA repair proteins, and/or alterations in histone architecture or structure like methylation, acetylation, sumoylation, activating or inactivating histone marks and/or alterations in chromatin architecture like for example in eu- or hetero-chromatin and the like. Accordingly, in the methods of the present invention a step of DNA methylation analysis may be included. It is known that epigenetic mechanisms play important roles during normal development, aging and a variety of disease conditions. Hypermethylation of CpG islands located in the promoter regions of tumor suppressor genes is firmly established as a frequent mechanism for gene inactivation in cancers (Hansen et al. 2011. Nat. Genet. 43, 768-775). Methylation of the 5' carbon of cytosine is a form of epigenetic modification that does not affect the primary DNA sequence, but affects secondary interactions that play a critical role in the regulation of gene expression. Aberrant DNA methylation may suppress transcription and subsequently gene expression. Methylation analysis as in the methods of the present invention may comprise selective modification of the target DNA. Such modification may comprise the addition of methylation-dependent restriction enzymes (MDREs) or methylation-sensitive restriction enzymes (MSREs), preferably MDREs. Selective modification of the target DNA may also comprise addition of a chemical agent that is able to selectively differentiate between methylated or unmethylated nucleotides. In particular, methylation analysis as employed in the methods of the present invention may be able to selectively identify methylated cytosines that may later be read-out using methods known in the art, e.g. methods described in WO 2015/118077 or WO 2000/017390. For example, treatment with bisulfite is known to convert unmethylated cytosines (C) to uracil (U) while methylated cytosines are not converted (Frommer et al. 1992. Proc. Natl. Acad.Sci. USA 89, 1827-1831). Sequencing of DNA subsequent to treatment with bisulfite may be used to identify methylated nucleotides, in particular cytosines. Treatment with MDREs leads to methylation-dependent restriction of DNA fragments, while treatment with MSREs leads to methylation-dependent inhibition of restriction. Sequencing of DNA subsequent to MDRE/MSRE restriction in addition to MseI restriction may be used to identify methylated nucleotides, in particular cytosines.

Additionally or alternatively, one of the most commonly used techniques for the detection of epigenetic alterations in DNA organized in chromatin may be used, i.e. chromatin immunoprecipitation (ChIP) and related techniques such as ChIP-chip, ChIP-PET, ChIP-Seq,

MeDIP, DamID or the like; see Minard et al. (2009) *Genesis* 47(8), pp.559-72 and references cited therein. For example, methylation of DNA may genome-wide be detected using MeDIP, MeDIP-chip or MeDIP-seq. Exemplary protocols comprise steps of subjecting purified DNA to sonication to shear it into random fragments. The resulting fragments may range from 300 to 1000 base pairs (bp) in length, preferably between 400 and 600 bp. The DNA fragments are then denatured to produce single-stranded DNA. Following denaturation, the DNA is incubated with 5-methylcytosine (5-mC) antibodies available in the art. The classical immunoprecipitation technique is then applied: magnetic beads conjugated to anti-mouse-IgG are used to bind the anti-5mC antibodies, and unbound DNA is removed in the supernatant. To purify the DNA, proteinase K is added to digest the antibodies and release the DNA, which can be collected and prepared for DNA detection. Subsequent DNA detection is done using methods described further below for detection of genetic alterations. Further experimental protocols are described by Weber M, Davies JJ, Wittig D, et al. (August 2005) *Nat. Genet.* 37 (8): 853–62; Pomraning KR, Smith KM, Freitag M (March 2009) *Methods* 47 (3): 142–50; Wilson IM, et al. (2005) *Cell Cycle* 5 (2): 155–8; and Zhang X, Yazaki J, Sundaresan A, et al. (September 2006) *Cell* 126 (6): 1189–201. For an exemplary MeDIP-chip protocol, the following steps are applied: A fraction of the input DNA obtained after the sonication step above is labeled with cyanine-5 (Cy5; red) deoxy-cytosine-triphosphate while the methylated DNA, enriched after the immunoprecipitation step, is labeled with cyanine-3 (Cy3; green). The labeled DNA samples are cohybridized on a 2-channel, high-density genomic microarray to probe for presence and relative quantities. The purpose of this comparison is to identify sequences that show significant differences in hybridization levels, thereby confirming the sequence of interest is enriched, i.e. methylated. There are additional standard steps required in signal processing to correct for hybridization issues such as noise, as is the case with most array technologies. Additionally or alternatively, the MeDIP-seq approach may be applied, i.e. the coupling of MeDIP with next generation, short-read sequencing technologies such as 454 or Illumina sequencing. The high-throughput sequencing of the methylated DNA fragments produces a large number of short reads (36-50 bp or 400 bp, depending on the technology). The short reads are aligned to a reference genome using alignment software such as Mapping and Assembly with Quality (Maq) which uses a Bayesian approach, along with base and mapping qualities to model error probabilities for the alignments. The reads can then be extended to represent the ~400 to 700 bp fragments from the sonication step. The coverage of these extended reads can be used to estimate the methylation level of the region. A genome browser such as ENSEMBL can also be used to

visualize the data. Validation of the approach to assess quality and accuracy of the data can be done using, inter alia, quantitative PCR. This is done by comparing a sequence from the MeDIP sample against an unmethylated control sequence. The samples are then run on a gel and the band intensities are compared. The relative intensity serves as the guide for finding enrichment.

ChIP techniques may also be used to detect other epigenetic alterations, such as histone modifications or modifications relating to other proteins bound to DNA. Such techniques are known in the art and extensively described in various standard reference books, such as “Chromatin Immunoprecipitation Assays – Methods and Protocols” by Philippe Collas, Humana Press (2009). However, the person skilled in the art is well-aware that other techniques may also be applied to detect epigenetic alterations of DNA. Accordingly, in the methods of the present invention, any technique suitable for detecting such modifications, in particular epigenetic alterations, may be applied.

However, in the context of the present invention, it is preferred that methods for detecting epigenetic alterations be used that are compatible with methods for detecting genetic alterations, in particular that allow simultaneous detection of genetic and epigenetic alterations. Such methods are described in e.g. WO 2015/118077 or WO 2000/017390. However, alternative methods relying on DNA sequence information in order to detect epigenetic alterations are known to the person skilled in the art. For example, next generation sequencing techniques as marketed by Illumina[®] are known to the person skilled in the art and are suitable for detecting epigenetic alterations such as methylation, in particular combined with further techniques such as next-generation sequencing, in particular methylation sequencing; and/or for detecting protein-DNA interactions, techniques such as chromatin immunoprecipitation (ChIP) and/or ChIP combined with next-generation sequencing, in particular ChIP-Seq (for example using protocols available for Illumina[®] sequencing machines).

A preferred example of a method suitable for isolated and/or simultaneous detection of epigenetic and/or genetic alterations is a method comprising the steps of providing a sample comprising DNA, in particular a sample derived from one or more lymph node(s) or a bone marrow sample comprising one or more DCC(s) or CTC(s); adding an agent to said DNA that selectively recognizes epigenetically modified nucleic acids, in particular methylated nucleic acids, e.g. bisulfite; digesting the DNA with a restriction endonuclease under conditions

suitable to obtain DNA fragments of similar length, wherein said restriction endonuclease is capable of providing 5' overhangs, wherein the terminal nucleotide of the overhang is phosphorylated or, wherein said restriction endonuclease is capable of providing 3' overhangs, wherein the terminal nucleotide of the overhang is hydroxylated on said DNA fragments; annealing a first oligonucleotide to said DNA fragments, wherein a first sequence of said first oligonucleotide is complementary to the 5' or 3' overhang, respectively, of said DNA fragment, and a second sequence of said first oligonucleotide is complementary to a first sequence of a second oligonucleotide, wherein said second oligonucleotide comprises a second and a third sequence, wherein said second sequence of said second oligonucleotide comprises a randomized sequence; ligating said second oligonucleotide to said DNA fragment; filling in of the generated overhangs; amplifying said DNA fragments using a third oligonucleotide comprising a sequence binding to said third sequence of said second oligonucleotide; sequencing said amplified DNA fragments; and identifying methylated nucleic acid residues, wherein when bisulfite is used as agent that selectively recognizes epigenetically modified nucleic acids, a cytosine (C) corresponds to a methylated residue in said DNA sample and an uracil (U) corresponds to an unmethylated residue in said DNA sample. Alternative agents are known to the person skilled in the art in order to detect other epigenetic modifications than methylation, i.e. agents that specifically recognize epigenetically modified nucleic acids. The sequence information obtained in the last step of the above described method may be used to simultaneously detect genetic alterations. The obtained sequence information may, for example, be used in methods for DNA sequence analysis like whole genome sequencing, whole exome sequencing, whole regulome sequencing, sequencing-based methylation analysis, sequencing-based breakpoint detection, ChIP sequencing, or targeted sequencing and variations thereof.

As explained above, the methods of the present invention comprise a step of detecting somatic alterations of the DNA of one or more DCC(s) or CTC(s), respectively, obtained from a tissue or body fluid sample, in particular a lymph node sample or bone marrow sample or liquor (in the case of DCC(s)), or blood (in the case of CTC(s)), respectively. In this regard, the person skilled in the art is well-aware of methods suitable for detecting somatic alterations of DNA, in particular genetic alterations. An overview of suitable methods is given by Schwartz et al. (2013) JMB 425(21), pp-3914-8. Basically, with completion of the human genome research project, a reference sequence was established to which genetic alterations can be compared. That is, subsequent to sequencing DNA, the resulting sequence may be

compared to reference sequences known in the art. With regard to methods for sequencing DNA, any method may be employed within the context of the present invention. However, it is preferred to use methods which are fast, efficient, reliable and only require low input amounts. This is particularly important for detecting DCCs or CTCs in an early phase of cancer spread where only one single cell may be present in a tissue or body fluid sample, in particular a lymph node sample or bone marrow sample or liquor sample, or blood respectively. Thus, in order to detect genetic alterations sequencing methods known in the art may be employed that are particularly suitable for sequencing of low amounts of input DNA, e.g. as described in Mardis (2008) *Annu Rev Genomics Hum Genet* 9, 387-402. Preferably, error-free sequencing methods of low amounts of input DNA are used, as e.g. described in WO 2015/118077 or WO 2000/017390. Accordingly, a preferred method to be employed in the present invention is a DNA sequencing method comprising the use of endonucleases, oligonucleotides specifically recognizing overhangs created by the endonucleases and further oligonucleotides for amplification. The oligonucleotides may, for example, comprise barcode sequences in order to distinguish between fragments generated by the endonucleases and in order to generate an internal sequencing reference for error-free sequencing. An exemplary and preferred method for DNA sequencing comprises the steps of providing a sample comprising DNA, in particular a sample derived from one or more lymph node(s) or a bone marrow sample or a liquor or blood sample comprising one or more DCC(s) or CTC(s); digesting the DNA with a restriction endonuclease under conditions suitable to obtain DNA fragments of similar length, wherein said restriction endonuclease is capable of providing 5' overhangs, wherein the terminal nucleotide of the overhang is phosphorylated or, wherein said restriction endonuclease is capable of providing 3' overhangs, wherein the terminal nucleotide of the overhang is hydroxylated on said DNA fragments; annealing a first oligonucleotide to said DNA fragments, wherein a first sequence of said first oligonucleotide is complementary to the 5' or 3' overhang, respectively, of said DNA fragment, and a second sequence of said first oligonucleotide is complementary to a first sequence of a second oligonucleotide, wherein said second oligonucleotide comprises a second and a third sequence, wherein said second sequence of said second oligonucleotide comprises a randomized sequence; ligating said second oligonucleotide to said DNA fragment; filling in of the generated overhangs; amplifying said DNA fragments using a third oligonucleotide comprising a sequence binding to said third sequence of said second oligonucleotide; and sequencing said amplified DNA fragments.

Subsequent to detecting somatic alterations, in particular genetic and/or epigenetic alterations, of the DNA of one or more DCC(s) of one or more lymph node(s) or a bone marrow sample or the DNA of one or more CTC(s) from blood, the methods of the present invention comprise a step of determining the somatic evolution based on the detected somatic alterations. As described above, the somatic evolution is determined based on the accumulated somatic alterations during the lifetime of a cell, in particular a DCC or CTC, preferably comprising further parameters such as the DCCD/CTCD. Thus, based on the detected somatic alterations, in particular genetic and/or epigenetic alterations, the somatic evolution may be determined and the stage/type of the cancerous disease, in particular cancer, may be determined, treatment methods may be adjusted and the like. The person skilled in the art will appreciate how to express the determined stage/type of a cancerous disease, using, for example, letter codes, number codes or the like.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The methods and techniques of the present invention are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. See, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989) and Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publishing Associates (1992), and Harlow and Lane *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1990).

While the invention is illustrated and described in detail in the drawings and foregoing description, such illustration and description are to be considered illustrative or exemplary and not restrictive. It will be understood that changes and modifications may be made by

those of ordinary skill within the scope and spirit of the following claims. In particular, the present invention covers further embodiments with any combination of features from different embodiments described above and below.

The invention also covers all further features shown in the figures individually, although they may not have been described in the previous or following description. Also, single alternatives of the embodiments described in the figures and the description and single alternatives of features thereof can be disclaimed from the subject matter of the other aspect of the invention.

Furthermore, in the claims the word "comprising" does not exclude other elements or steps, and the indefinite article "a" or "an" does not exclude a plurality. A single unit may fulfill the functions of several features recited in the claims. The terms "essentially", "about", "approximately" and the like in connection with an attribute or a value particularly also define exactly the attribute or exactly the value, respectively. Any reference signs in the claims should not be construed as limiting the scope.

The present invention is also illustrated by the following figures.

Figure 1: Dissemination of melanoma cells as function of tumor thickness

(A) Staged function: estimated cumulative probability of dissemination as a function of tumor thickness (Turnbull) (n = 1027 patients). Continuous line: Weibull distribution incorporating a fraction of patients without long-term dissemination (95% CI lower dashed lines). Upper dashed line: only 63.5% of melanomas disseminate lymphatically (95% CI 53.5-73.4 %). Fifty percent of this value (31.75%) provides the median thickness (0.40 mm, 95% CI 0.04-0.75 mm) of disseminating melanomas (straight dashed line).

(B) Comparative analysis of histopathological and immunocytological lymph node halves. Displayed are representative examples with immunocytological scores of $DCCD \leq 100$, $100 < DCCD \leq 1000$ and $DCCD > 1000$. Samples LN 72 and LN 89 are stained against melan A LN 10, LN 135 and LN 168 against S100. LN 154 shows a highly pigmented melanoma in H&E staining.

(C) Percentage of DCC-positive patients (n = 525) with colonization ($DCCD > 100$) according

to the Turnbull estimate (staged line). The percentage of colonization (continuous curve, 95% CI dashed curves) is described by a cumulative Weibull distribution function (median 8.9 mm; 95% CI 6.8-14.3 mm).

(D) Hazard functions for dissemination, and colonization describing the instantaneous risk per unit thickness for an event (dissemination, colonization) for those tumors, for which it has not yet occurred.

(E) Survival analysis of melanoma patients ($n = 1027$) according to T-stage (T1: ≤ 1 mm; T2: 1.01-2.0 mm; T3: 2.01-4.0 mm; T4: >4 mm thickness).

Figure 2: gp100-positive cells from SLNs display multiple CNAs.

Ninety DCCs from the patient collective were selected according to QC criteria (see main text) for CGH and mutation analysis (see below). Histograms depict the genomic gains (light grey) or losses (dark grey) per cell and confirm malignant origin. The identifiers indicate cell ID.

Figure 3: Genetic comparison of DCCs and primary tumors

(A) Cluster analysis of paired primary tumors and DCCs for chromosomal aberrations (gain = +1; loss = -1). Only the ten most variable regions are included. Bottomline identifiers indicate patient ID, sample type (PT, primary tumor; DCC, disseminated cancer cell) and sample index; sideline labels indicate chromosomal regions. Black and white filled squares indicate examples of PT-DCC pairs for which several areas of the primary tumor were available. Examples of paired DCCs and PTs with varying PT-thickness are indicated by squares.

(B) Comparison of paired PTs and DCCs for chromosomal aberrations. Displayed are the 18 chromosomal regions that differ significantly (FDR-adjusted p -value ≤ 0.05) between paired PTs ($n=23$) and DCCs ($n=24$) regarding aberration frequency. Gains and losses are given in percent.

(C) Single cell WGA reliably captures wild type and mutated alleles. Exon 15 mutation c1799T>A (*BRAF*) and Exon 2 mutation c181C>A (*NRAS*) were detected in all single cells (lanes 1-15) of cell lines with *BRAF* (cell lines 70-61 and MelHo) or *NRAS* (cell line 102-4)

mutation. The allelic ratio of wt vs. mt alleles of each cell line is provided by pooled DNA. Note that this ratio is preserved in most single cells.

(D) Mutation analysis of *BRAF* and *NRAS* for paired PT-DCC samples (n=32 patients). Different mutations (either *NRAS* or *BRAF*) are indicated by mut1 and mut2. Fisher's exact test *p*-values indicate differences in *BRAF* mutational status between PT and DCC.

(E) Percentage of patients with homogeneous (all cells harboring the mutation) and heterogeneous *BRAF* *NRAS* mutational status among DCCs. DCCs were detected using two markers, gp100 or MCSP.

(F) Oncogenic mutations in *BRAF* and *NRAS* of paired PT-DCC-metastases triplets, pairs of PT-metastases or pairs of DCC-metastases. Squares indicate areas (PT and metastases) or individual cells (DCCs). Squares indicate areas (PT), individual cells (DCCs) or individual metastases (Met). Black squares indicate mutation was detected and white squares indicate wild type sequence.

Figure 4: Molecular analysis of DCCs at dissemination.

(A) Top: Samples with DCCD<24 were tested for genomic aberrations that can split DCCs into two groups according to the thickness of their matched PTs. Displayed are lowest (across chromosomal loci) FDR-adjusted *p*-values as a function of the limiting PT thickness that defines the low and high PT thickness groups. Low *p*-values (corresponding to high values of $-\log_{10}(p)$) would indicate thickness limits for which there is a significant difference in aberration frequency between DCCs from patients with thin and thick PTs. 5% significance is indicated by $-\log_{10}(0.05)=1.30$. Red points refer to all 31 cells. Blue clouds represent subsampling-based robustness estimates (Supplemental Methods). **Mid:** Individual adjusted *p*-values for the top two loci 18q21-q23 ($p=0.051$ [1.8mm] and 0.10 [1.7mm]) and 1q24-q44 ($p=0.26$ [0.8mm]) as a function of limiting PT thickness. **Bottom:** Aberration status (gain= +1, loss= -1) per cell for both loci listed according to sample PT thickness.

(B) Kaplan-Meier survival analysis of patients with DCCs that display loss (n = 5) or no loss (n=56) of 18q21-q23. .

(C) Upper: Thickness of thin (<1.8 mm) and thick (≥ 1.8 mm) tumors, from which DCCs were analyzed. Thin-thick splitting was performed according to the PT thickness 1.8 mm as

determined by Figure 4A. **Lower:** Number of chromosomal aberrations per cell in DCCs from thin and thick tumors, respectively.

Figure 5: Colonization-associated changes in DCCs.

(A) Samples were tested for genomic aberrations that can split DCCs into two groups according to patient DCCD. Displayed are lowest (across chromosomal loci) FDR-adjusted p-values as a function of the limiting DCCD that defines the low and high DCCD patient groups. Low p-values indicate DCCD limits for which there is a significant difference in aberration frequency between DCCs from patients with low and high DCCD. Red points refer to all 90 cells. Blue clouds are robustness estimates.

(B) Individual, adjusted p-values for all loci reaching 5% significance (indicated by $-\log_{10}(0.05)=1.30$) for at least one DCCD as a function of limiting DCCD (lowest $p = 0.0002$ for *BRAF*). In addition, *NRAS* is displayed (lowest $p = 0.29$).

(C) Aberration status (gain= +1, loss= -1) per cell for all panel B genetic loci plus *BRAF* and *NRAS* mutational status, listed according to increasing patient DCCD. For *BRAF* and *NRAS* aberration status +1 indicates mutation.

(D) Proliferation of DCCs in sentinel nodes. Immunofluorescence of Melan A⁺ cells in G0-, G1-, G2-phase and mitosis (from left to right). Nucleus, Melan A and Ki-67 are displayed.

Figure 6: Tumor-forming ability of DCCs before and after colonization and patient survival

(A) Left to right. Isolated MCSP⁺ DCCs from a patient-SLN: DCC-derived sphere; H&E-staining of a patient DCC-derived xenograft (DCC-PDX); DCC-PDX (7 s.c. injected DCCs).

(B) Side-by-side transplantation of paired MCSP⁺ DCCs and DCC-derived spheres from the same patient into NSG-mice. Left: Kaplan-Meier analysis of tumor-free mice ($p<0.0001$, log-rank test). Right: number of injected MCSP⁺ DCCs and DCC-derived spheres per injection site ($p=0.86$, Mann-Whitney U test). Black filled circles indicate tumor formation.

(C) Number of MCSP⁺ DCCs (DCCD>100: n=24 injection sites) or DCC-derived spheres (DCCD>100: n=12 injection sites; DCCD≤100: n=14 injection sites) that were transplanted into NSG-mice. Each circle represents one injection site. Black filled circles indicate tumor formation (engraftment). The p-value (Fisher's exact test) indicates a significant difference in

the engraftment rate for samples with DCCD>100 (pooled cells and spheres, n=36) vs. DCCD≤100 (spheres, n=14).

(D) Colonization signature (genetic loci identified in Figure 5 A-C), *BRAF* and *NRAS* mutational status of patient-derived DCCs and their respective xenografts.

(E) Left: Kaplan-Meier survival analysis of patients with DCCs that display at least one of the colonization signature changes (n=40) or not (wt, n=21) or. Right: Kaplan-Meier survival analysis of patients with DCCs that display *BRAF* mutation (*BRAF*mut, n=15) or wild type sequence (wt, n=46).

Figure 7: Model of melanoma progression from local to metastatic disease

Histologic appearance, patient-derived dissemination estimate, proliferation rate and *BRAF* mutational state are integrated into the scheme. Data are taken from this study and from references (Dong (2003) Cancer research 63, 3883-3885; Gimotty (2005) Journal of clinical oncology: official journal of the American Society of Clinical Oncology 23, 8048-8056; Verlinden (2014) Medicine 93, e285).

Figure 8 CGH profiles of 30 control cells isolated from blood, lymph node or bone marrow. Cells were identically isolated and amplified as gp100-positive cells. All chromosomes are depicted. In some cases, we used sex-mismatch control DNA to demonstrate successful hybridization. Colored bars indicate relative gains or losses of the X-chromosome (red, underrepresentation in test cells; green, overrepresentation in test cells) bars next to the ideogram. Chromosomal regions (centromeric regions) marked by gray bars are excluded from analysis as they contain repetitive regions. All cells displayed normal genomes.

Figure 9 Overview of analyzed patient samples

Our study focuses on melanoma patients in their earliest disease stages; therefore only non-metastasized (M0) patients with clinically node-negative disease were included. For

molecular analysis, samples were included according to availability or DNA-quality. Our criteria for selection of patients/cells for further genomic or functional analysis included:

- 1) Patients had gp100⁺ cells in their SLN.
- 2) Gp100+ DCCs were isolated.
- 3) DCC-derived DNA passed quality control for CGH analysis (see Polzer et al. 2014).
- 4) Paraffin-blocks of primary tumors (PTs) could be received from external dermatologists. (Note, that acquisition of primary tumors is extremely difficult as almost all patients are operated outside of the University hospital).
- 5) Sufficient material of PTs was left and not completely used for diagnosis.
- 6) DNA from many paraffin-embedded tissues passed the quality control.
- 7) Sufficient SLN material to allow short-term culture or xenotransplantations.
- 8) Follow-up was available and of sufficient length.

Figure 10 Microdissection of paraffin-embedded melanoma

Upper: H&E skin staining containing an area of primary melanoma. **Lower:** Same area after lasermicrodissection. Note the excised area of the melanoma.

Figure 11 Genetic comparison of DCCs and primary tumors. Cluster analysis of paired primary tumors (PT) and disseminated cancer cells (DCCs) for chromosomal aberrations (gain = +1; loss = -1). All variable regions are included. The identifiers indicate patient ID, sample type and sample index. Black and white filled squares indicate PT-DCC pairs of which several areas of the primary tumor were available.

Figure 12 Oncogenic mutations in *BRAF* and *NRAS* of paired primary tumors and DCCs. Squares indicate areas (PT) or individual cells (DCCs). Black filled squares indicate that the mutation was detected and white filled squares indicate wild type sequence. Blue and red filled squares indicate *NRAS*mut Q61K and *NRAS*mut Q61R, respectively. Grey lined squares indicate samples of which no sequence could be obtained.

Figure 13 (A) **Top:** Lowest FDR-adjusted p-values that identify genetic loci separating DCCs (n = 30) from patients with DCCD<19 into two groups according to observed PT thickness. **Mid:** Adjusted p-values for the top two loci 18q21-q23 (p = 0.065 [1.8mm] and 0.13 [1.7 mm]) and 1q24-q44 (p = 0.29 [0.8 mm]). The 5% significance level is indicated by $-\log_{10}(0.05) = 1.3$. **Bottom:** Aberration status (gain = 1, loss = -1) per cell for both loci listed according to PT thickness. (B) Same as (A) but for DCCD<32 (n=32). Top two loci: 18q21-q23 (p=0.083 [1.8 mm] and p=0.15 [1.7 mm]) and 10q23-q26 (p = 0.19 [2.3, 2.4mm]). (C) Same as (A) but for DCCD<95 (n= 40). Top two loci: 11q14-q23 (p = 0.082 [2.3, 2.4 mm]) and 1q24-q44 (p = 0.16) [0.8 mm].

Figure 14 **Xenotransplantation of single cells and spheres from melanoma cell lines.**

(A) groups of 3-5 spheres with or without matrigel or groups of 5 single cells with matrigel were s.c. transplanted into NSG-mice. The percentage of injection sites with tumor growth was determined. (B) Mice with s.c. injections of groups of 3-5 spheres plus matrigel or groups of 5 single cells plus matrigel were weekly palpated and the time-point of first palpation of the s.c. growing tumor was documented. p-values indicate statistical significance (log-rank test). Numbers of injection sites are given in the figures. (C) Confirmation of patient-origin of spheres/xenografts by STR analysis. All 4/7 xenografts are shown. Xenograft from LN 154 was erroneously FFPE-fixed and could not be analysed thereafter. Tri- as well as quattro-allelic patterns at D21S11, D16S538 and vWA loci indicate repeat number at these loci. n.d. = not detectable

Figure 15 **Colonization of lymph nodes is associated with functional impairment of CD8 T cells.**

(A) Lymph nodes were analyzed by flowcytometry and the percentage of antigen-experienced (CD45RA-CCR7+/-) CD8 T cells in lymph nodes of melanoma was determined as a function of DCCD of the LN. The number of antigen-experienced CD8 T cells increases with increasing DCCD (depicted on a log-scale). (B) Lymph nodes were analyzed by flowcytometry and the percentage of PD-1 and Tim-3 expressing CD8 T cells in lymph nodes of melanoma patients was determined as a function of DCCD of the lymph node. Lymph nodes from healthy controls (patients without cancer) were used as controls. The y-axis cut-

off was set according to the highest percentage of PD-1 high expressing CD8 T cells in control lymph nodes. At a DCCD ≥ 2000 a significantly increased number of samples showed a higher percentage of PD-1 high expressing CD8 T cells than in healthy controls (y-axis cut off; $p < 0.004$, fisher's exact test). The flowcytometric dot plots illustrate representative examples of PD-1 and Tim-3 expression in lymph nodes with a DCCD < 2000 and a DCCD ≥ 2000 (C) Single cell suspensions of lymph nodes were stimulated with PMA/ionomycin and the percentage of TNF and IFN γ expressing PD-1 high and PD-1 intermediate expressing CD8 T cells and their respective median fluorescence intensity (MFI) for TNF and IFN γ was determined by flowcytometry. PD-1 high cells show a reduced percentage of IFN γ and TNF producing cells as well as a reduced MFI for both cytokines. Unstimulated CD8 T cells were used as control and were found to be negative for TNF and IFN γ production. (D) The IFN γ or TNF score combines the ratio of the percentage of PD-1 intermediate plus negative CD8 T cells to PD-1 high CD8 T cells multiplied with the ratio of the percentage of cytokine producing PD-1 high to PD-1 intermediate CD8 T cells and the ratio of the median cytokine fluorescence intensity of cytokine expressing PD-1 high CD8 T cells to the median fluorescence intensity of PD-1 intermediate expressing cells. IFN γ score = ratio % CD8 PD-1 int+neg/%PD-1 high x % IFN γ PD-1 high/% IFN γ PD-1 int cells x ratio MFI IFN γ + cells of PD1hi/ MFI IFN γ + cells of PD-1 int CD8 T cells. The lower the score the more PD-1 high CD8 T cells with reduced IFN γ or TNF secretion are present. Lymph nodes from healthy controls (patients without cancer) were used as a control.

Figure 16 Colonization is associated with an increase in MDSCs and a decrease in cytolytic NK cell numbers

(A) The percentage of MDSCs in the lymph nodes (n=39) of melanoma patients was determined by flowcytometry with regard to the respective DCCD (on a log-scale). Lymph nodes from healthy controls (two patients without cancer) are included. The y-axis cut-off was set according to the highest percentage of MDSCs in lymph nodes with a DCCD of zero (on a log-scale for DCCD+1=1), as the number of control lymph nodes was too low. At a DCCD ≥ 2000 a significantly increased number of samples showed a higher percentage of MDSCs in lymph nodes with a DCCD of zero (y-axis cut off; $p < 0.007$, fisher's exact test). (B) Analysis of NK cells in several lymph nodes of one patient. The respective DCCD of each lymph node is given and the ratio of the percentage of CD56^{bright} over cytolytic CD56^{dim} cells

is shown. A decrease in the ratio indicates a reduction in the percentage of cytolytic CD56^{dim} cells versus non-cytolytic CD56^{bright} NK cells.

The present invention is additionally described by way of the following illustrative non-limiting examples that provide a better understanding of the present invention and of its many advantages. The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques used in the present invention to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should appreciate, in light of the present disclosure, that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

A number of documents including patent applications, manufacturer's manuals and scientific publications are cited herein. The disclosure of these documents, while not considered relevant for the patentability of this invention, is herewith incorporated by reference in its entirety. More specifically, all referenced documents are incorporated by reference to the same extent as if each individual document was specifically and individually indicated to be incorporated by reference.

Example 1 Tumor thickness when cells disseminate and form colonies

It was investigated at what tumor thickness melanomas disseminate to the sentinel lymph node(s) (SLN) in patients with clinically node-negative disease as assessed by palpation and ultrasound. A highly sensitive and quantitative gp100-based detection method for single melanoma cells in sentinel nodes (Ulmer *et al.* (2005), Clin Cancer Res. 11, 5425-5432) was applied in a prospective study on 1027 melanoma patients (Ulmer *et al.* (2014) PLoS Med. 11:e1001604. Of these, 51% harbored gp100-positive cells (Ulmer *et al.* (2014) PLoS Med. 11:e1001604, whereas not a single gp100-positive cell among 70 control samples (average number of cells screened per patient 2.3×10^6). Upon comparative genomic hybridization

(CGH) analysis it had been seen that 98% of randomly selected gp100-positive cells from lymph nodes harbor copy number alterations (Ulmer *et al.* (2014) PLoS Med. 11:e1001604). For control, 30 single leukocytes were isolated and CGH analysis was performed with none of the control cells displaying any aberration ($p < 0.0001$; Fisher's exact test; Figure 8).

There was a weak positive correlation (Spearman's $\rho = 0.18$, $p < 0.0001$, $n = 1027$) between primary tumor thickness and disseminated cancer cell density (DCCD; defined as the number of gp100-positive cells per million cells in disaggregated lymph nodes). The percentage of patients with gp100-positive lymph nodes increased only marginally from T1 (≤ 1 mm) to T4 (> 4 mm) tumors (T1: 45.8 %, T2: 47.4 %, T3: 54.9 %, T4: 59.4 %), suggesting that dissemination occurs preferentially early. The Turnbull method and a Weibull function were used (see Supplemental Information) to determine thickness at dissemination. These analyses revealed that lymphatic dissemination was restricted to 63.5% of all patients and that in 50% of cases cancer cell spread had occurred before tumors reached a thickness of 0.4 mm (95% CI 0.04-0.75 mm) (Figure 1A). In sum, these data show that $\sim 1/3$ of melanomas disseminate lymphatically at a tumor thickness of < 0.4 mm, $\sim 1/3$ at a thickness ≥ 0.4 mm, and $\sim 1/3$ are not capable of lymphatic spread.

It was then investigated at what tumor thickness disseminated melanoma cells would have grown to a colony in SLN. To establish a DCCD representative for colonization as compared to early dissemination (first arrival) the two halves of a patient's lymph node were compared. In all cases, had lymph nodes were split and one half was analyzed by histopathology (preserving the architecture) and the other by gp100 immunocytology after disaggregation (destroying the architecture but enabling quantification; for details, see (Ulmer *et al.* (2014) PLoS Med. 11:e1001604). Then the DCCD was compared to histomorphological appearance of the corresponding section. Samples with a $DCCD \leq 100$ displayed spotted single cells or small nests of cells in the outer lymphatic sinus, if melanoma cells were detected at all, while at $DCCD > 100$ melanoma cells usually expanded into inner zones of lymph nodes (Figure 1B). Thus, colonization had probably occurred at a DCCD of ~ 100 whereas samples with lower DCCD comprise melanoma cells before colony outgrowth. Using a DCCD of 100 as a definition for colony formation it was found that the median tumor thickness of patients with a colonized lymph node was 22 times higher than that at seeding ($DCCD \leq 100$; Figure 1C; 8.9 mm; 95% CI 6.8 to 14.3 mm). The risk of *de novo* tumor seeding steadily decreased as tumors grew, while the risk of *de novo* colonization increased (Figure 1D).

Example 2 Dissemination and survival

To explore how the marginal difference (13.6%) in dissemination rates between thin T1 and thick T4 tumors is linked to survival, it was determined how many patients had died during the median follow-up period of 49 months (range 3 to 123 months), with 370 (36%) patients having a follow-up of ≥ 5 years. Although 38/83 (46%) of T1 stage melanomas harbored DCCs in the sentinel, only one patient died, consistent with previous studies (Balch *et al.* (2009), JCO 27, 6199-6206); Leiter *et al.*, (2004) JCO 22, 3660-3667). In contrast 47/133 (35%) patients with T4 melanomas died (Figure 1E; 9-year survival 88.9% for T1, and 45.9% for T4; $p < 0.0001$, log-rank test). Thus, there is a discrepancy between T1 and T4 melanomas regarding seeding and death.

To address this difference, the genomic evolution of disseminating melanoma cells in a subset of 61 patients was assessed. To focus on melanoma patients in their earliest disease stages, only patients with clinically node-negative disease (no lymph node involvement by palpation and ultrasound) and no distant metastasis were included. From these patients samples for molecular analysis were taken according to availability or DNA-quality. Specifically, the selection criteria for patients and cells to be subjected to genomic and functional analysis were: (i) successful isolation and whole genome amplification of gp100+ DCCs, (ii) DCC-derived DNA passed quality control for comprehensive genomic analysis (Polzer *et al.* (2014) EMBO Mol Med. 6:1371-138), and (iii) sufficient follow-up time for survival analysis. The clinical baseline characteristics of these patients are provided in Table 1 (for further details on clinical sample acquisition see Figure 9). From these patients, 90 individual cells were obtained and c their malignant origin was confirmed by CGH (Figure 2). These DCCs displayed a large range of copy number variations ranging from 1 to 52 per cell (median = 14; interquartile range = 14.8). It was noted that genomic gains per cell (median = 9; range = 0 to 39) were more frequent than losses (median 3.5; range = 0 to 21).

Example 3 Genetic lineages of primary tumors and DCCs

The standard approach to addressing outcome-associated differences between T1 and T4 melanomas employs primary tumor tissue. It assumes that the molecular characteristics of metastasis-initiating DCCs can be identified within the primary tumor, because primary

tumors and DCCs are thought to be largely identical. To test this assumption, the genomic profiles of primary tumors and their matched DCCs were investigated.

Primary tumors were isolated by laser microdissection (Figure 10) and whenever possible several areas were analyzed. However, compared to other cancers such as kidney cancer (Gerlinger *et al.*, (2012) *The New England journal of medicine* 366:883-892), early stage melanomas are very small, mostly precluding the assessment of subclones from different areas. Microdissected primary tumor samples (n=23, 19 patients) and micromanipulator-isolated single DCCs (n=24, 19 patients) were analyzed by CGH. Regardless of melanoma thickness, there was a striking disparity between primary tumors and matched DCCs. Unexpectedly, primary tumors from different individuals clustered closer together than individual pairs of primary tumors and their matched DCCs (Figure 3A and Figure 11). Primary tumors contained significantly more deletions than DCCs (Figure 3B; $p=0.003$, Mann-Whitney U test) while the corresponding difference in gains was clearly non-significant ($p=0.66$, Mann-Whitney U test). When several areas from the same primary tumors were available, genomic heterogeneity was noted, while still clustering together (for example T28, T30 in Figure 3 and Figure 11) apart from their paired DCCs. These data show that DCCs disseminated before most deletions occurred and that unrelated melanomas growing in the skin converge on similar chromosomal losses.

Since *BRAF* and *NRAS* mutations are frequent (in respectively 40% and 21% of cases on average) in melanoma (Platz *et al.*, (2008) *Mol Oncol* 1:395-405), it was investigated whether these mutations are transmitted from the primary tumor to DCCs. Both alleles (wild type and mutant) could reliably be retrieved from single cells with heterozygous *BRAF* and *NRAS* mutations (Figure 3C). In paired patient samples, *BRAF* was mutated more frequently in primary tumors (34%) than in DCCs (15%; $p=0.012$, Fisher's exact test; n=32 patients; Figure 3D and Figure 12), whereas no significant difference was observed for *NRAS* mutations (15% mutated primary tumors and 11% DCCs; $p=0.58$; n=29 patients). For these two oncogenes, a shared wild type in 47%, a shared mutated status in 16%, and disparate mutational states in 37% of cases (Figure 3D) was found. Among patients with mutated primary tumors, matched DCCs were mostly not sharing these mutations (shared in 3/11 for *BRAF* and 3/6 for *NRAS*), indicating that they had disseminated before fixation within the primary site.

BRAF and *NRAS* mutations have been suggested to initiate melanoma (Shain, *et al.*, (2015) *The New England Journal of Medicine* 373, 1926-1936) and consequently to be fully clonal. We therefore sequenced individual DCCs from patients with *BRAF* or *NRAS* mutant gp100⁺

DCCs where we had isolated more than one DCC and tested if all sibling cells harbor the mutation. We found that gp100⁺ DCCs are heterogeneous in 45% and 80% for *BRAF* and *NRAS* mutations, respectively (Figure 3E). To rule out a selective effect of the detection marker gp100, additional MCSP⁺ melanoma DCCs we analyzed and similar results were obtained. These findings were compared with control cells (Figure 3C) and it was noted that the expected mutant allele was not detected in a significant number of gp100⁺ DCCs (*BRAF* (n=43 cells): one-sided Fisher's exact test p=0.02; *NRAS* (n=44): p<0.0001) and MCSP⁺ DCCs (*BRAF* (n=61): p=0.003; *NRAS* (n=30): p=0.02). Finally, when the mutational state for primary tumor-DCC-metastases triplets or pairs of primary tumors-metastases or pairs of DCC-metastases was compared, it was found that DCCs with and without *BRAF/NRAS* mutations were able to form manifest metastases (Figure 3F).

In summary, both copy number alterations and targeted mutation analysis demonstrated that primary melanomas and their paired DCCs are largely genetically disparate implying early evolutionary branching.

Example 4 Molecular characteristics of DCCs at early lymphatic arrival

DCCs from thick melanomas could harbor characteristic alterations responsible for the prognostic relevance of high T stage, which are absent in DCCs from thin melanomas. Therefore, a focus was set on DCCs before evident lymph node colonization as being representative of the time of arrival and their genomic profile was investigated.

Colony formation became clearly apparent at a DCCD>100 (Figure 1B). To determine a DCCD representative for the time of arrival, i.e. clearly before colony formation, all patient samples with DCCD values below 100 were tested to define a thickness threshold at which genetic alterations characteristic for DCCs from "thick" (defined by the tested threshold) could be identified. In other words, for all samples available at a tested DCCD ranging from 1-99, it was searched for genomic aberrations that could split DCCs into two groups characterized by origin no statistical differences (p>0.05, Fisher's exact test) could be found for any tumor thickness. The lowest p-value (p=0.051; loss of 18q21-23; Figure 4A, Figure 13) was obtained for a DCCD of 24 and a thickness of 1.8 mm (Figure 4A, upper), suggesting a DCCD of up to 24 that defines early arriving DCCs and 1.8 mm indicating the thickness at which melanoma cells had acquired loss of 18q21.23 within the primary site. However,

survival did not correlate with loss of 18q21-23 (Figure 4B), indicating that this change is unrelated to the impact of T stage on survival. It was also noted that the number of copy number alterations (CNAs) did not differ between DCCs arriving from thick and thin melanomas (Figure 4C).

Example 5 Molecular characteristics of colonizing DCCs

These data are difficult to reconcile with a model in which dissemination occurs late and genetic aberrations acquired at high tumor thickness render DCCs more metastatic. It was therefore considered the possibility that DCCs that disseminate early to the SLN, are genetically “immature”, and acquire further genetic alterations during metastatic colony formation. Thus, the genetic alterations that mark the transition from immature DCCs to colony-forming DCCs in the sentinel node was investigated. It was analyzed at which DCCD DCCs could be classified in two groups according to their genetic alterations. The greatest significance ($p < 0.001$, Fisher’s exact test) was achieved for $77 \leq \text{DCCD} \leq 95$ (Figure 5A), close to the DCCD of 100, at which colonization became evident in histopathological analyses (Figure 1B). DCCs acquired three genetic alterations including *BRAF* mutations, loss of chromosome 9p11-13 and loss of chromosome 9p21-24, which comprises the melanoma suppressor gene, *p16* (Figure 5B). Strikingly, *BRAF* mutations were observed in 1/43 (2%) cells with $\text{DCCD} < 95$ and in 20/47 cells (42%) for $\text{DCCD} \geq 95$ (Figure 5C; $p < 0.0001$). Two additional significant splits were observed: at $\text{DCCD} = 3$, involving chromosome Xq25-28 and at $\text{DCCD} = 19$, involving 7q21-36. While Xq25-28 was frequently altered in a non-directional way, i.e. either gain or loss, in DCCs from patients with $\text{DCCD} < 3$ (Figure 5C), samples with a $\text{DCCD} \geq 19$ enriched the amplification (gain) of 7q21-36 (Figure 5B, C), which harbors the *MET* oncogene.

Since acquisition of genetic alterations often occurs during cell division, labeling indices for the proliferation marker Ki-67 in sentinel node DCCs from patients with $\text{DCCD} \leq 100$ and $\text{DCCD} > 100$ were compared, i.e. before and after colony formation (Figure 5D). As double staining of MIB-1 (anti-Ki-67) and HMB45 (anti-gp100) was unsuccessful, gp100 was replaced with the melanoma-associated marker MelanA (Ulmer *et al.* (2005), Clin Cancer Res. 11, 5425-5432). Ki-67 expression was assessed in 37 nodes with $\text{DCCD} \leq 100$ (Group A) and 21 nodes with $\text{DCCD} > 100$ (Group B) involving evaluation of 937 cells in total. In Group A 11.4% of cells were clearly mitotic, whilst in Group B 22.0% were dividing ($p = 0.0005$;

Fisher's exact test, Figure 5D). Thus, a basal proliferation rate of 11% may initially enable acquisition of genetic alterations, which subsequently accelerate outgrowth.

Example 6 Colonization-associated alterations, xenotransplantation and patient survival

Cancer cells forming a lymph node colony displayed a characteristic signature of alterations. To test whether DCCs have tumor-initiating ability, they were transplanted into NSG-mice. First conditions for xenotransplantation of rare melanoma cells were evaluated (Quintana (2008) Nature 456, 593-598). For cell line cells and patient DCCs two approaches were compared: direct transplantation of groups of DCCs and transplantation of DCC-spheres after brief culture under melanosphere conditions. Melanospheres formed tumors in immunodeficient NSG mice more frequently than groups of single cells ($p < 0.0001$, log-rank test; Figure 6A, B and Figure 14). The applied conditions supported growth from as few as one transplanted sphere (Figure 6B) or a group size of 7 DCCs (Figure 6C). Therefore, when the tumor-initiating ability of DCCs from SLNs was compared with a $DCCD \leq 100$ to those with a $DCCD > 100$, spheres from samples with a $DCCD \leq 100$ and spheres or groups of single DCCs from samples with a $DCCD > 100$ were transplanted. The number of spheres per injected site was similar for both, $DCCD \leq 100$ and > 100 ($p = 0.27$, Mann-Whitney U test; Figure 6C). Strikingly, it was found that $DCCD > 100$ was predictive for successful xenotransplantation (9/36 transplantations gave rise to tumors in 4/7 patients; Figure 6C) while samples with $DCCD \leq 100$ never established tumors (0/14 injection sites in 0/5 patients). Genetic fingerprinting confirmed patient origin in all cases (Figure 14). Furthermore, in all patient-derived xenografts either *BRAF* mutation, loss of 9p11-13 or 9p21-24, or gain of 7q21-36 was present (Figure 6D). In one case, successful outgrowth was linked to the presence of a *NRAS* mutation.

Finally, it was investigated whether the genetic aberrations associated with colony-formation in the SLN or with tumor formation in mice were associated with clinical outcome. For this, we tested whether *BRAF* mutation, loss of 9p11-13/9p21-24, gain of 7q21-36, or *NRAS* mutation in single DCCs increased the risk for death. Indeed, this combined colonization and engraftment signature was present in 8/9 (89%) patients dying from melanoma ($p = 0.048$, log-rank test; Figure 6E), with *BRAF* mutation being the most relevant single indicator ($p = 0.031$).

Example 7

Colonization-associated alterations of the immune cell microenvironment

Flowcytometric analysis of lymph node suspensions showed a correlation (Spearman's $\rho = 0.58$, $p < 0.002$) between the percentage of antigen-experienced CD8 lymphocytes and the DCCD indicating the presence of a tumor-cell related CD8 T cell response (Figure 15A). However, detailed analysis of the phenotype of CD8 T cells with regard to the DCCD of the respective lymph node revealed a significantly increased percentage of PD-1 high expressing CD8 T cells at a DCCD ≥ 2000 (Figure 15B; $p < 0.0004$, fisher's exact test, $n=50$ lymph nodes from melanoma patients, 6 control lymph nodes from healthy controls). PD-1 is induced on T cells in response to T cell activation, but is down-regulated once the immune response has eliminated the antigen. If the antigen does not vanish, as is the case during chronic viral infections, PD-1 is not downregulated and T cells lose effector functions in a hierarchical manner: IL-2 production, high proliferative capacity and cytolytic activity are lost first, followed by impaired cytokine production. In this regard, CD8 T cells expressing high levels of PD-1 are considered as terminally exhausted T cells. These cells co-express Tim-3 (Figure 15B), another marker of exhausted T cells and have an impaired ability for cytokine production. Furthermore, the percentage of cells that are able to produce IFN γ and TNF was reduced in the PD-1 high population as compared to the PD-1 intermediate expressing population, demonstrating that PD-1 high cells are exhausted CD8 T cells (Figure 15C). Analysis of 50 lymph nodes from 26 melanoma and 6 lymph nodes from 6 non-tumor patients, showed that the score for IFN γ and TNF was significantly decreased at a DCCD ≥ 2000 (Figure 5D; both $p < 0.0001$, fisher's exact test). The IFN γ or TNF score combines the ratio of the percentage of PD-1 intermediate and negative CD8 T cells to PD-1 high CD8 T cells multiplied with the ratio of the percentage of cytokine producing PD-1 high to PD-1 intermediate CD8 T cells and the ratio of the median cytokine fluorescence intensity of cytokine expressing PD-1 high CD8 T cells over the median fluorescence intensity of PD-1 intermediate expressing cells. A decrease in the score indicates an increased number of exhausted CD8 T cells and consequently a loss of CD8 T cell function. The presence of PD-1 high CD8 T cells was directly linked to the local number of DCCs in the lymph node as several lymph nodes of the same patient with a DCCD < 2000 did not show this increase in exhausted CD8 T cells (Figure 15E). In addition to the loss of CD8 T cell function, the local functional impairment of anti-tumoral immune responses was reflected at the level of recruitment of immune suppressive immature MDSCs (Figure 16A) and loss of cytolytic NK

cell function (Figure 16B). Specifically a significantly increased percentage of immature MDSCs was observed in lymph nodes with a DCCD ≥ 2000 (Figure 16A; $p < 0.007$, fisher's exact test, $n=39$ lymph nodes from melanoma patients, 2 control lymph nodes from healthy controls). As for the loss of CD8 T cell function, the loss of cytolytic NK cell function evident by a decrease in the ratio of non-cytolytic CD56bright to cytolytic CD56dim cells, was directly linked to the local DCCD in the respective lymph node, as revealed by side-by-side comparison of several lymph nodes of one patient. In summary these data show that the observed changes in the CD8 T cell and NK cell function as well as the recruitment of MDSCs resemble a local reaction to the local tumor cell load.

Methods

Patients

We used data from 1027 melanoma patients from Tübingen with clinically node negative (as assessed by palpation and ultra-sound) melanoma who underwent sentinel node biopsy to describe the association between thickness and melanoma spread (Ulmer (2014) PLoS Med 11, e1001604). Molecular studies and *BRAF/NRAS* mutational survival analysis included patients recruited in Tübingen and Regensburg. Informed written consent was obtained from all patients. The study was approved by the ethics committees of the Universities Tübingen (ethics vote number 5/99) and Regensburg (07-079).

For assessment of microenvironmental changes data was obtained from patients from Regensburg who underwent complete lymph node dissection.

Cell lines

The melanoma cell lines A375 and MelHo were used (obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell cultures). MelHo is listed in the ICLAC-database for mis-identified cell lines due to unclear patient-origin, but was used as the cell line is heterogenic for the exon 15 mutation c1799T>A (*BRAF*). The origin of the cell lines was verified by short tandem repeat (STR) analysis (Cell-ID™, Promega). The cell lines 70-61 and 102-4 were developed from DCC-derived xenografts and are exon 15 mutation c1799T>A (*BRAF*) and exon 3 mutation c181C>A (*NRAS*) mutated, respectively, as determined by Sanger sequencing (Sequissime, Vaterstetten, Germany). Their patient-origin was verified by short tandem repeat (STR) analysis (Cell-ID™, Promega), their melanoma-origin by a human pathologist and their aberrant genotype by CGH. A375 and MelHo were

maintained in DMEM, 10%FCS, 0.5 x Pen/Strep; 102-4 and 70-61 in RPMI, 10% FCS, 0.5xPen/Strep. All cell lines were routinely tested for mycoplasma and were found to be negative.

Controls

Control lymph nodes (n = 70) were obtained from 60 non-melanoma patients (47 skin-draining nodes from non-malignant conditions, 6 sentinel nodes from non-melanoma skin cancer patients, and 17 nodes from non-small cell lung cancer patients) and disaggregated, stained and evaluated identically to the melanoma-derived lymph nodes. After screening 2×10^6 lymphocytes, the control lymph node status was revealed to the observer, and screening of the samples was continued until completion, unlike to the melanoma patient samples.

Lymph node disaggregation and immunocytology

Quantitative immunocytology was performed as described (Ulmer (2014) PLoS Med 11, e1001604 and Ulmer (2005) Clinical cancer research : an official journal of the American Association for Cancer Research 11, 5425-5432) after sentinel node biopsy using unfixed lymph node tissue. Briefly, the lymphatic tissue was cut into 1-mm pieces and disaggregated mechanically into a single-cell suspension by rotating knives (DAKO Medimachine, DAKO), washed with HBSS (Life Technologies, Heidelberg, Germany) and centrifuged on a density gradient made of a 60% Percoll solution (Amersham, Uppsala, Sweden). Cells were counted using a Neubauer counting chamber. Per slide, 10^6 cells from the interphase were then given onto adhesion slides (Menzel, Braunschweig, Germany) in a volume of 1 mL PBS. After sedimentation for 1 hour, the slides were air-dried overnight. Immunocytological staining was carried out with the alkaline phosphatase/anti-alkaline phosphatase method using primary antibodies against gp100 (HMB45, DAKO) and as primary antibody and 5-bromo-4-chloro-3-indolyl phosphate/NBT (DAKO) as substrate, yielding a blue reaction product. A lymph node was defined as gp100 positive if it contained at least one gp100-positive cell. The number of positive cells per million lymphocytes was recorded. Positive samples were stored for a maximum of 4 days in PBS at 4 °C until cell isolation for whole genome amplification. For the isolation of living DCCs, single cells were stained with an anti-human MCSP (melanoma

chondroitin sulfate proteoglycan, clone 9.2.27, BD Pharmingen) according to the manufacturers recommendations and detected by indirect immunofluorescence (goat anti-mouse-Cy3, Jackson). After washing, MCSP⁺ cells were isolated using a micromanipulator (Eppendorf PatchMan NP2) and transplanted.

Flowcytometric assessment of microenvironmental changes

Single cells of disaggregated lymph nodes were stained with viability dye eFlour 780 (ebioscience) for live/dead cell discrimination. To reduce non-specific binding single cell suspensions were incubated for 10 min at 4°C with PBS/10% AB-serum (Bio-Rad, subsequently stained with fluorescence-labeled antibodies for 30 min at 4°C, washed two times with PBS/2% FCS/0.01% NaN₃ and fixed with Fluoro-Fix buffer (Biolegend). Cells were analyzed on a LSR II machine equipped with FACS DIVA 5.03 software (BD Bioscience,) and data was analyzed with FloJo 8.8.6 (Treestar). The cells were stained using the following antibodies (Biolegend). For MDSC identification: CD45 (HI30), CD3 (HIT3a), CD19 (HIB19), CD56 (HDC56), HLA-DR (L243), CD33 (WM53), CD11b (ICRF44). For NK cell identification: CD45 (HI30), CD3 (Sk7), CD56 (HDC56), CD161 (HP-3610). For CD8 T cell identification: CD45 (HI30), CD3 (Sk7), CD8 (HIT8a), CD45RA (HI100), CCR7 (G043H7), PD-1 (EH12.2H7), Tim-3 (F38-2E3), TNF (Mab11), IFN γ (4S.B3).

For measurement of IFN γ and TNF production single cells of disaggregated lymph nodes were stimulated for 4 hours with PMA (10ng/ml, Sigma) and ionophore (1 μ g/ml, Sigma) in the presence of 1xBrefeldin A (Biolegend). Single cells of disaggregated lymph nodes were stained with viability dye eFlour 780 (ebioscience) for live/dead cell discrimination. To reduce non-specific binding single cell suspensions were incubated for 10 min at 4°C with PBS/10% AB-serum (Bio-Rad), subsequently stained with fluorescence-labeled antibodies for 30 min at 4°C, washed two times with PBS/2% FCS/0.01% NaN₃, fixed and permeabilized with Fixation buffer (Biolegend) and Perm Wash Buffer (Biolegend), respectively. To reduce non-specific binding single cell suspensions were incubated again for 10 min at 4°C with PBS/10% AB-serum and subsequently stained with fluorescence-labeled antibodies for IFN γ and TNF in Perm Wash Buffer for 30 min at 4°C and washed two times with Perm Wash buffer.

Comparison between single cells and sphere transplantations

Single cells of disaggregated sentinel lymph nodes were plated in 6 cm poly-HEMA (12mg/mL, Sigma-Aldrich) coated cell culture plates (Sigma-Aldrich) at a density of 200,000 viable cells/mL. Cells were grown in a serum-free DMEM/Ham's F12 basal medium (PAN Biotech GmbH), supplemented with 0.5 x Pen/Strep (PAN Biotech GmbH), 0.5 % BSA (VWR-Biochemical), 10 µg/mL insulin (Sigma-Aldrich), 10 nM HEPES (Sigma-Aldrich), 1 x B27 (Life Technology GmbH), 10 ng/mL EGF (Sigma-Aldrich) and 10 ng/mL bFGF (Sigma-Aldrich), 4 µg/mL heparin (Sigma-Aldrich), 5 ng/mL GRO- α (R&D Systems), 20 ng/mL HIL-6 (kindly provided by S. Rose-John) and 0.2% Methylcellulose (Sigma-Aldrich). Cultures were incubated at 37 °C and 5% CO₂ and 7% O₂. Sphere growth was weekly monitored. To generate spheres from melanoma cell lines (MelHo, A375 maintained in DMEM, 10%FCS, 0.5 x Pen/Strep), single cells were plated at a density of 10,000 viable cells/mL on poly-HEMA coated cell culture plates in the same medium as for sentinel lymph node cells, but without HIL-6 and GRO- α . Spheres were isolated manually.

Xenotransplantation

Spheres or MCSP⁺ cells from disaggregated sentinel lymph nodes were collected using a micropipettor or micromanipulator and pooled in a microwell (volume 10-15 µl, Terasaki). Microwells were pre-coated over night with 12 mg/mL poly-HEMA (Sigma-Aldrich) at RT. Single cells were transplanted in a final volume of 30 µl and 25% high-concentration matrigel (BD Biosciences) as published before (Quintana (2008) Nature 456, 593-598). Cells were injected with an insulin syringe (Microfine, 29G, U-50, BD Biosciences) sub-cutaneously into NOD.Cg-*Prkdc*^{scid} *IL2r γ* ^{tmWjl/Sz} (NSG, 6-8 weeks old, males and females). Mice were purchased from the Jackson Laboratory and maintained under specific-pathogen free conditions, with acidified water and food ad libitum in the research animal facilities of the University of Regensburg, Germany. All approved experimental animal procedures were conducted to German federal and state regulations. Mice were palpated every week at the site of injection. Melanoma-origin of xenografts was verified by a human pathologist and patient-origin was authenticated using short tandem repeat (STR) analysis (Cell-ID™, Promega). Due to the whole genome amplification (Klein et. al. 1999) of samples prior to STR analysis, which includes restriction digest by Mse I, only the STR loci TH01, D21S11, D5S818, D13S317, D16S538 and vWA can be used for detection. Amplified fragments were detected

using 3100-Avant Genetic Analyzer (Applied Biosystems). Fragment sizes were determined manually using Cell™ ID Allelic Ladder and Cell™ ID Bins 1.0 provided by Promega.

Ki-67 labeling of DCC

For immunofluorescence staining cells were incubated with primary antibodies against Melan A / MART-1 (Epitomics rabbit monoclonal dilution 1:100) and Ki-67 (DAKO MIB-1 mouse monoclonal 1:50) overnight at 4°C. As secondary antibodies we used Alexa Fluor 555 (Invitrogen, donkey anti-rabbit) and Alexa Fluor 488 (Invitrogen, donkey anti mouse). The nucleus was stained with DAPI (blue), Melan A with Alexa Fluor 555 (red) and Ki-67 with Alexa Fluor 488 (green). Counterstaining was performed with 4'-d-diamidino-2-phenylindole (DAPI) in mounting medium (Vector, Vectashield).

DNA extraction and microdissection of primary tumor areas

DNA was extracted from paraffin-embedded tumor blocks after highlighting tumor areas by a pathologist (P.R.). The PALM Microbeam system (Bernried) was used for microdissection and catapulting. DNA was processed as previously described (Klein (2002) *Lancet* 360, 683-689; Klein (2002) *J Exp Med* 196, 359-368).

Whole genome amplification and single cell comparative genomic hybridization

Whole genome amplification (WGA) was performed as previously described by Klein (2002) *Lancet* 360, 683-689; Klein (2002) *J Exp Med* 196, 359-368 and Klein (1999) *PNAS* 96, 4494-4499). The method is now commercially available as kit (*Ampli1*, Silicon Biosystems).

Single cell comparative genomic hybridization

Single cell CGH was performed as previously described by Czyz (2014) *PloS one* 9, e85907; Klein (2002) *Lancet* 360, 683-689; Klein (2002) *J Exp Med* 196, 359-368). For most samples we used chromosomal CGH, as it is a very robust method, well established for single cells. We carefully compared both methods. We found a good agreement between array CGH and chromosomal CGH when applied to same samples (see Czyz et al., 2014). While aCGH may detect more changes (mainly for aberrations <10 Mb), the overall picture for aCGH and

cCGH is very similar. For cases where we used aCGH, the resolution was adjusted to that of cCGH.

Mutation analysis of *BRAF* and *NRAS*

Mutations in *NRAS* and *BRAF* genes were detected using Sanger sequencing (Sequiaserve, Vaterstetten, Germany) after gene specific amplification from WGA samples. The primers for *BRAF* exon 15 analysis were as follows: forward 5'-TCCAGACAACTGTTCAAAGT and reverse 5'-CTCTTCATAATGCTTGCTCTG, encompassing the mutations of codon 600 (V600E, previously called V599E; V600K, V600R). Cycling temperatures were set to 94 °C (2 min), 60 °C (30 sec) and 72 °C (2 min) for one cycle; 94 °C (15 sec), 60 °C (30 sec) and 72 °C (20 sec) for 14 cycles; 94 °C (15 sec), 60 °C (30 sec) and 72 °C (30 sec) for 24 cycles and an additional final extension step at 72 °C (2 min). The PCR primers for *NRAS* exon 3 codon 61 analysis were: forward 5'-GGCAAATACACAGAGGAAGC and reverse 5'-ACCCCCAGGATTCTTACAGA encompassing the common mutations of codon 61: Q61K and Q61R. The PCR cyclers were set to 94 °C (2 min), 63 °C (30 sec) and 72 °C (2 min) for one cycle; 94 °C (15 sec), 63 °C (30 sec) and 72 °C (20 sec) for 14 cycles; 94 °C (15 sec), 63 °C (30 sec) and 72 °C (30 sec) for 24 cycles and an additional final extension step at 72 °C (2 min). PCR products were sent for sequencing to Sequiaserve, Vaterstetten. The mutation assay was established using single cells or genomic DNA of cell lines with known exon 15 mutation c1799T>A (*BRAF*) and exon 3 mutation c181C>A (*NRAS*). The mutant *BRAF* allele was detected in 62% (70-61), 84% (MelHo) of detected sequences over all analyzed single cells and in 61% (70-61) and 86% (MelHo) in bulk genomic DNA. The mutation *NRAS* allele was present in 59% of all single cells and 46% of the bulk genomic DNA. When several areas of the primary tumor were microdissected or several DCCs were isolated, the primary tumor or DCCs were called positive if one of the areas or DCCs harbored the *BRAF* or *NRAS* mutation.

Statistical analysis

Unless otherwise stated, statistical significance was assumed for $p < 0.05$, with all tests performed two-sided.

Thickness when tumor cells disseminate

Using Turnbull's method we determined that 42.3% of all melanomas had disseminated before reaching a thickness of 0.4 mm (Fig. 1B). Regardless of tumor thickness, dissemination was restricted to 63.5% of melanoma. A modified Weibull model with an upper limit less than 100% (Fig. 1B) predicted an asymptote of 63.5% (95% CI 53.5 to 73.4%) and revealed that 50% of seeding tumors had spread before 0.4 mm (95% CI 0.04-0.75 mm).

To determine the proportion of disseminating and colonizing tumors as a function of tumor thickness the data were fitted non-parametrically by maximum-likelihood according to the iterative method of Turnbull (42) for interval-censored data. Since it is unknown, at which tumor thickness an event occurred or will occur, all data are either left or right censored. For dissemination the obtained estimate is fitted with the maximum-likelihood-method by a Weibull distribution with a fraction of patients without dissemination in order to obtain interpretable parameter estimates for the asymptotic proportion of disseminating tumors and the median thickness of disseminating tumors.

Tumor thickness when DCCs colonize

Of the 525 DCC-positive patients, the number of samples with a DCCD>100 increased with tumor thickness as a Weibull cumulative distribution function with a median of 8.9 mm (95% CI 6.8 to 14.3 mm), i.e. 22 times higher than the median thickness at seeding (Fig. 1C). For colonization the obtained estimate is fitted by a Weibull distribution. Explicit formulas for the Weibull distribution were used to calculate the hazard rates. The hazard function describes the instantaneous risk per unit thickness for an event (dissemination, colonization) for those tumors, for which the event has not yet occurred. For example, the hazard rate of 1 per mm (0.2 per mm) indicates that the tumor needs to grow 1 mm (5 mm, respectively) on average for the event to occur.

Comparison of primary tumors and DCCs

The frequency statistics of gains and losses between primary tumors and DCCs were determined with a Mann-Whitney U test. Statistic significance for *BRAF/NRAS* mutations in paired primary tumors and DCCs was determined with Fisher's exact test.

Identification of mutational patterns

Mutational patterns that discriminate between primary tumors and DCCs, DCCs from patients with thin and thick melanomas and low and high DCCD, as well as with and without *BRAF/NRAS* mutations were identified by Fisher's exact test. Only loci with high enough cross-sample standard deviation (>0.25) allowing for sufficient class discrimination were considered. In Figure 2A only the ten most variable loci in terms of the maximum variance across samples without accounting for primary tumor/DCC class labels were included. Multiple testing corrections were derived according to Benjamini and Hochberg (FDR).

Identification of discriminating mutations

To identify the thickness at which critical alterations may have been acquired within the primary tumor, we split DCCs of samples with a $DCCD \leq 100$ into two groups according to observed different thickness values and identified genomic alterations that clearly showed non-random distributions across these groups, i.e. lead to low Fisher's test p-values. DCCD- and thickness thresholds associated with low p-values might indicate primary tumor sizes and genomic alterations that facilitate direct dissemination to lymph nodes.

We tested all DCCD thresholds below 100 to define the population of DCCs before colonization and found evidence for statistical differences for certain thickness thresholds for DCCD limits 19, 24, 32, and 95; however, none reached 5% significance.

A similar approach was taken to determine limiting DCCD thresholds and genetic alterations that mark the transition from early DCCs to colony-forming DCCs in the sentinel node. We asked for all genetic regions at which DCCD they would split the DCCs into two groups. This DCCD would then separate DCCs with and without that specific alteration and indicate the number of cells at which an alteration critical for disease progression has been acquired.

Robustness of results with respect to sample size was estimated by systematically excluding two cells from the analysis (subsampling with enumeration of all cases). The respective adjusted p-value distributions were kernel density-smoothed and plotted in 2D. Hierarchical cluster analyses were performed using euclidean distance and complete linkage. Analyses were conducted using R (<http://www.R-project.org>) or JMP (<http://www.jmp.com>).

Survival analysis

All survival statistics and tumor-free time of xenografts were calculated using a log-rank test (JMP, IBM SPSS Statistics 20 for Windows or GraphPad Prism 6.0 software for OSX).

Results

This study provides a compelling molecular model that accounts for the ectopic evolution in the spread of early systemic cancer. For the first time the the tumor extent at which metastatic dissemination of a human cancer occurs is reported; it is based on highly sensitive, *direct* detection of DCCs rather than being inferred from tumor growth rates (Engel (2003) European journal of cancer 39, 1794-1806; Friberg (1997) Journal of surgical oncology 65, 284-297; and Yachida (2010) Nature 467, 1114-1117}. The median thickness of seeding melanomas was 0.4 mm (95% CI 0.04 - 0.75 mm), much earlier than previously thought. However, the 9-year death rate for T1 melanomas was 11%, which was much lower than the seeding rate at this stage (46%), whereas seeding and death rates in T4 melanomas were similar (59% vs. 54%). This observation indicates that, while dissemination might occur early, additional factors are needed to generate lethal metastatic disease.

To address the discrepancy between seeding and death rates of thin melanomas primary tumors and matched DCCs were compared, which were clearly different for CNAs independent of tumor thickness. Primary tumors displayed a differential loss of chromosomal material strongly suggesting that DCCs disseminated before these losses had occurred. This conclusion is unaffected by potential limitations of the used technology, i.e. CGH as compared to next-generation sequencing (NGS). Extensive controls (Figure 3C and Figure 8) demonstrate that the applied single cell technology retrieves cellular karyo- and genotypes from clinical samples with much higher reliability and robustness (Polzer (2014) EMBO molecular medicine 6, 1371-1386} than NGS approaches for single cells which are prone for random allelic dropouts (Lohr (2014) Nature biotechnology 32, 479-484; Ni (2013) PNAS 110, 21083-21088}. Thus, it was shown that melanoma cells disseminated before specific CNAs and specific mutations (such as *BRAF*, discussed below) were acquired in the primary irrespective of the notion that some aberrations (shared or not shared) remained undetected by our approach. CNAs were analyzed as they best reflect cancerous progression as opposed to point mutations (Bauer (2006) Dermatologic therapy 19, 40-49; Shain (2015) The New England journal of medicine 373, 1926-1936}, which are frequently detected also in benign

lesions in contrast to CNAs (Hafner (2010) PNAS 107, 20780-20785; Klein (2013) Nature 501, 365-372; Shain (2015) The New England journal of medicine 373, 1926-1936). For this reason, the study did not address dissemination relative to the acquisition of non-CNA alterations except of *BRAF* or *NRAS*.

The conclusion of cancer cell spread occurring early in the somatic progression of a melanoma is also consistent with the possibility that minor sub-clones exist in the primary tumor that are more similar to DCCs but escaped detection by analysis of microdissected samples. Such subclones, if existing, are genomically immature as compared to the analyzed predominant clone; existence and dissemination (even if occurring at high T stage) of such subclones would then indicate that genomically immature cells display a higher propensity to spread than parallel existing mature cells. However, combined with the documented early seeding at 400 μm , it is more likely that melanomas often disseminate early in tumor formation *and* early in genomic maturation.

It was tested whether late-disseminating, genetically more mature cells are detected that may be more successful founder cells of a metastasis than early-disseminating cancer cells. For this, DCCs were analyzed before colony formation from patients with thick melanomas. They were compared with pre-colonizing DCCs from thin melanomas, but no differentiating CNAs could be identified. Also the number of CNAs did not differ between pre-colonizing DCCs from thin vs. thick melanomas.

This led to the conclusion that DCCs acquired critical alterations within lymph nodes. Indeed, growth beyond a DCCD of 77-95 was associated with losses on chromosome 9p, comprising the region of *p16*, and acquisition of *BRAF* mutations. Before that point, at DCCD=19 there was enrichment for the gain of chromosome 7q, comprising the *MET* oncogene. DCCD~100 was further associated with colony formation in the lymph node and significant increase in mean Ki-67 proliferation index from 11% (DCCD $\leq$ 100) to 22% (DCCD>100), confirming basal proliferation and indicating the acquisition of advantageous changes. Interestingly, the mean proliferation index of 11% before colonization resembles that of T1 melanomas at transition to the tumorigenic vertical growth phase (VGP) previously found to range between 9-13% (Gimotty (2005) Journal of clinical oncology : official journal of the American Society of Clinical Oncology 23, 8048-8056). Thus, early lymph node-DCCs are non-dormant and display progression-enabling growth rates.

Taken together, a striking difference between early, pre-colonizing DCCs and matured, colonizing DCCs was identified. Survival data and xenotransplantation support the conclusion

that DCCs form metastasis only after acquisition of critical colonization-enabling alterations. Therefore, metastases will genomically differ from early DCCs. These findings are fully supported by the largest sequencing study comparing primary tumors and matched metastasis from various cancers (Brastianos (2015) Cancer discovery). In all 86 cases (including melanoma), primary tumors and metastasis differed to a large degree and had acquired mutations private to both the primary and the distant sites. No example was found where the metastasis-founding clone could be identified within the primary tumor, as would be expected from linear progression. Another study, confined to melanoma, also supports branching evolution, however postulated (“deduced”) ancestor mutations that were not sequenced in the primary lesion in an attempt to rescue the late dissemination model (Sanborn (2015) PNAS 112, 10995-11000). It should be noted that mathematical models for cancer phylogeny apply the “infinite sites assumption” (Deshwar (2015) Genome biology 16, 35; Jiao (2014) BMC bioinformatics 15, 35; Strino (2013) Nucleic acids research 41, e165) stating that each mutation is generated maximally once (Ma (2008) PNAS 105, 14254-14261). However, this assumption is not justified in cancer, as opposed to its original application to genome evolution of species (Ma (2008) PNAS 105, 14254-14261), because, for example, melanoma of *unrelated* patients converge on the classical *BRAF*-V660E mutation in 40% of cases. Therefore, it is unreasonable to exclude *a priori* that two clones of the same cancer acquire the *BRAF* V600E mutation independently. The herein presented single cell analysis indicates that current models of branching evolution as deduced from sequencing studies of bulk tumors underestimate the complexity of cancer evolution because they rely on the infinite sites model.

BRAF mutations are found in DCCs at colony formation in sentinel lymph nodes but rarely before. Although benign nevi harbor *BRAF* mutant cells in 70-88% (Pollock (2003) Nature genetics 33, 19-20; Shain (2015) The New England journal of medicine 373, 1926-1936), it is unclear whether *BRAF* mutations (or generally MAPK pathway mutations) initiate melanoma and are transmitted linearly. Several reasons argue against this scenario for many melanomas. First, primary tumors display generally lower rates of *BRAF* mutations than benign nevi (40% vs. 80%, (Platz (2008) Molecular oncology 1, 395-405; Pollock (2003) Nature genetics 33, 19-20), indicating different routes to malignancy; second, between 0 and 10% of *in situ* and early radial growth phase (RGP) melanomas harbor *BRAF* mutations (Dong (2003) Cancer research 63, 3883-3885; Verlinden (2014) Medicine 93, e285). A higher rate of *BRAF* mutations in such lesions is only seen in studies using samples with neighboring advanced melanoma (Omholt (2003) Clinical cancer research : an official journal of the American

Association for Cancer Research 9, 6483-6488; Shain (2015) The New England journal of medicine 373, 1926-1936), but not when in situ melanomas were resected before invasive growth (Dong (2003) Cancer research 63, 3883-3885; Verlinden (2014) Medicine 93, e285), suggesting that contamination may have occurred in the former. Fourth, among patients with *BRAF* mutant melanoma DCCs, readily cancer cells without this mutation could be identified, disproving full clonality for this alteration in primary lesions as well.

The herein presented genetic findings cohere with the patient data suggesting that dissemination often occurs at the beginning of the VGP (0.04-0.75 mm depth). When VGP melanomas expand in the dermis (i.e. become tumorigenic), they often acquire *BRAF* mutations (Dong (2003) Cancer research 63, 3883-3885; Verlinden (2014) Medicine 93, e285), and increase their proliferation rate - with Ki-67 frequencies of >20% being a marker of poor outcome (Gimotty (2005) Journal of clinical oncology : official journal of the American Society of Clinical Oncology 23, 8048-8056). DCCs in SLN and possibly other metastatic sites re-capitulate this process during colonization. The herein presented genetic data and mathematical progression modeling indicate that after acquisition of a proliferative phenotype, dissemination becomes increasingly unlikely. Hazard rates for dissemination diminished with increasing tumor thickness and *BRAF* and *NRAS* mutations in primary tumors were rarely shared by matched DCCs, indicating that *BRAF/NRAS* mutant clones were less likely to seed. Together, dissemination from early lesions, published data of *BRAF* mutations in primary melanoma progression and the selection of specific alterations during lymph node colonization all cohere to a model of a largely parallel passage through the “Vogelgram” (Fearon (1990) Cell 61, 759-767) of melanoma cells at the primary and secondary sites (Figure 7). It is tempting to speculate that melanoma cells receive cues to disseminate from the stromal compartment as they invade the dermis (Figure 7) and continue to evolve after lodging to distant sites. Finally, the initial disparity between primary tumors and DCCs regarding *BRAF* mutations in addition to the strong selective advantage the mutations endow during colony formation, explain both the observed disparity for *BRAF* mutations between primary tumors and metastases in our and other studies (Colombino (2012) Journal of clinical oncology : official journal of the American Society of Clinical Oncology 30, 2522-2529; Saint-Jean (2013) J Invest Dermatol; Yancovitz (2012) PloS one 7, e29336; Verlinden (2014) Medicine 93, e285) as well as the increased frequency of *BRAF* mutations in metastases compared to early RGP melanomas.

It remains to be explored in more detail why high T stage is a risk factor in melanoma. The correlation between tumor thickness and colonization (as opposed to dissemination) may either reflect the lead-time (DCCs in patients with thick melanomas had more time to grow within the node than DCCs from thin melanomas) or suggest that primary tumors facilitate colonization by secreted factors (Peinado (2012) *Nature medicine* 18, 883-891). Such factors may act in a dose-dependent manner either directly upon DCCs or indirectly by altering the microenvironment locally or systemically. The fact that T1 melanomas are mostly cured by surgery (Balch (2009) *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 27, 6199-6206) strongly supports proliferation-stimulating factors secreted more abundantly by thick melanomas, which then promote the acquisition of genetic changes required for colony formation. Formal proof of this scenario would require model systems that enable genomic *in vitro* progression of immature to mature cancer cells triggered by supporting factors. Such models are currently not available. Unstimulated and consequently slow *in vivo* progression over years as observed in some patients may account for late recurrences (Ossowski (2010) *Pigment cell & melanoma research* 23, 41-56) and argue at least partially for a lead-time effect.

The lack of adequate supporting signals from the primary tumor may explain why the study failed to observe engraftment of pre-colonizing cells in NSG mice. Pre-colonizing DCCs rarely thrive, either in mice or in humans after early melanoma removal. On the other hand, colonizing DCCs engrafted in about 60% of cases, which is in agreement with the reported engraftment rate of 16% -75% for tumor cells from primary melanomas (Boiko (2010) *Nature* 466, 133-137; Quintana (2008) *Nature* 456, 593-598). DCCs generally needed between 20 and 50 weeks to form xenografts, which is much longer than cell line cells or primary tumor cells took to do so (Quintana (2008) *Nature* 456, 593-598}. This suggests that additional, including non-genetic, differences exist between primary tumor cells and DCCs with newly-acquired colonizing ability.

Analysis of the immune cell microenvironment of lymph nodes revealed a loss of CD8 T cell and NK cell function as well as recruitment of immunosuppressive immature MDSCs, which altogether impair the execution of an anti-tumoral response and result in a failure in tumor surveillance which otherwise could possibly limit metastatic growth. The functional impairment of tumor surveillance occurs at a DCCD > 2000 and follows the acquisition of genetic aberrations at a DCCD > 100. Furthermore it is dependent on the local tumor cell load

in the lymph node, i.e. the DCCD and therefore can only be deduced from the target organ of dissemination, but not from corresponding parameters in the primary tumor.

The herein presented findings have implications for the development of adjuvant therapies. First, although the classical approach has been to determine the molecular characteristics of advanced cancer cells (for example by comparing low and high T stages) and then target progression-associated alterations, this may be irrelevant to early systemic disease and therefore unproductive at best. Rather, it may become critically relevant to determine whether systemically spread cancer cells have already formed. If yes, there is a greater chance that genetic alterations supporting colony formation are shared with primary tumors and may be targeted. Secondly, novel drugs may be required to eradicate the metastatic seed prior to colonization as pre-colonizing DCCs lack typical drug targets. Finally, molecular evolution necessitates novel tools for monitoring latent disease activity. As colonization-associated alterations in DCCs were found in all but one of nine patients dying from melanoma during follow-up, diagnosis of this colonization signature before manifestation of metastasis may present novel opportunities for adjuvant therapy selection and timing. Administering agents targeting mutant *BRAF* in patients lacking mutant *BRAF* not only lacks benefit, but could stimulate the MAPK pathway to promote tumor growth (Poulikakos (2010) *Nature* 464, 427-430). Therefore, the risk of unintended disease promotion in patients with *BRAF* mutant primary tumors but wild type DCCs in stage IIC and stage III patients might be higher than previously thought. On the other hand, given the recently appreciated importance of neo-antigen diversity for the success of immune-checkpoint blockade therapies (Snyder (2014) *The New England journal of medicine* 371, 2189-2199), the continuing evolution of DCCs argues that the identification of these mutations and antigens in DCCs is imperative to enable and prevent lethal metastasis. With regard to immune-checkpoint blockade and other immunotherapies, the dependency of the local impairment of tumor surveillance mechanisms on the local colonization calls for its implementation in therapeutic decision making and patient-stratification.

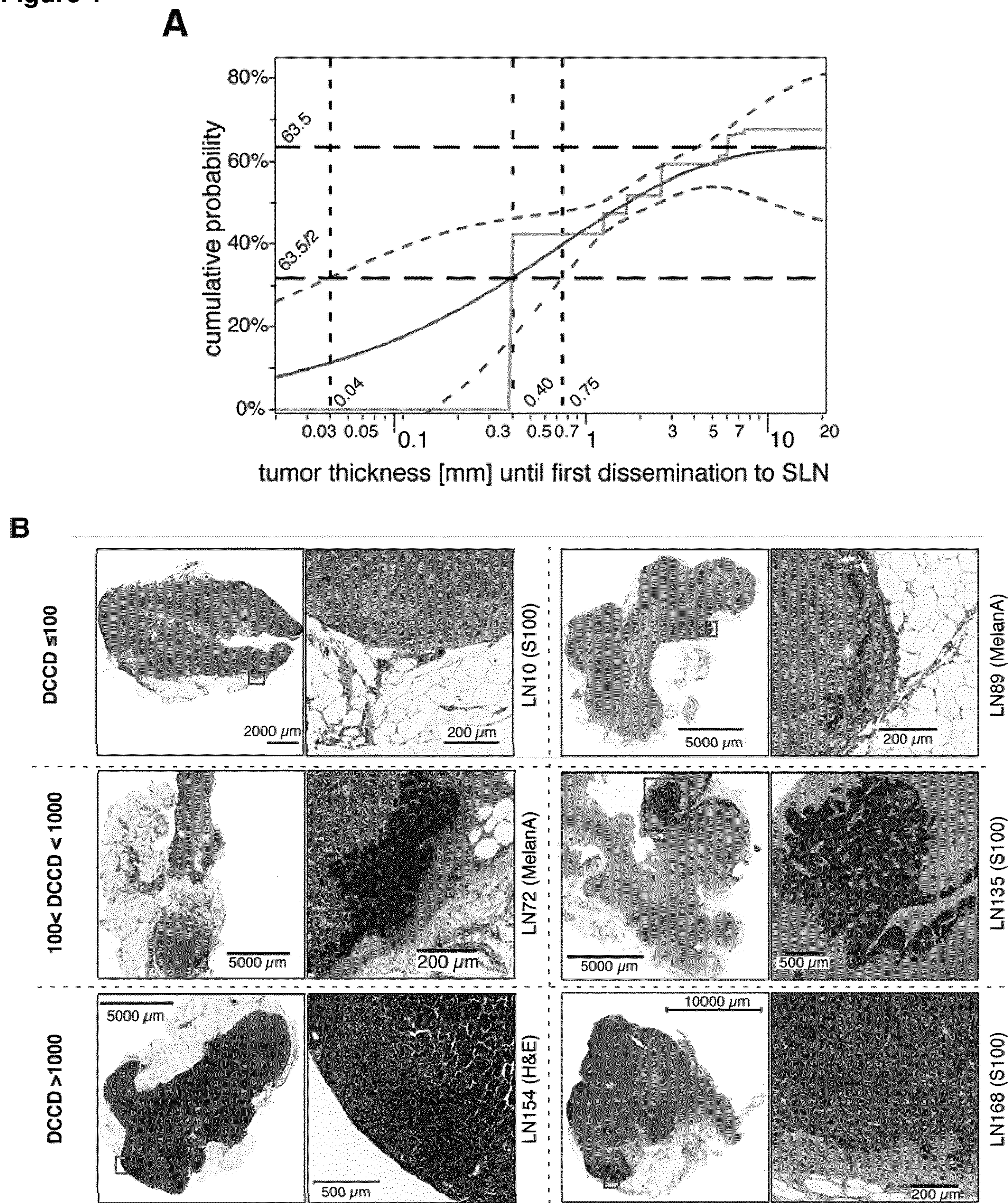
Claims

1. A method for staging and/or typing of a cancerous disease, said method comprising the following steps:
 - (a) detecting somatic alterations of the DNA of one or more disseminated cancer cell(s) (DCC(s)) obtained from one or more lymph node(s); and
 - (b) determining the somatic evolution of the DCC(s), based on the detected somatic alteration(s) in step (a),wherein the somatic evolution of the DCC(s) is indicative of the stage/type of the cancerous disease and wherein the cancerous disease is melanoma.
2. A method for treating a cancerous disease, said method comprising the following steps:
 - (a) detecting somatic alterations in the DNA of one or more DCC(s) obtained from one or more lymph node(s) of a subject;
 - (b) determining the somatic evolution of the DCC(s) based on the detected somatic alteration(s) in step (a); and
 - (c) determining the stage/type of the cancerous disease based on the somatic evolution of the DCC(s) determined in step (b),wherein the stage/type of the cancerous disease is used to initiate, continue or discontinue therapy of said cancerous disease and wherein the cancerous disease is melanoma.
3. A pharmaceutical composition for use in treating a cancerous disease in a subject, wherein treatment is initiated, continued or discontinued based on the stage/type of said cancerous disease, wherein said stage/type of said cancerous disease is determined by:
 - (a) detecting somatic alterations in the DNA of one or more DCC(S) obtained from one or more lymph node(s) of a subject;

- (b) determining the somatic evolution of the DCC(s) based on the detected somatic alteration(s) in step (a); and
 - (c) determining the stage/type of the cancerous disease based on the somatic evolution of the DCC(s) determined in step (b)
- and wherein the cancerous disease is melanoma.
4. The method of any one of claims 1 or 2 or the pharmaceutical composition of claim 3, wherein the DCC(s) are obtained from a sentinel lymph node.
 5. The method of any one of claims 1, 2, 3 or 4, or the pharmaceutical composition of any one of claims 3 or 4, further comprising determination of the DCC density (DCCD), wherein the DCCD is the number of DCCs per million cells in the lymph node used to obtain the DCCs, wherein the DCCD is indicative of the stage/type of the cancerous disease.
 6. The method of claim 5, or the pharmaceutical composition of claim 5, wherein a DCCD of more than about 50 to about 100 is indicative for the development of metastases.
 7. The method of any one of claims 1, 2 and 4-6, or the pharmaceutical composition of any one of claims 3-6, wherein the somatic alterations comprise at least one of the somatic alterations selected from the group consisting of a *BRAF* mutation, a loss of chromosome 9p11-13, a loss of chromosome 9p21-24, a gain of chromosome 7q21-36, and a *NRAS* mutation.
 8. The method of any one of claims 1, 2 and 4-7, or the pharmaceutical composition of any one of claims 3-7, wherein a *BRAF* mutation, a loss of chromosome 9p11-13, a loss of chromosome 9p21-24, a gain of chromosome 7q21-36, and/or a *NRAS* mutation indicates that the DCC(s) will develop to metastases.
 9. The method of any one of claims 1, 2 and 4-8, or the pharmaceutical composition of any one of claims 3-8, wherein step (a) further comprises evaluating the proliferation of the DCC(s), wherein an increased proliferation of the DCC(s) indicates that the DCC(s) will develop to metastases.

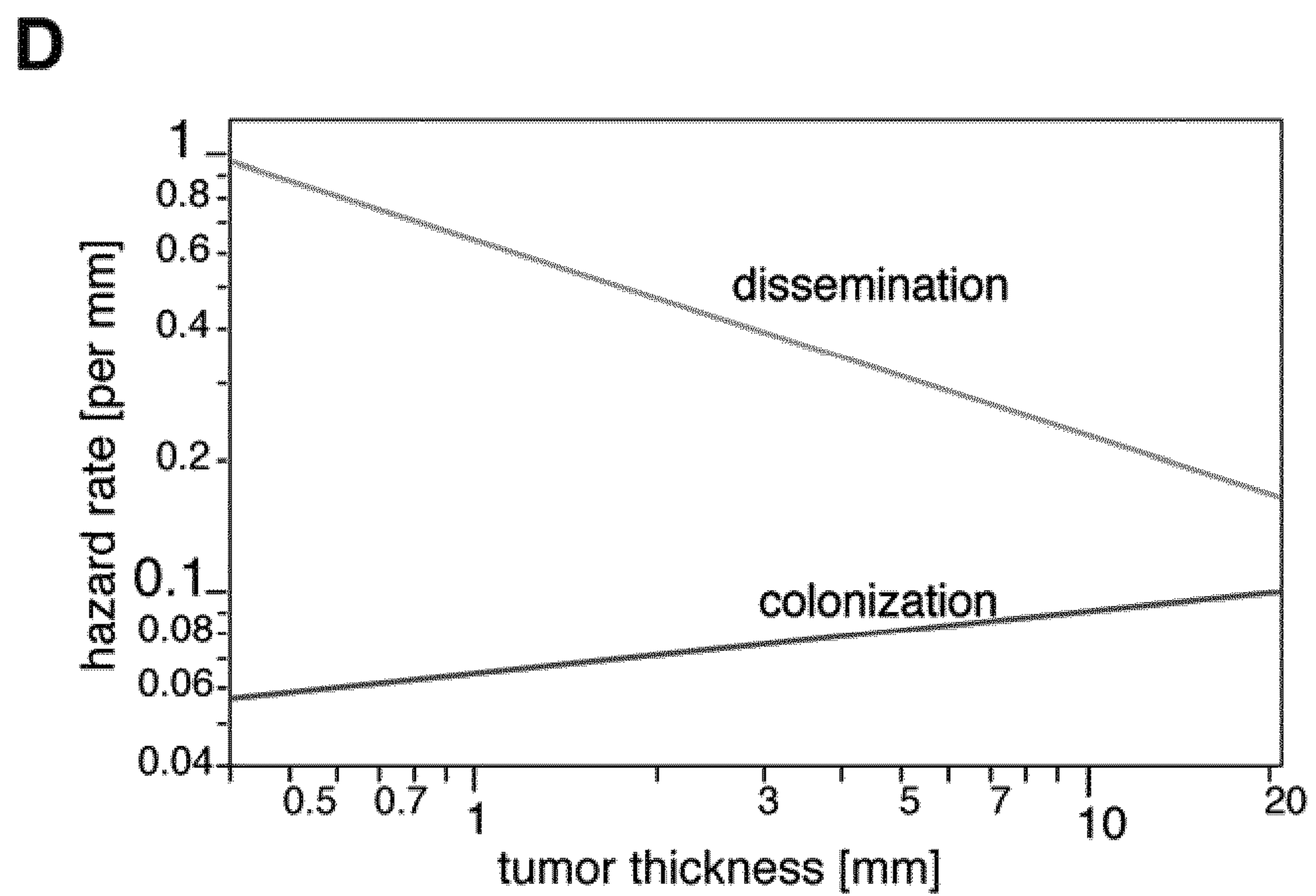
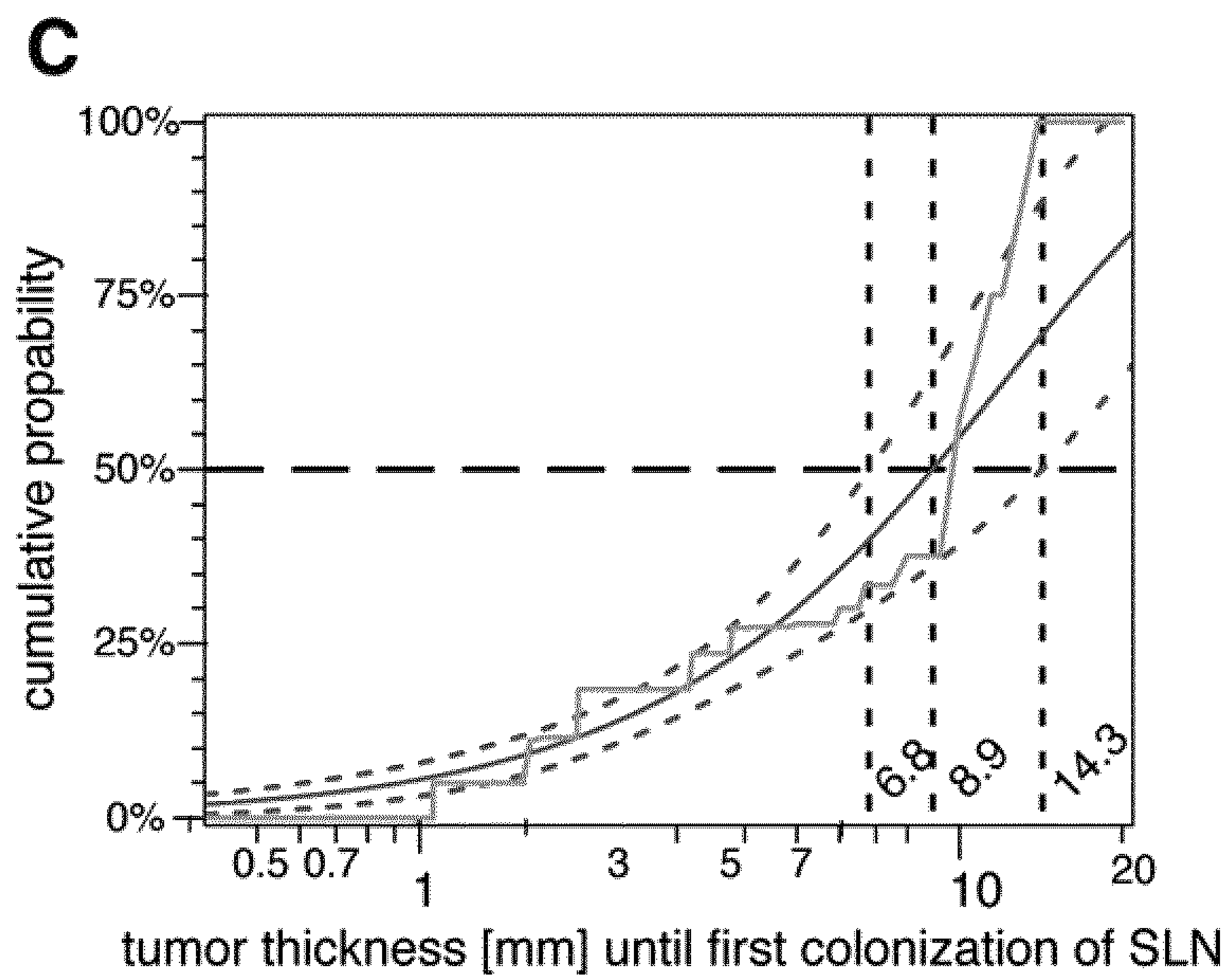
10. The method of any one of claims 2 and 4-9, or the pharmaceutical composition of any one of claims 3-9, wherein if the DCC(s) are found to carry a *BRAF* mutation, a treatment that is directed against cells carrying said mutation is to be initiated or continued; and wherein if the DCC(s) were found to not carry a *BRAF* mutation, a treatment that is directed against cells carrying said mutation is to be discontinued.
11. The method of claim 10, or the pharmaceutical composition of claim 10, wherein treatment directed against cells carrying a *BRAF* mutation comprises administration of a *BRAF* inhibitor.
12. The method of claim 11, or the pharmaceutical composition of claim 11, wherein the *BRAF* inhibitor is sorafenib or vemurafenib.
13. The method of any one of claims 2 and 4-10, or the pharmaceutical composition of any one of claims 3-10, wherein if the DCC(s) are found to carry a *NRAS* mutation, a treatment that is directed against cells carrying said mutation is to be initiated or continued; and wherein if the DCC(s) are found to not carry a *NRAS* mutation, a treatment that is directed against cells carrying said mutation is to be discontinued.

Figure 1



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Figure 1 cont.



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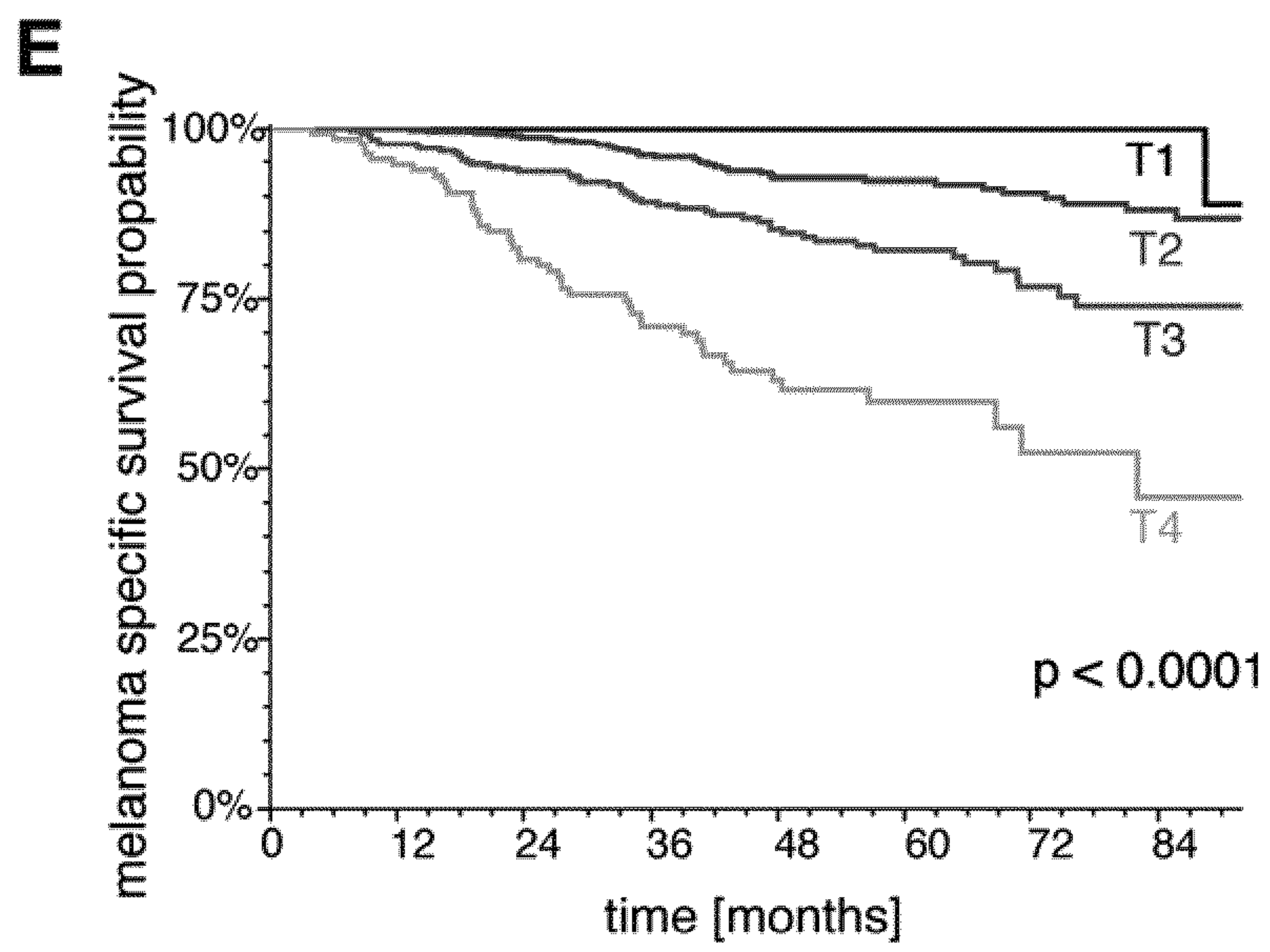


Figure 2

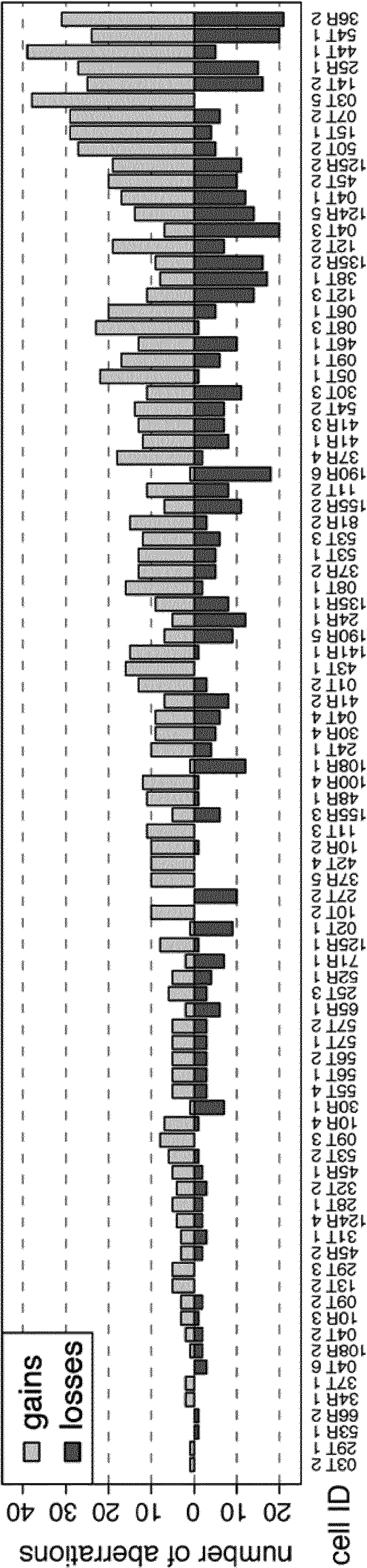


Figure 3

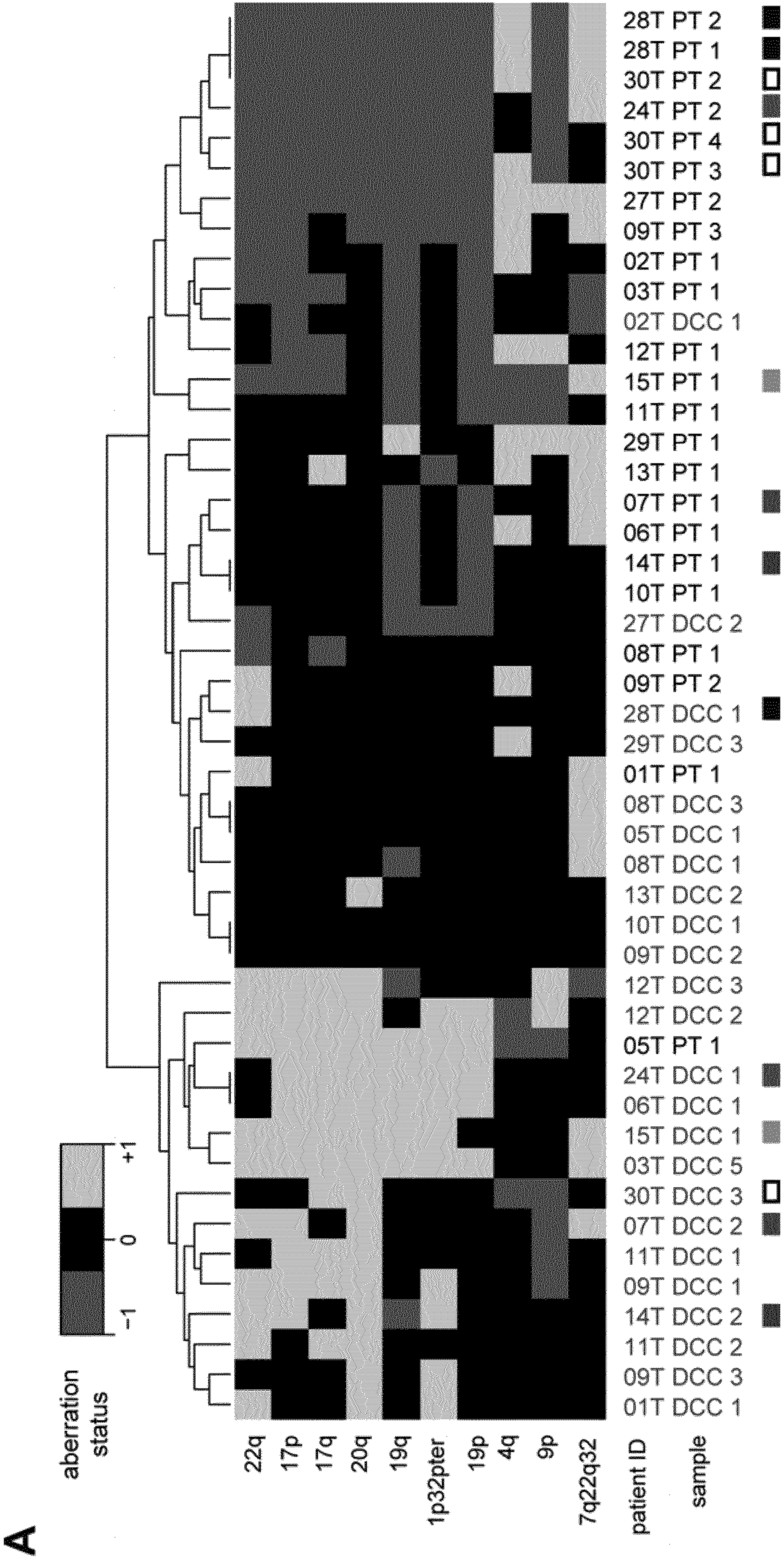
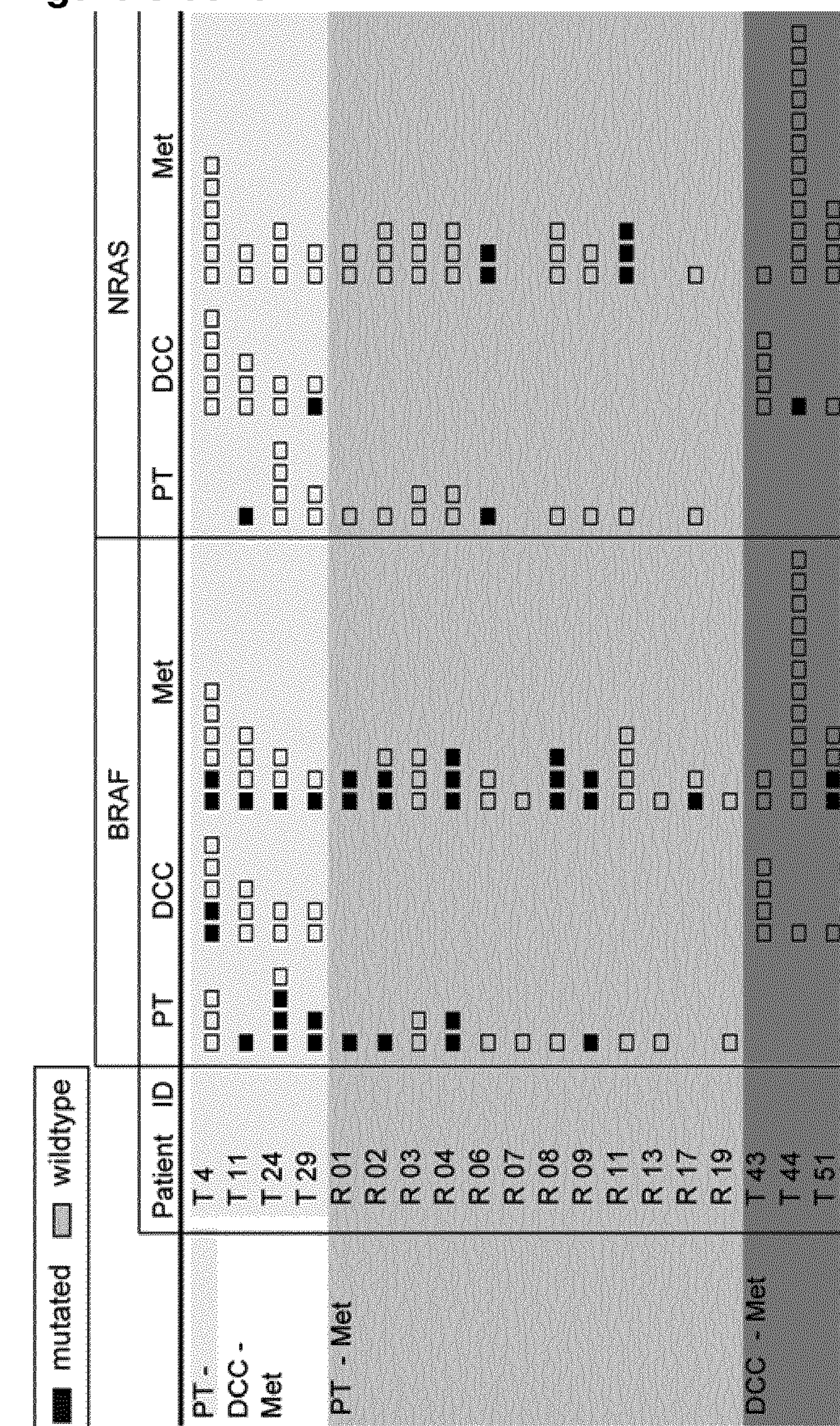
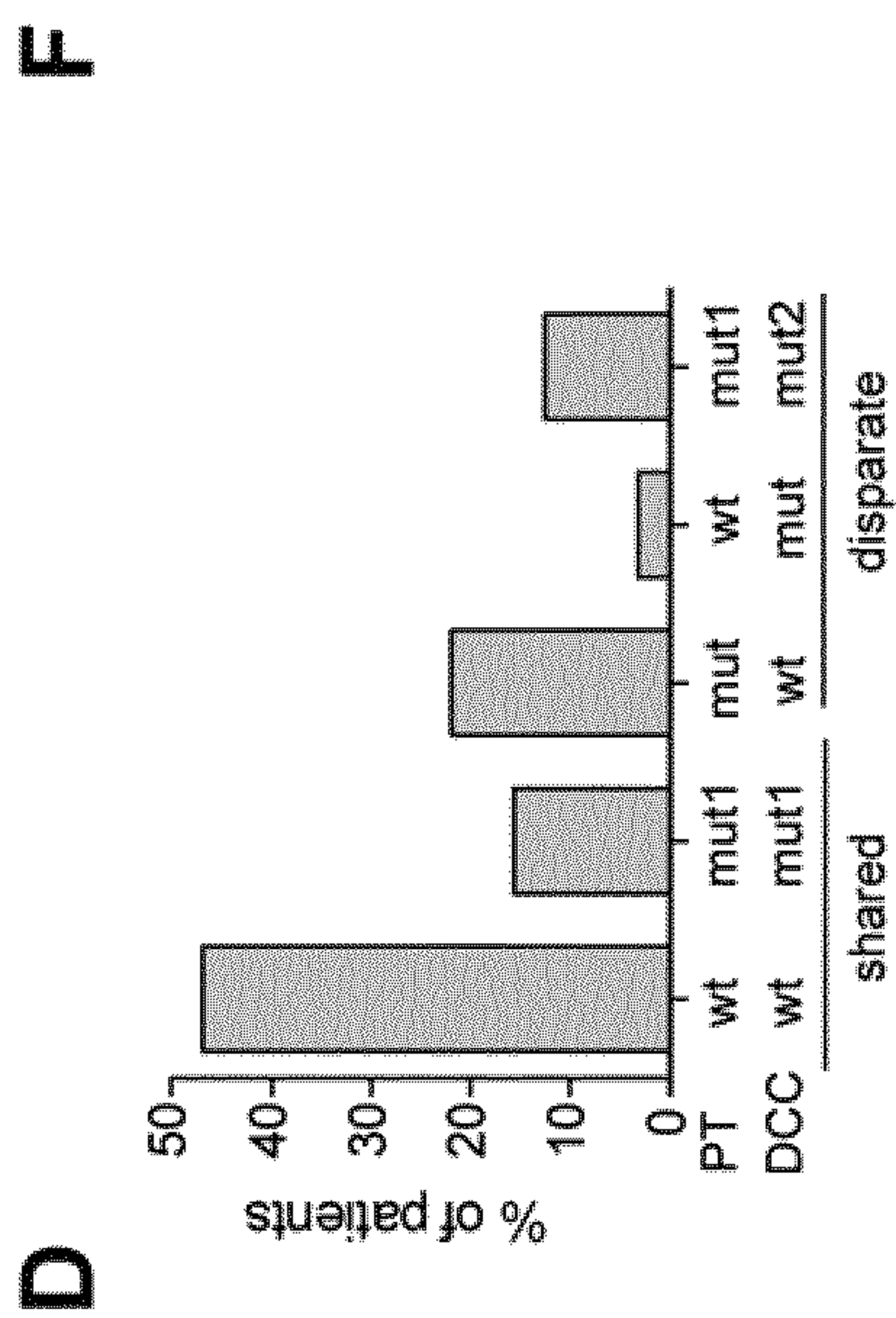


Figure 3 cont.



patients with heterogenous DCCs:

	DATA	NINOS
gp100	5/11 45%	8/10 80%
MCSP	10/18 56%	6/7 86%



paired samples:

	BRAF	NRAS
PT	18/53 34%	7/47 15%
DCC	13/86 15%	9/84 11%
p-value	0.012	0.580

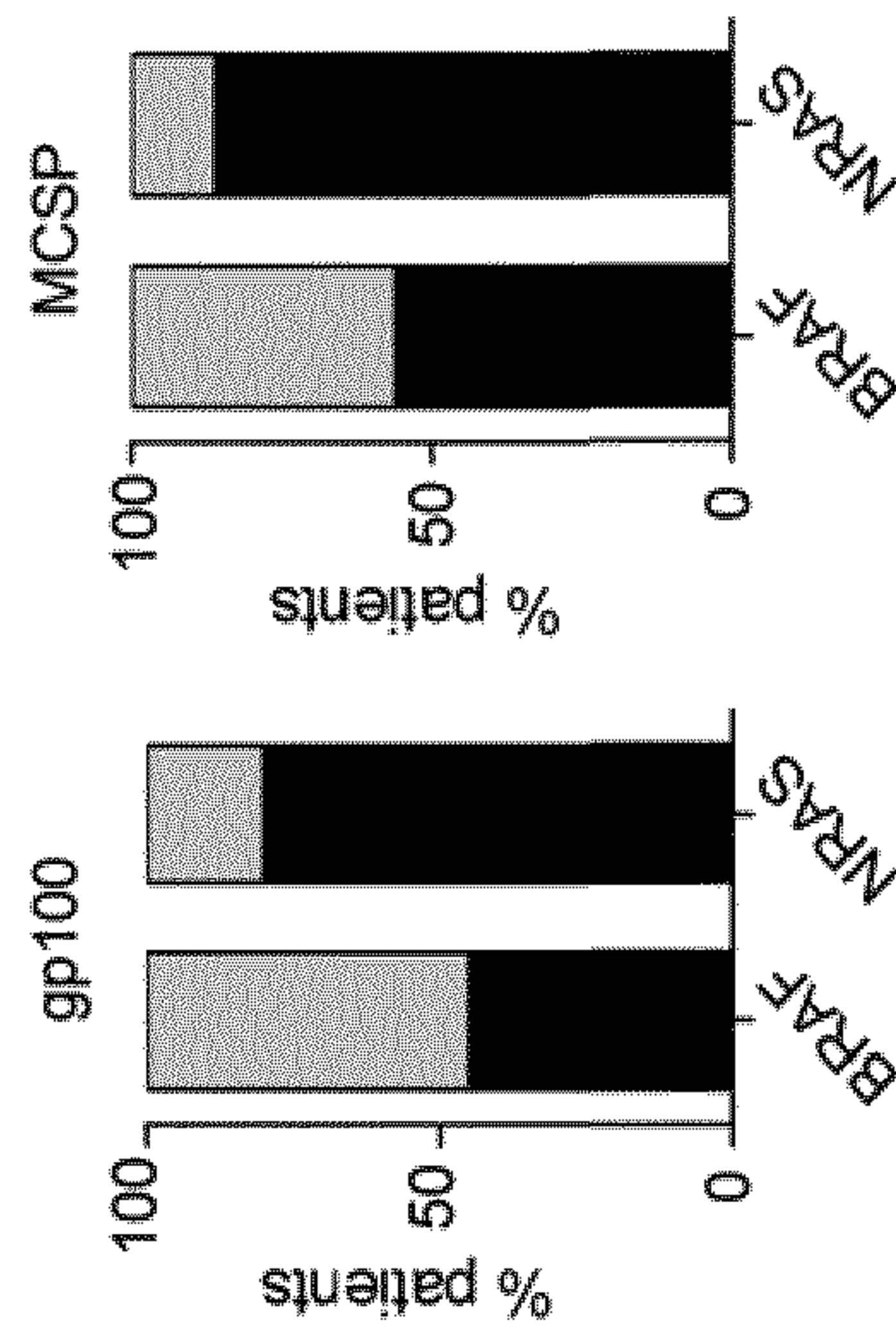
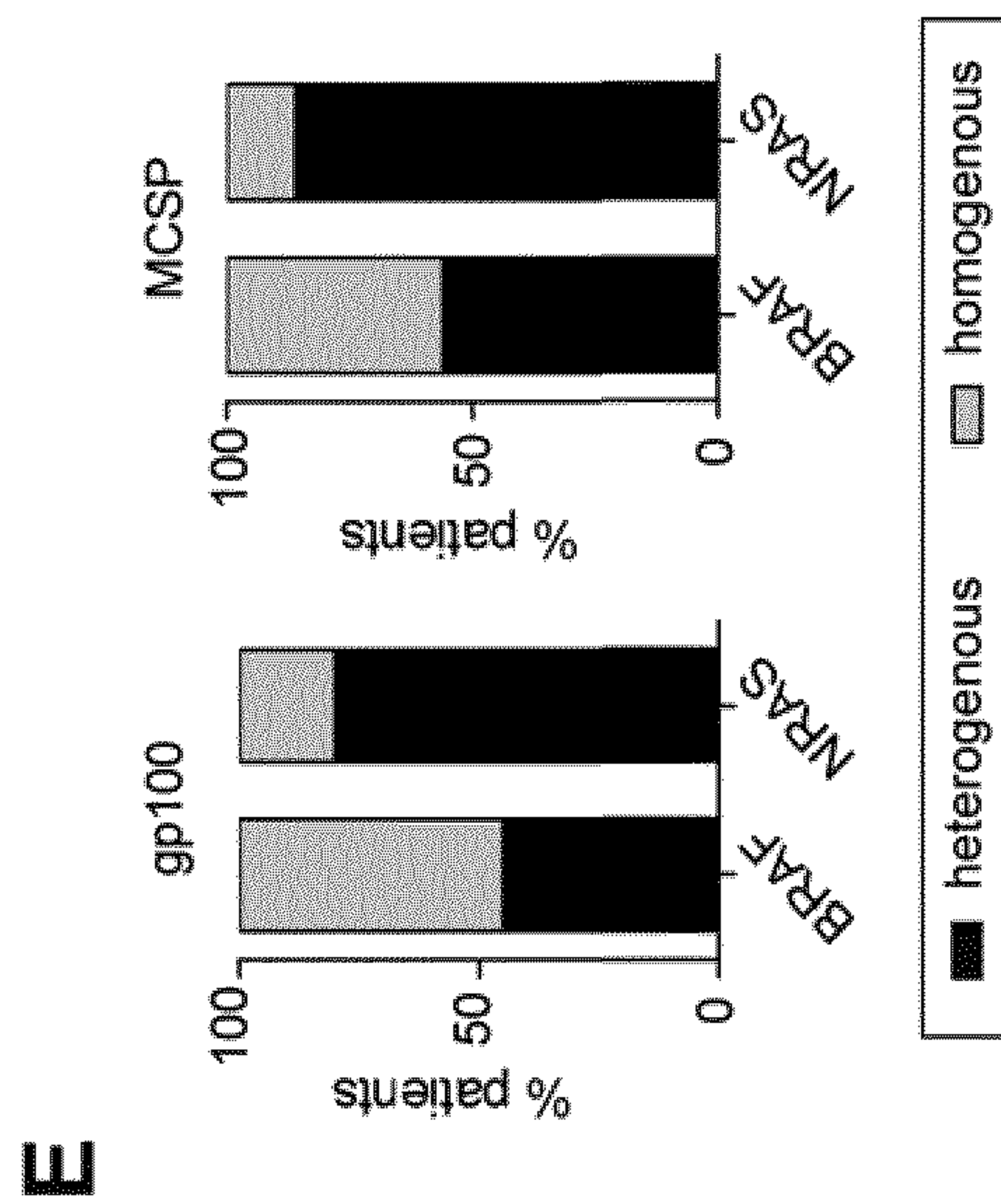
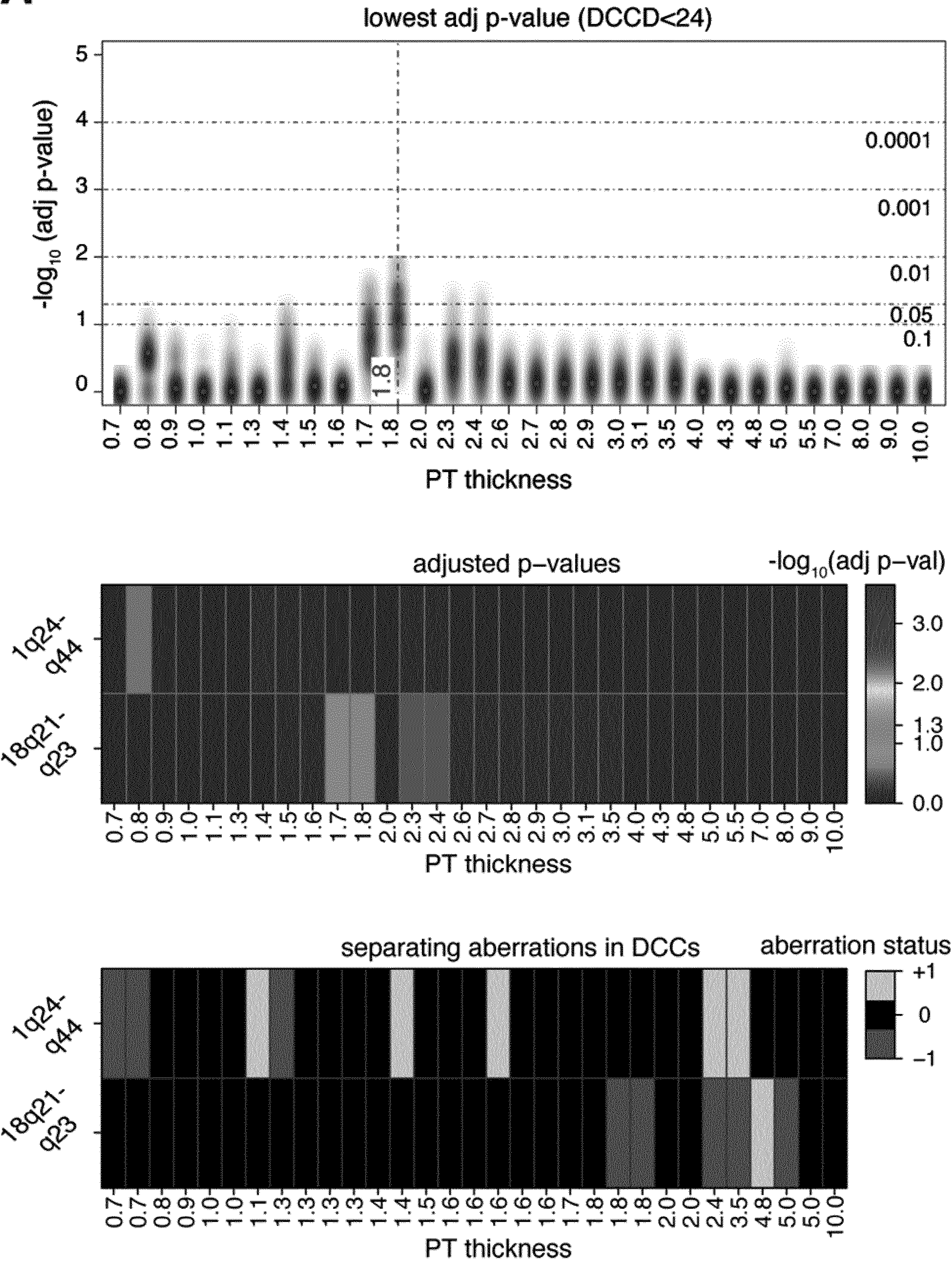


Figure 4
A



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Figure 4 cont.

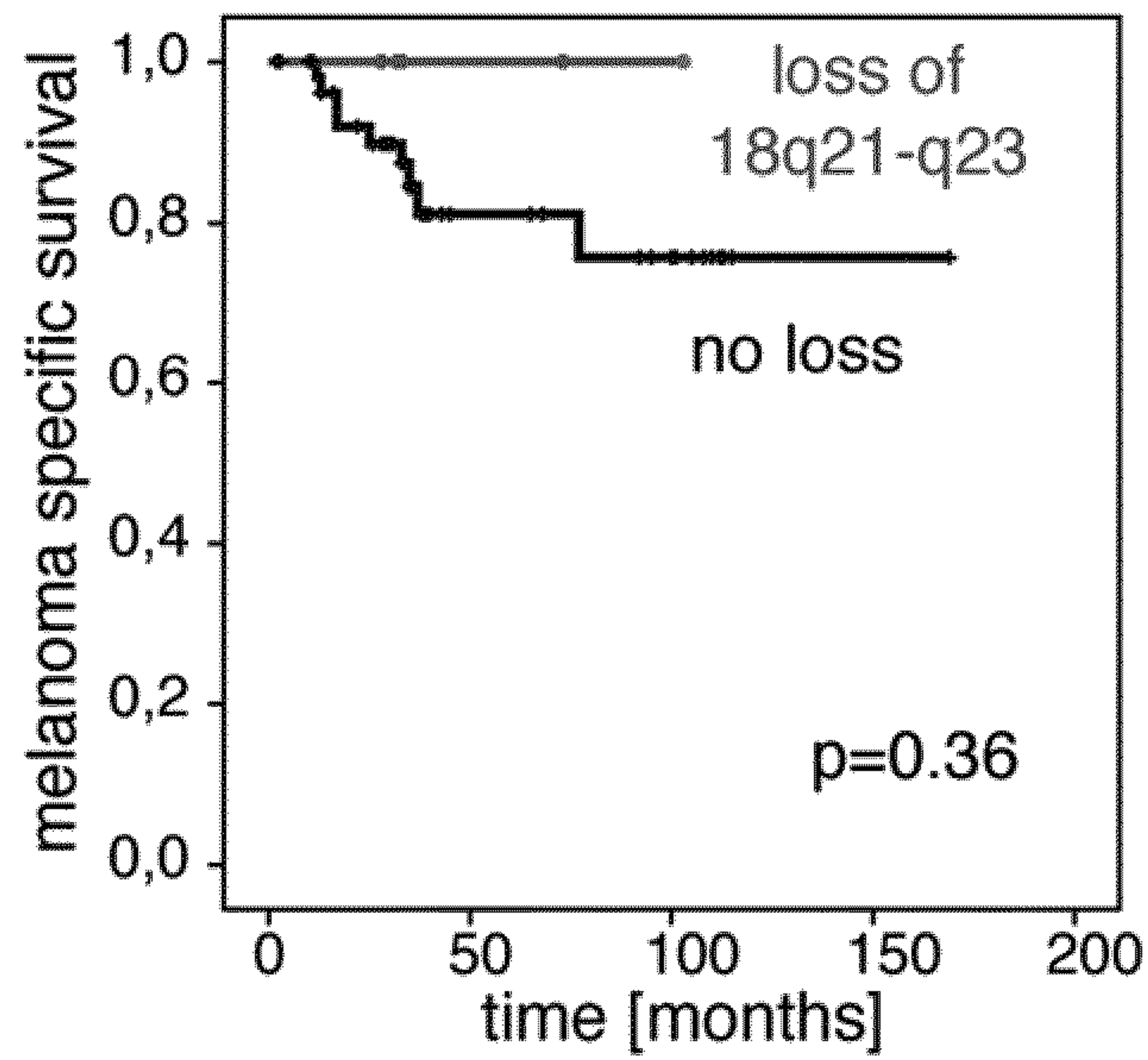
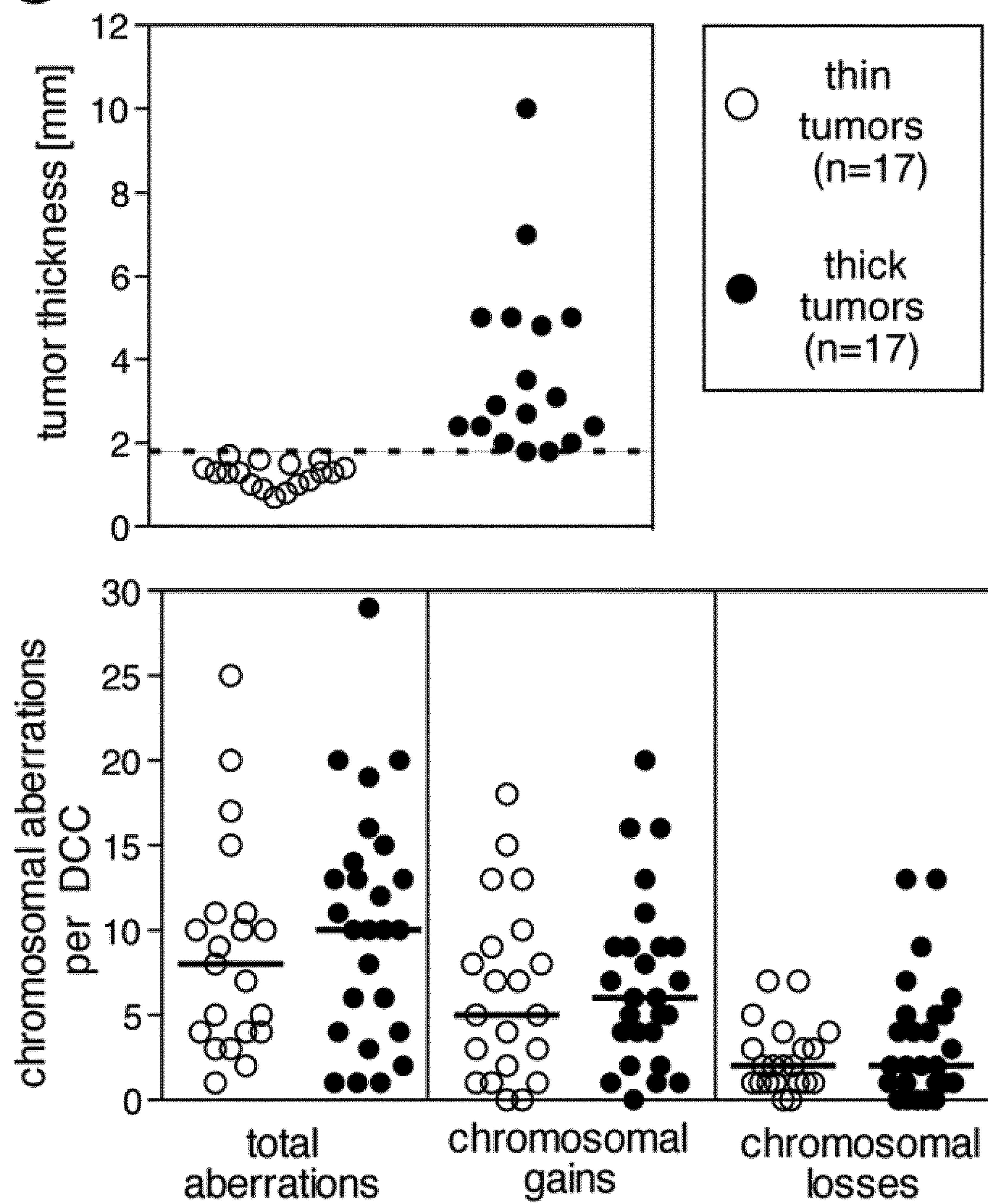
B**C**

Figure 5

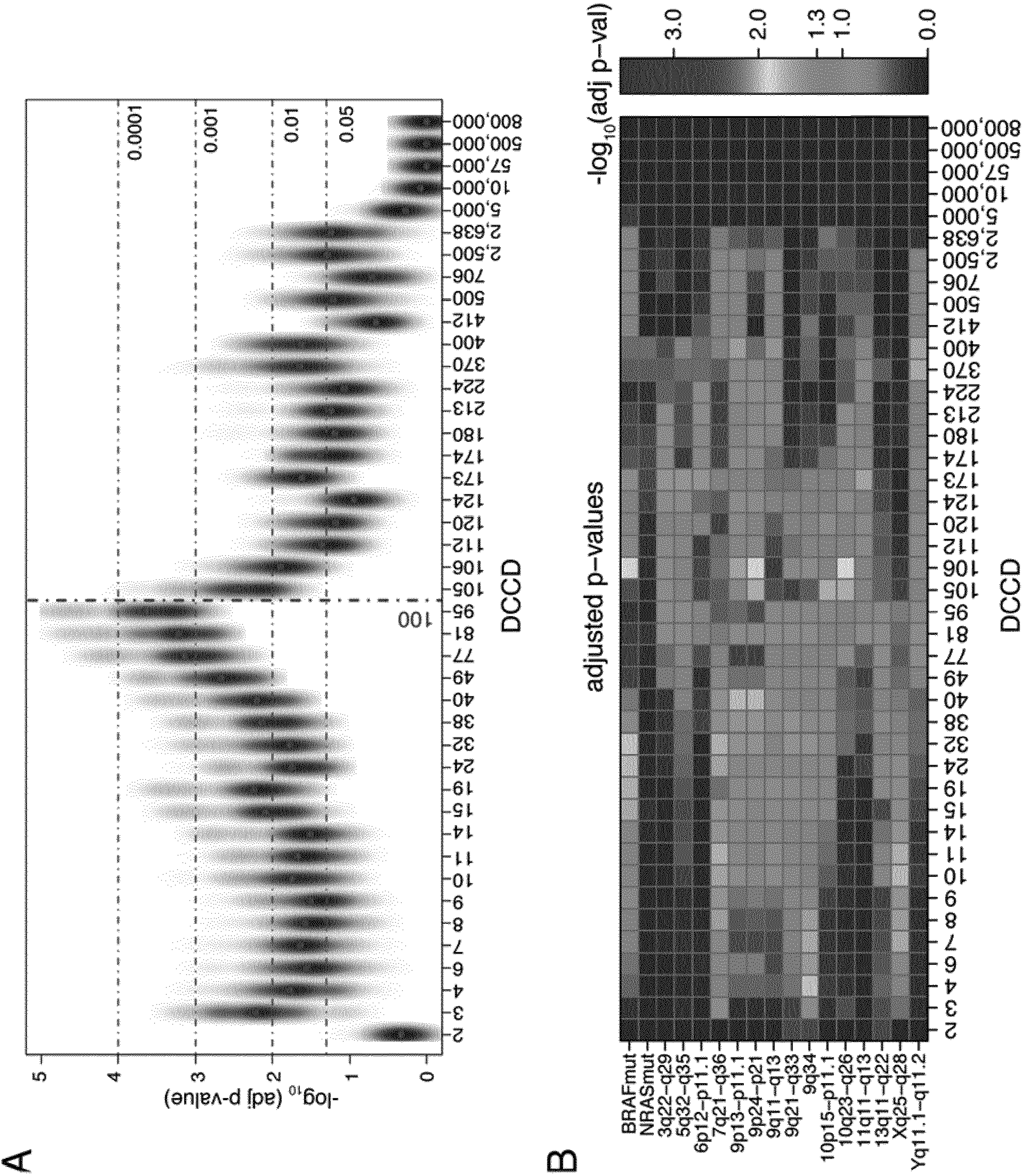


Figure 5 cont.

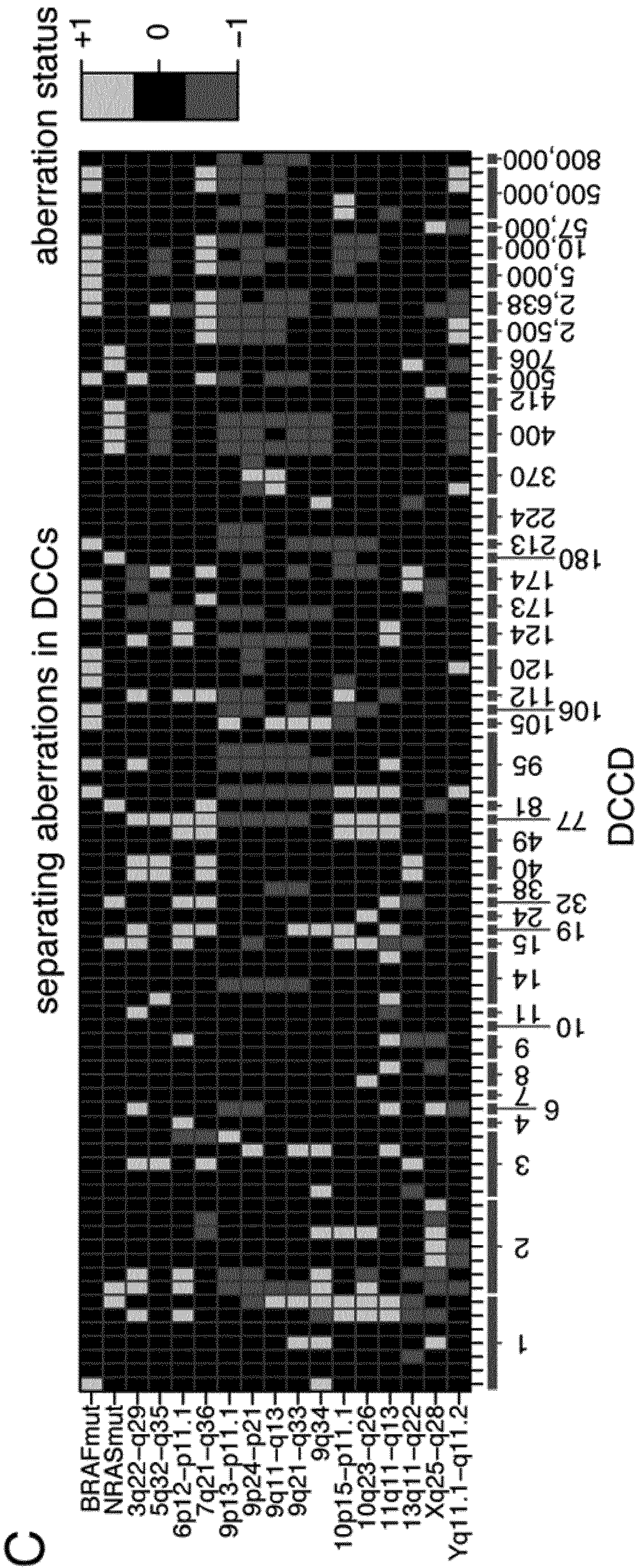


Figure 5 cont.

D

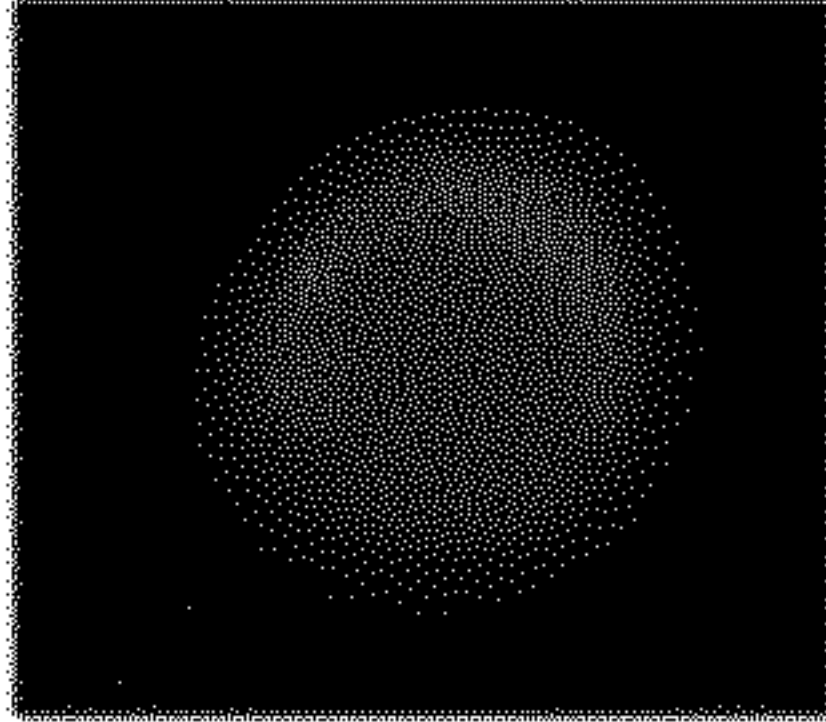
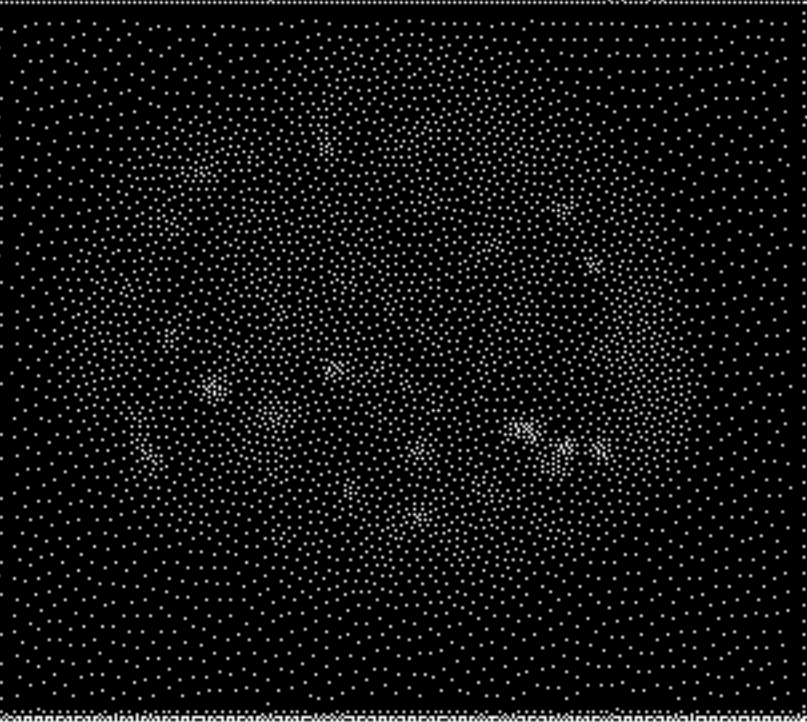
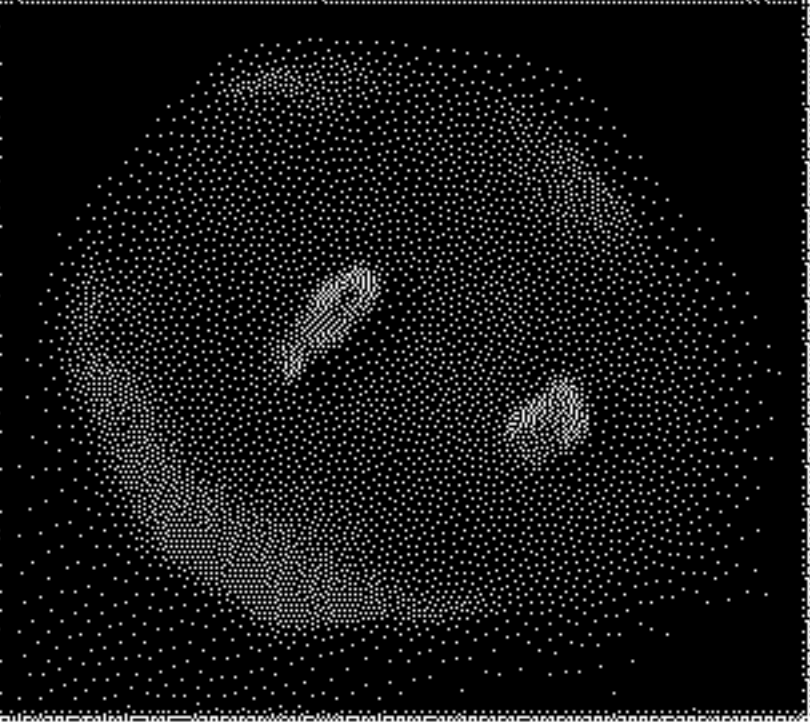
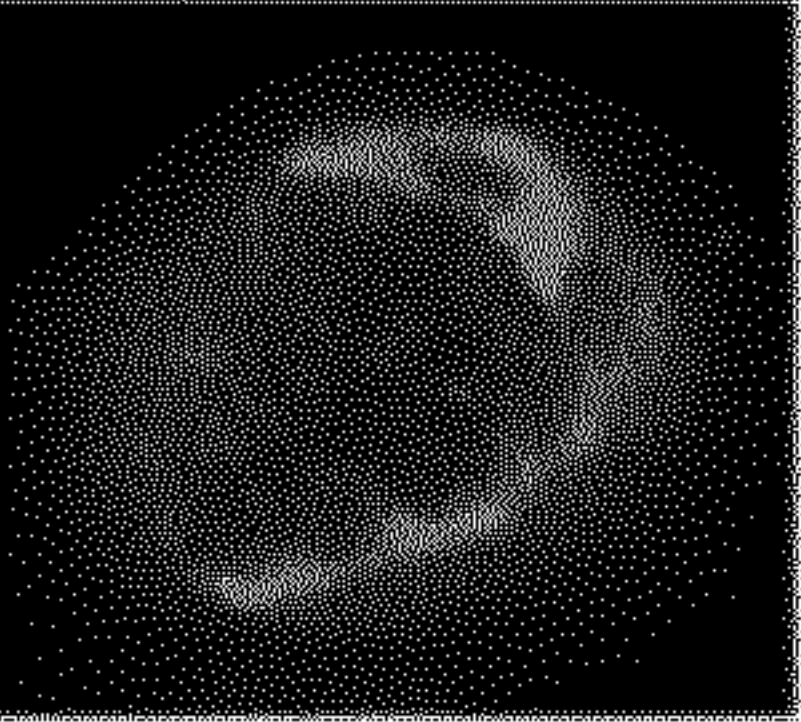
G0	G1	G2	mitosis
			
Ki-67 staining pattern	Group A (DCCD≤100) n cells	Group B (DCCD>100) n cells	
Mitosis	3 (1.4%)	46 (6.3%)	
Early G1	8 (3.8%)	68 (9.4%)	
G2	13 (6.2%)	46 (6.3%)	
G0	186 (88.6%)	567 (78.0%)	
n MelanA+ cells	210 (100%)	727 (100%)	
n lymphnodes (cells/lymphnode)	37 (1-28)	21 (10-50)	

Figure 6

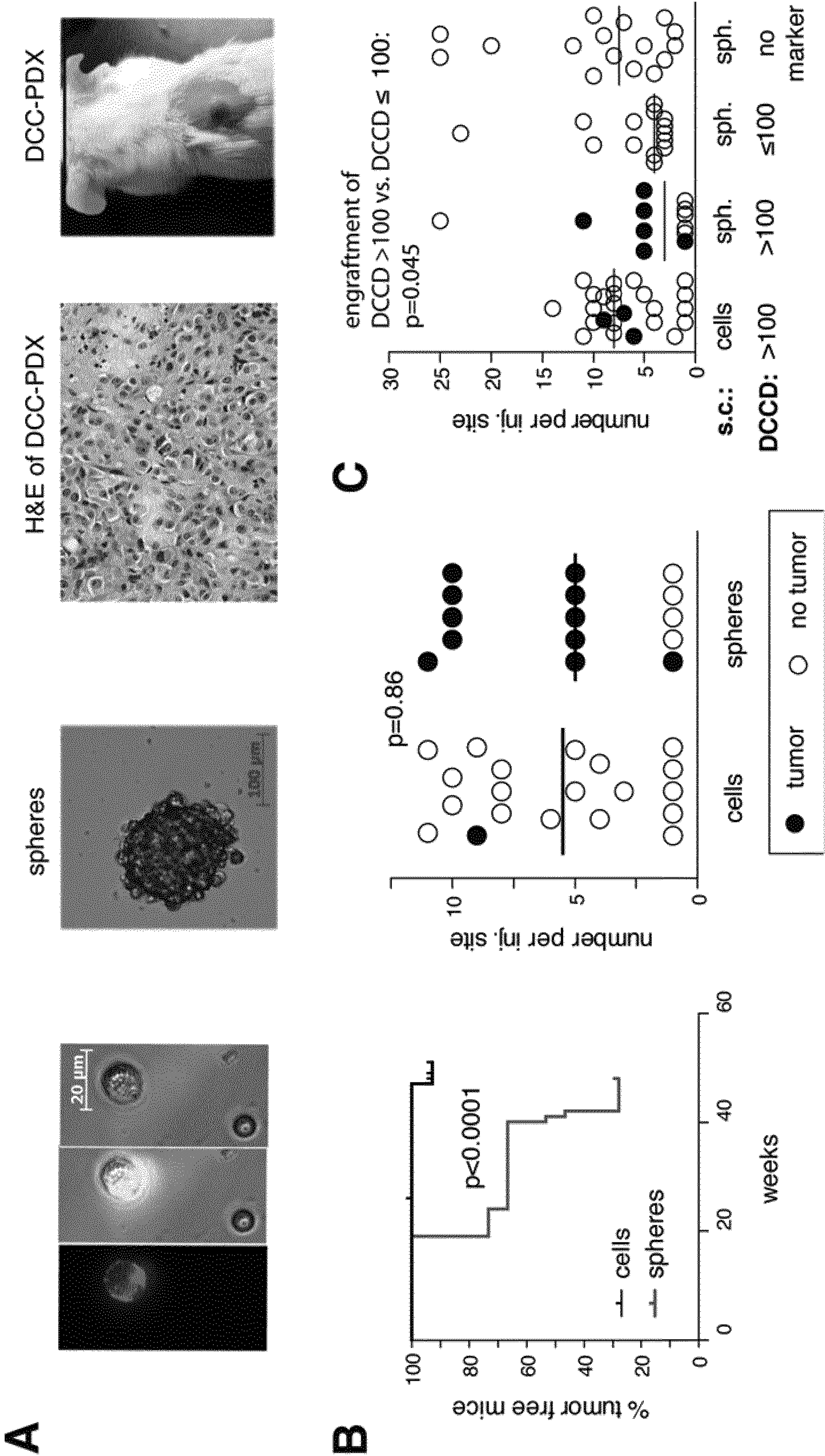


Figure 6 cont.

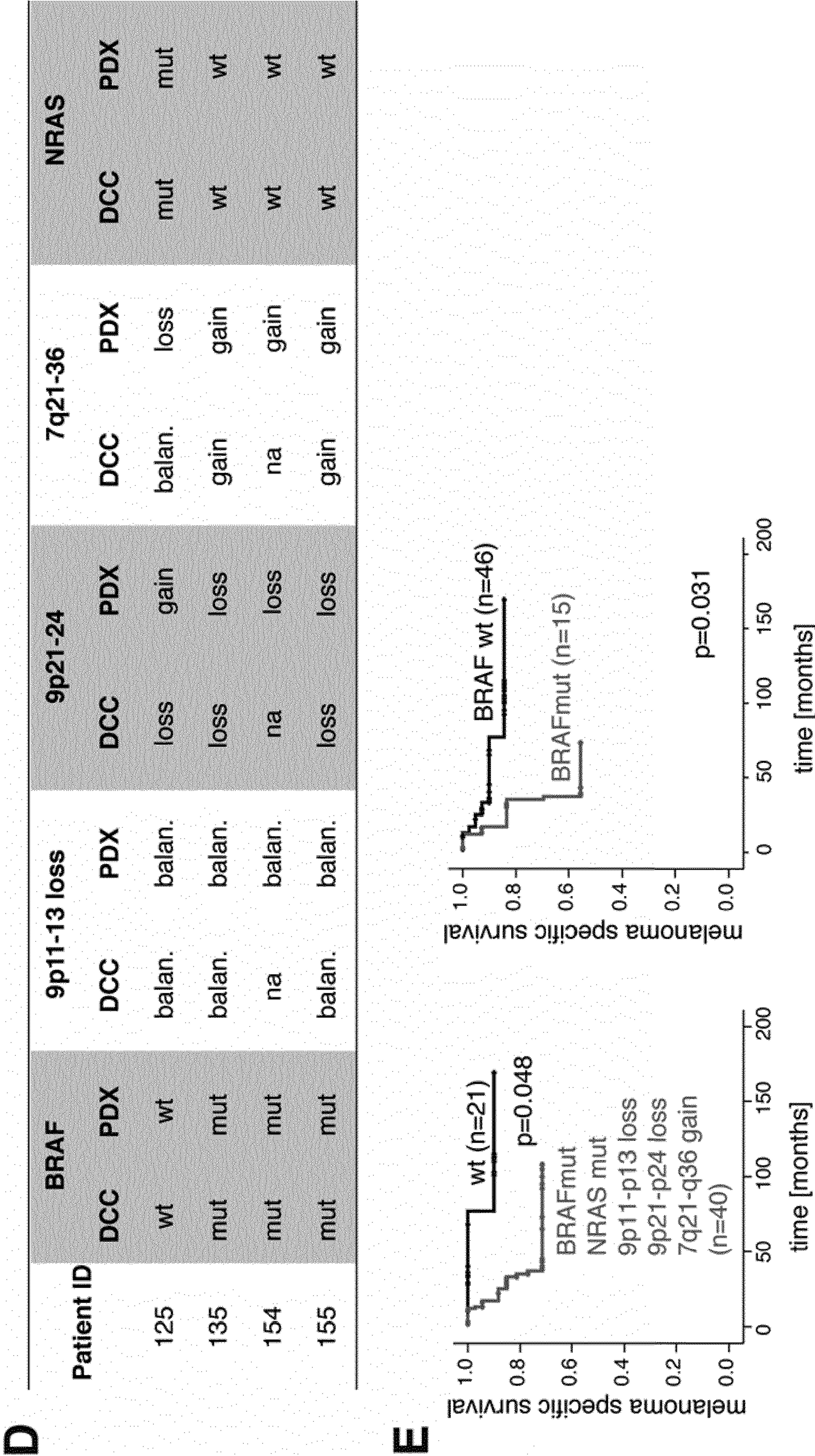


Figure 7

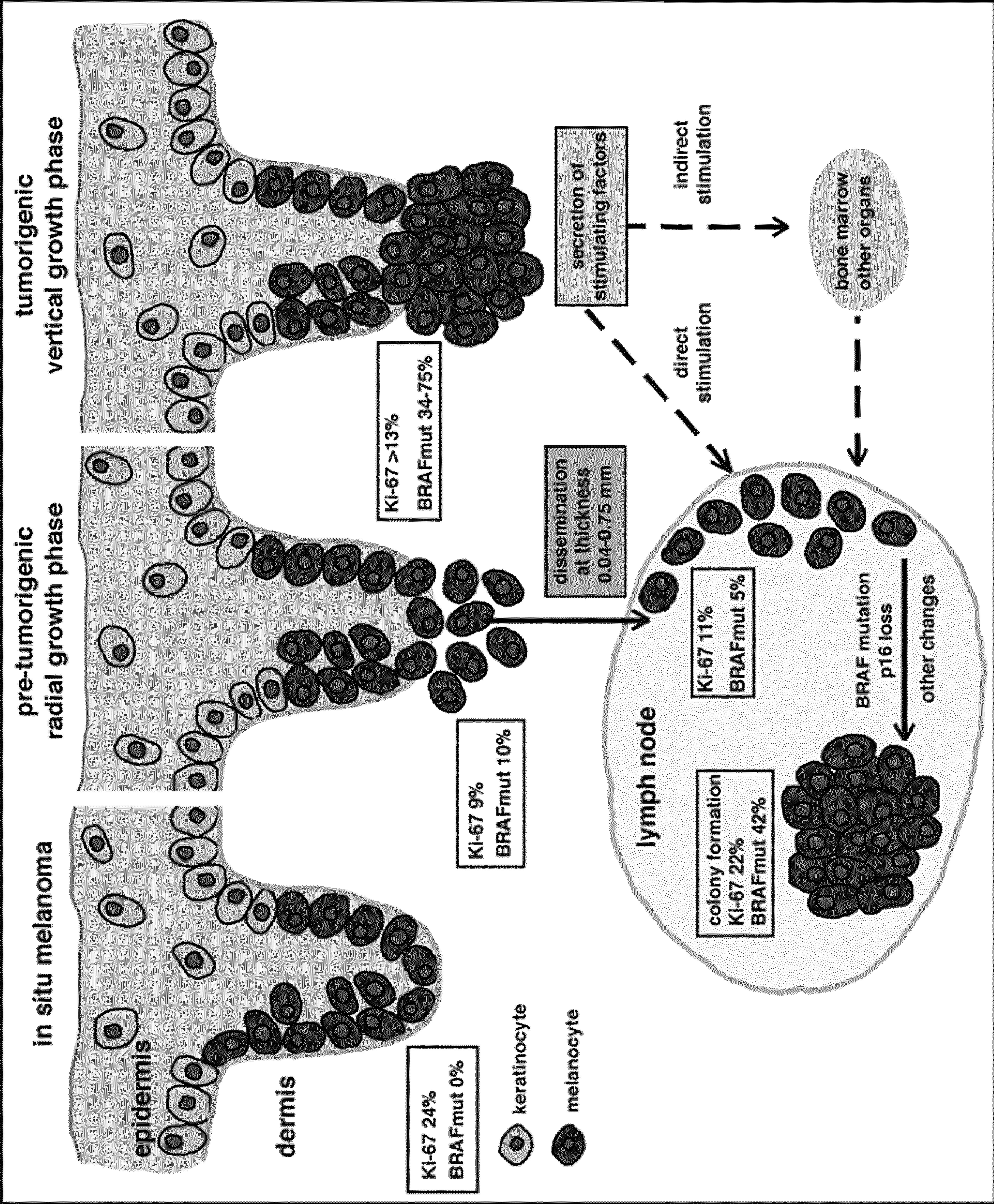
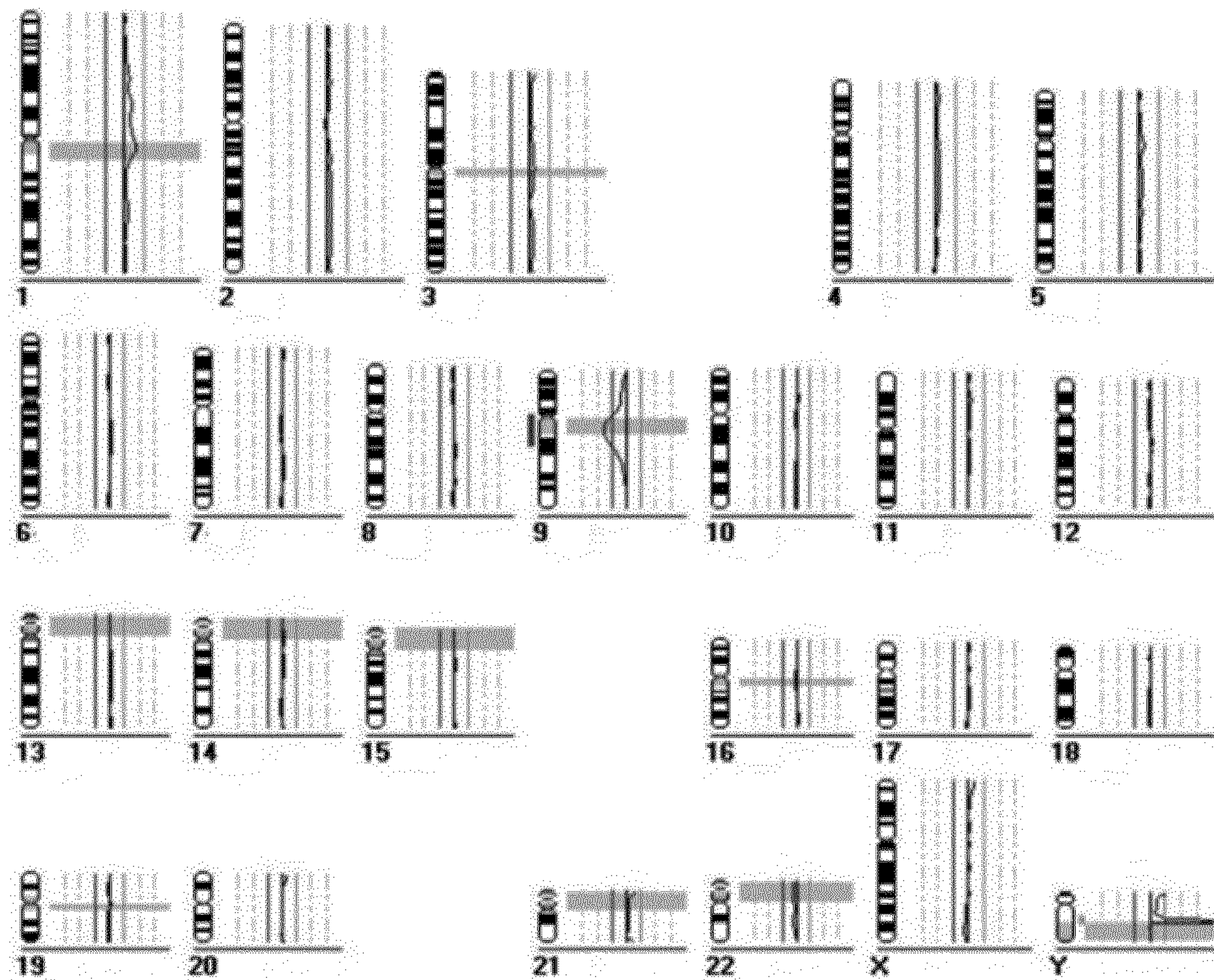
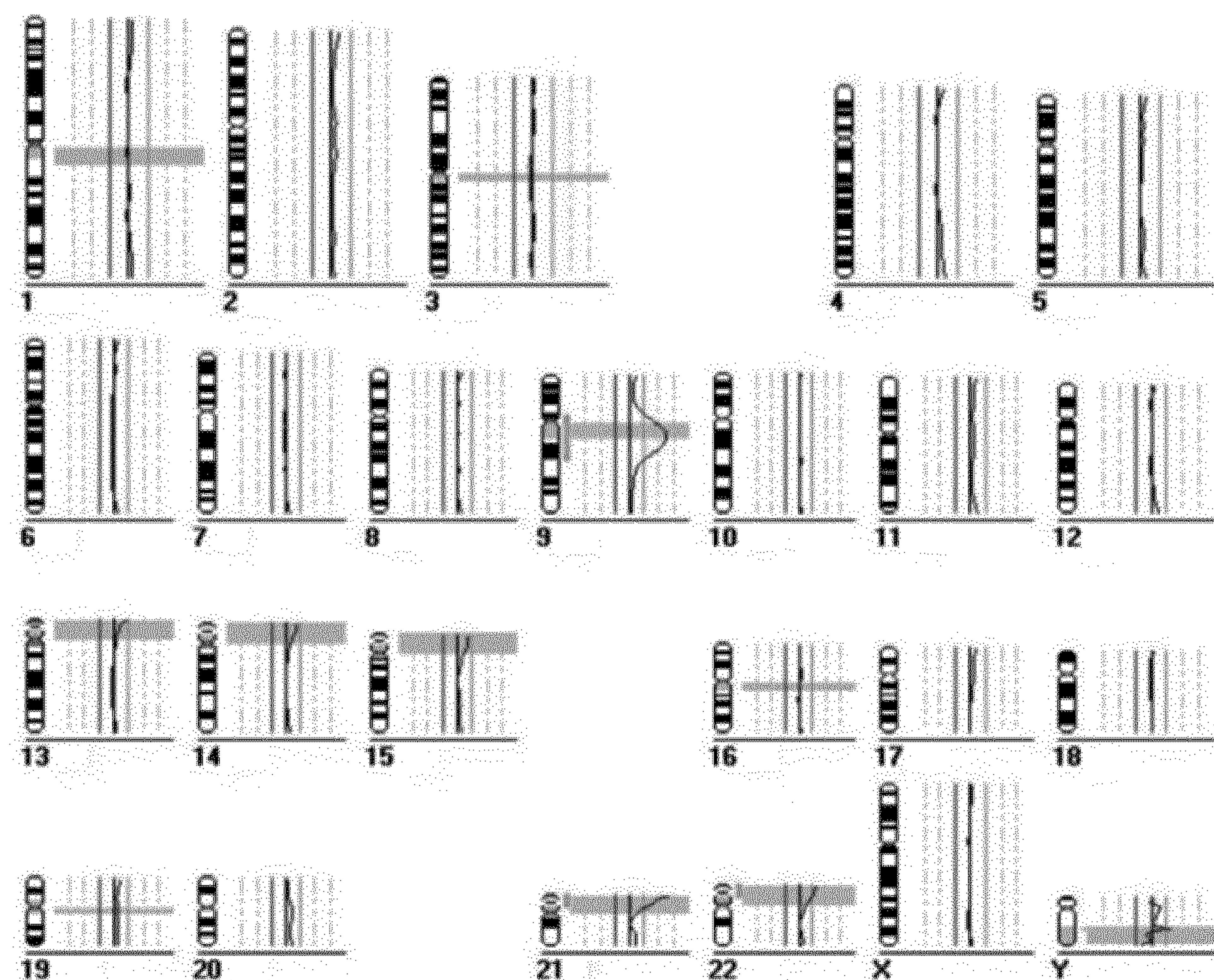


Figure 8

2316 KMN1**2349 KMN1**

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Figure 8 cont.

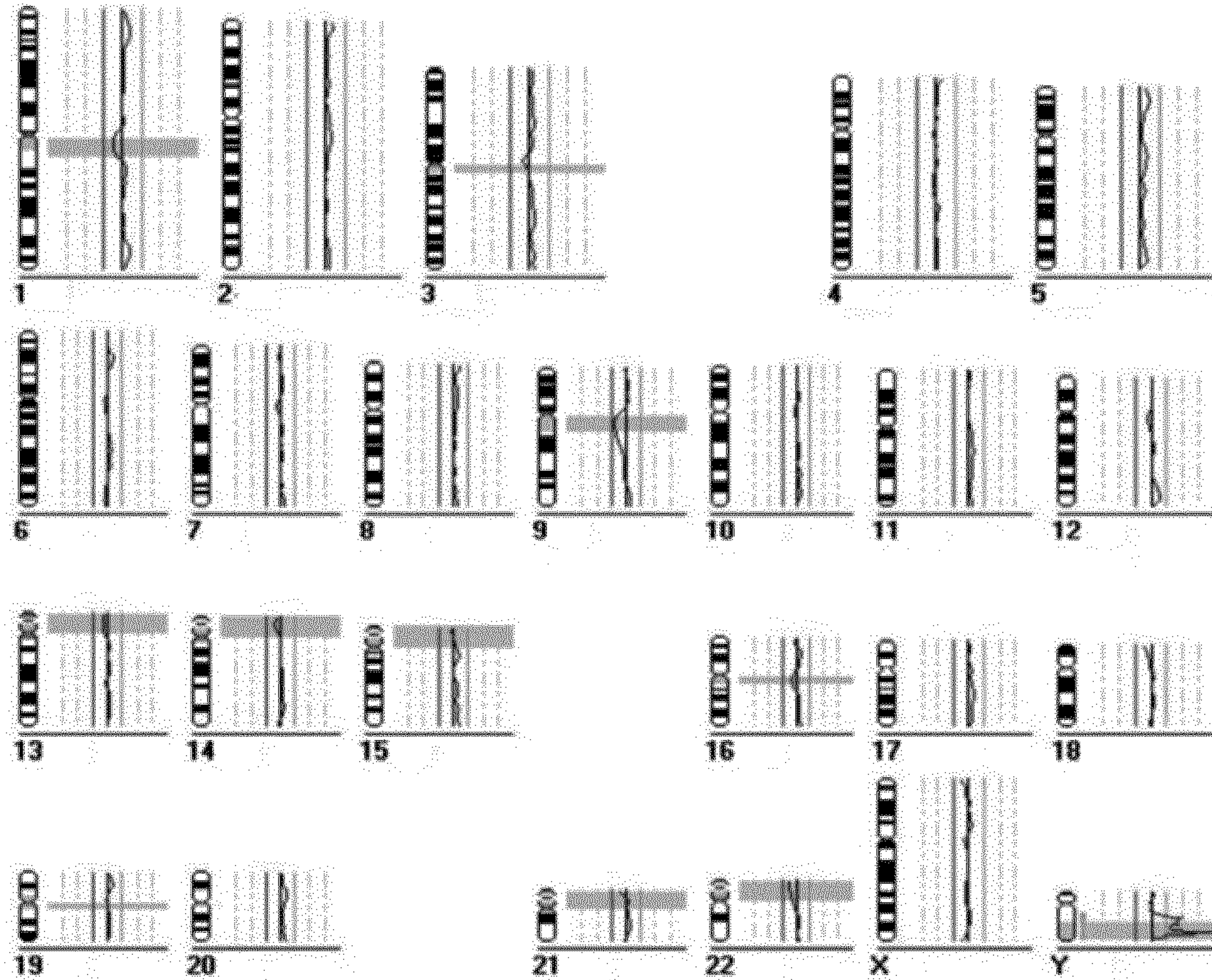
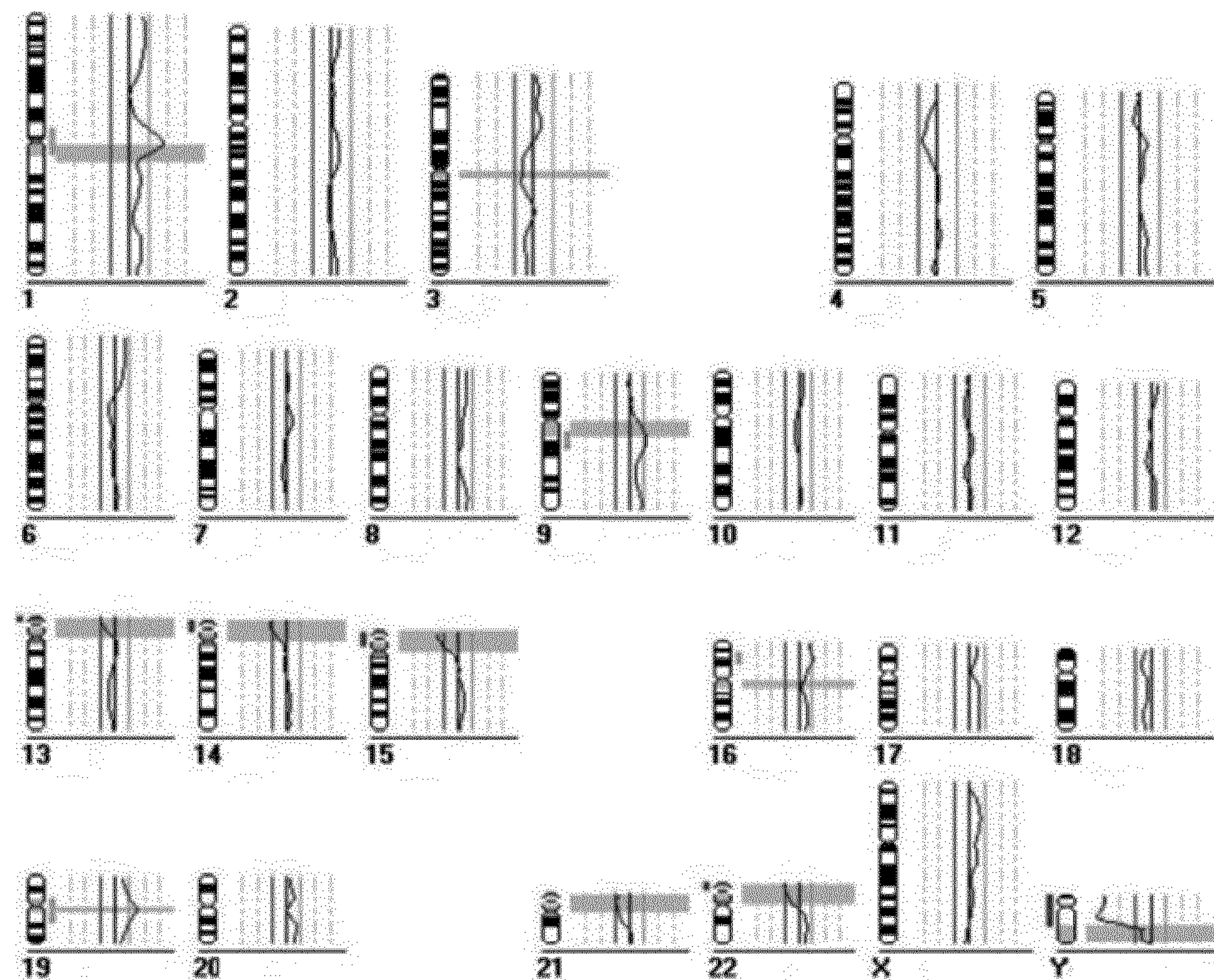
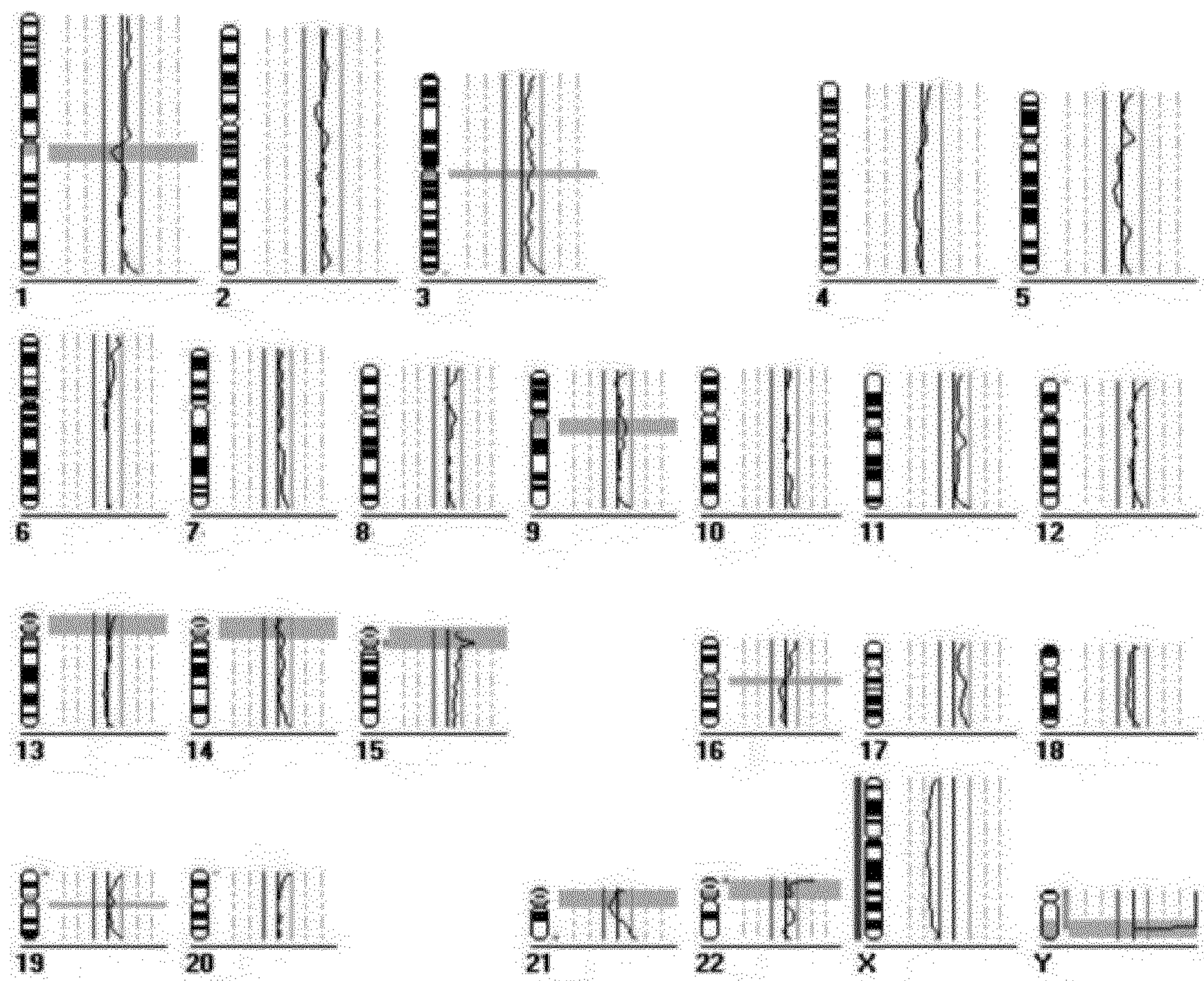
2337 KMN1**2349 KMN2**

Figure 8 cont.
2436 KMN1



2436 LKN1

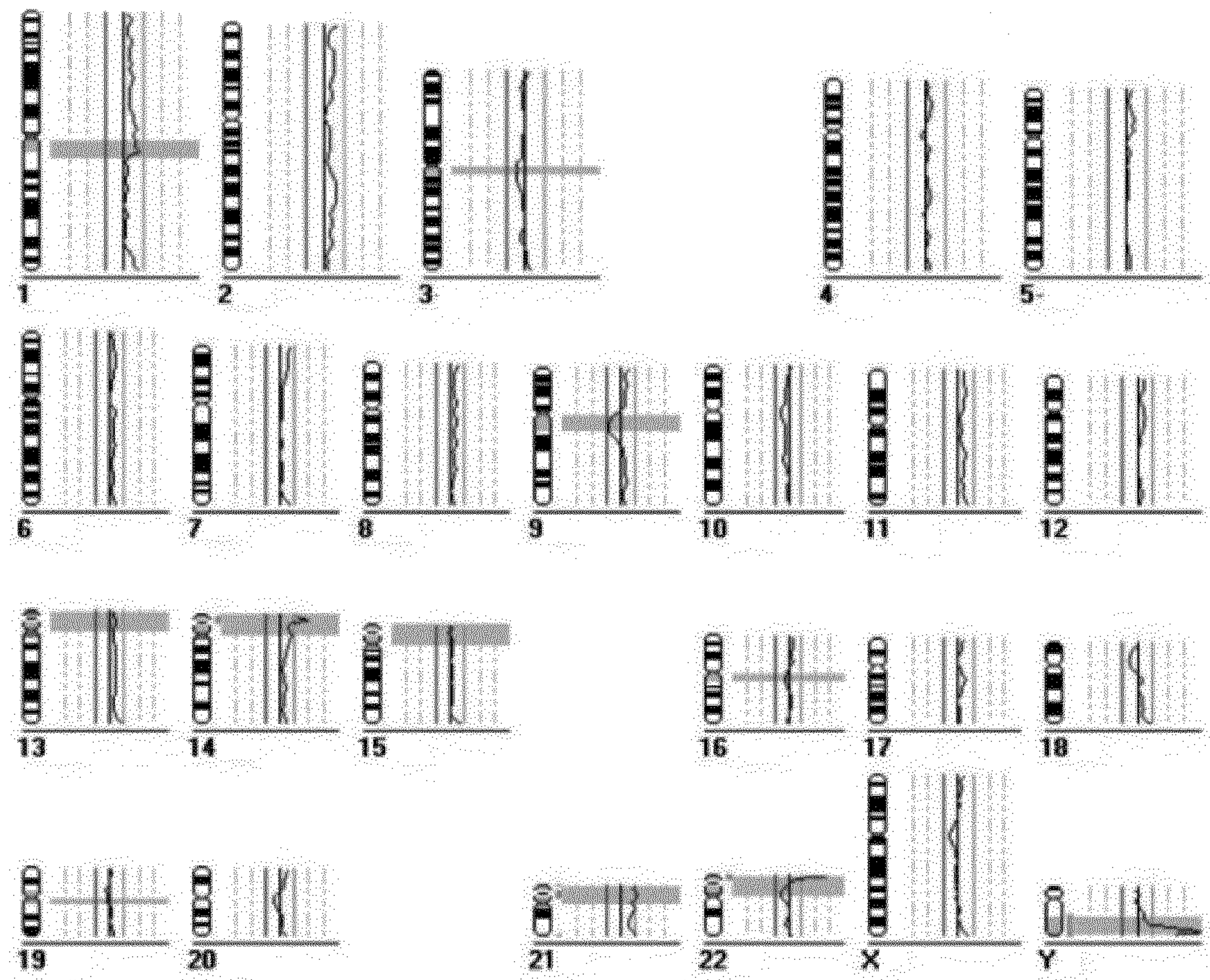
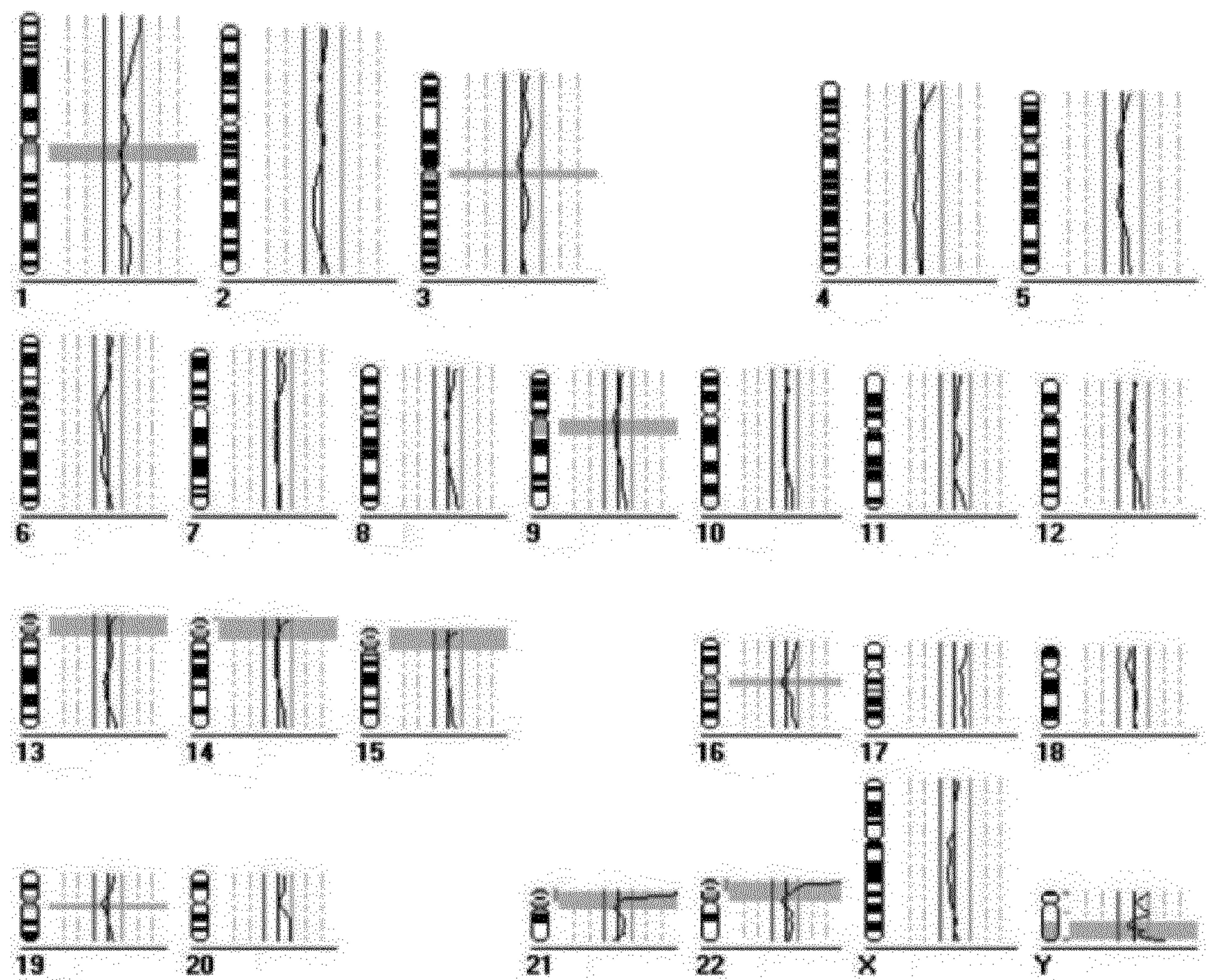
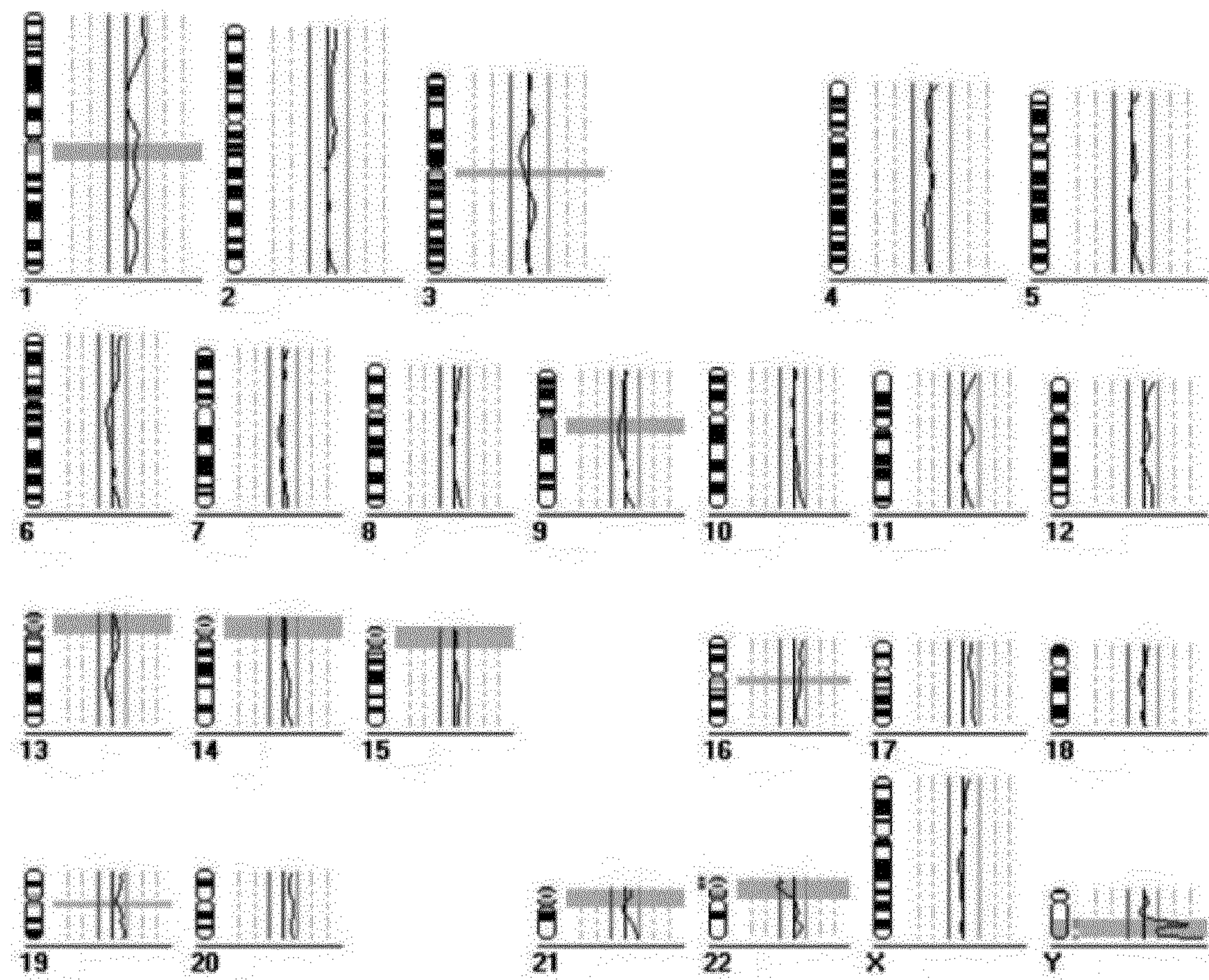


Figure 8 cont.
2436 KMN2

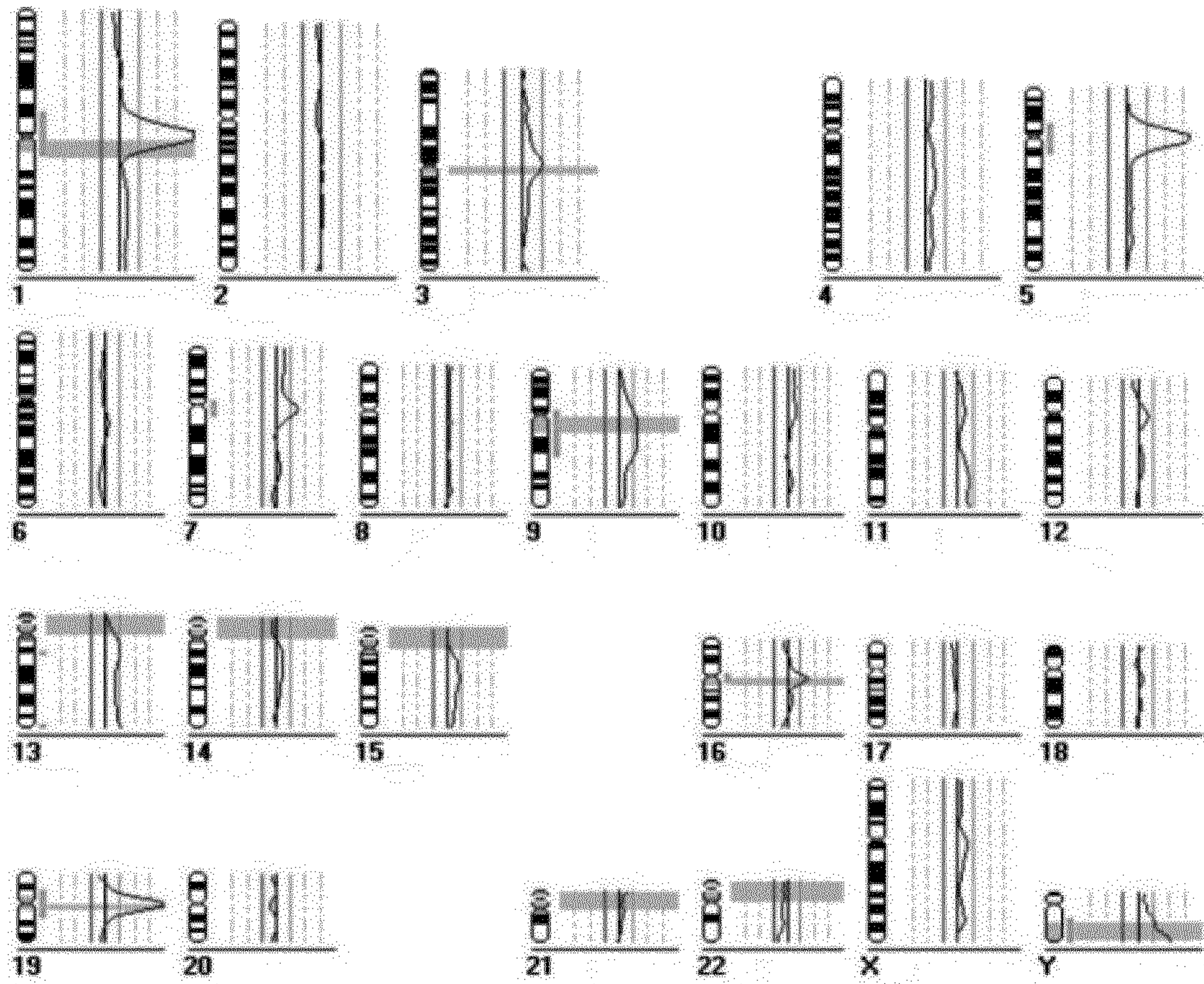
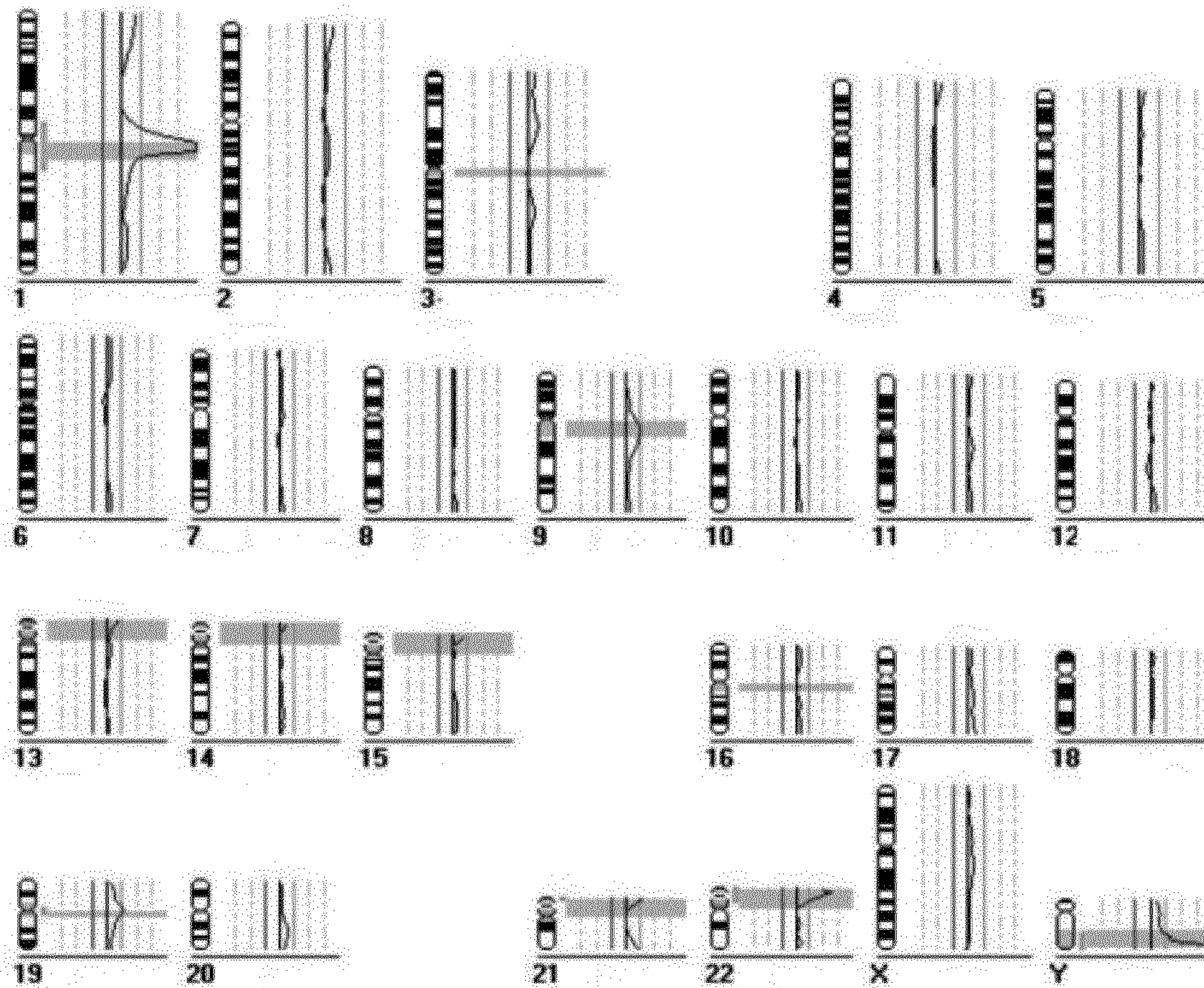


2501 KMN1



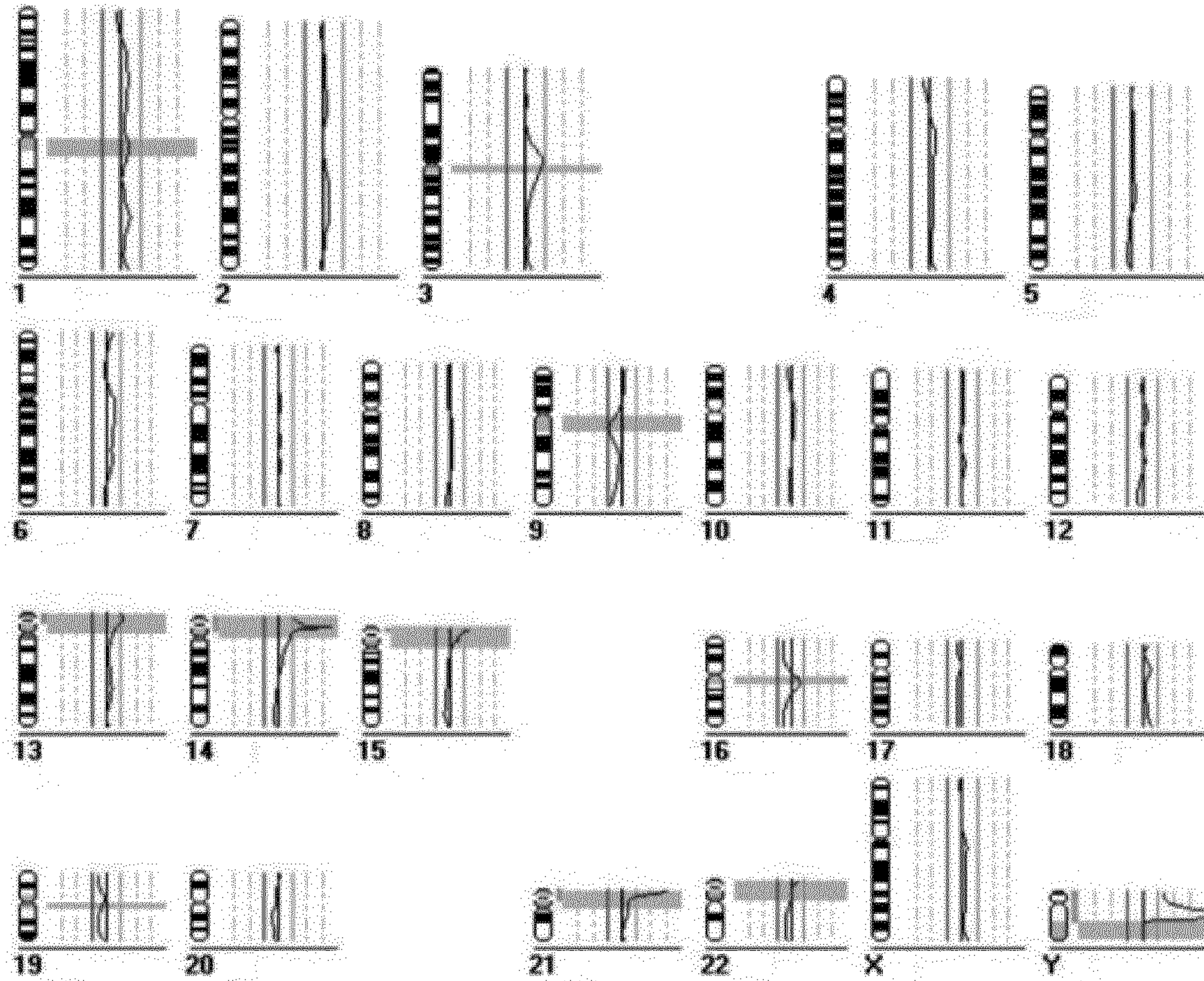
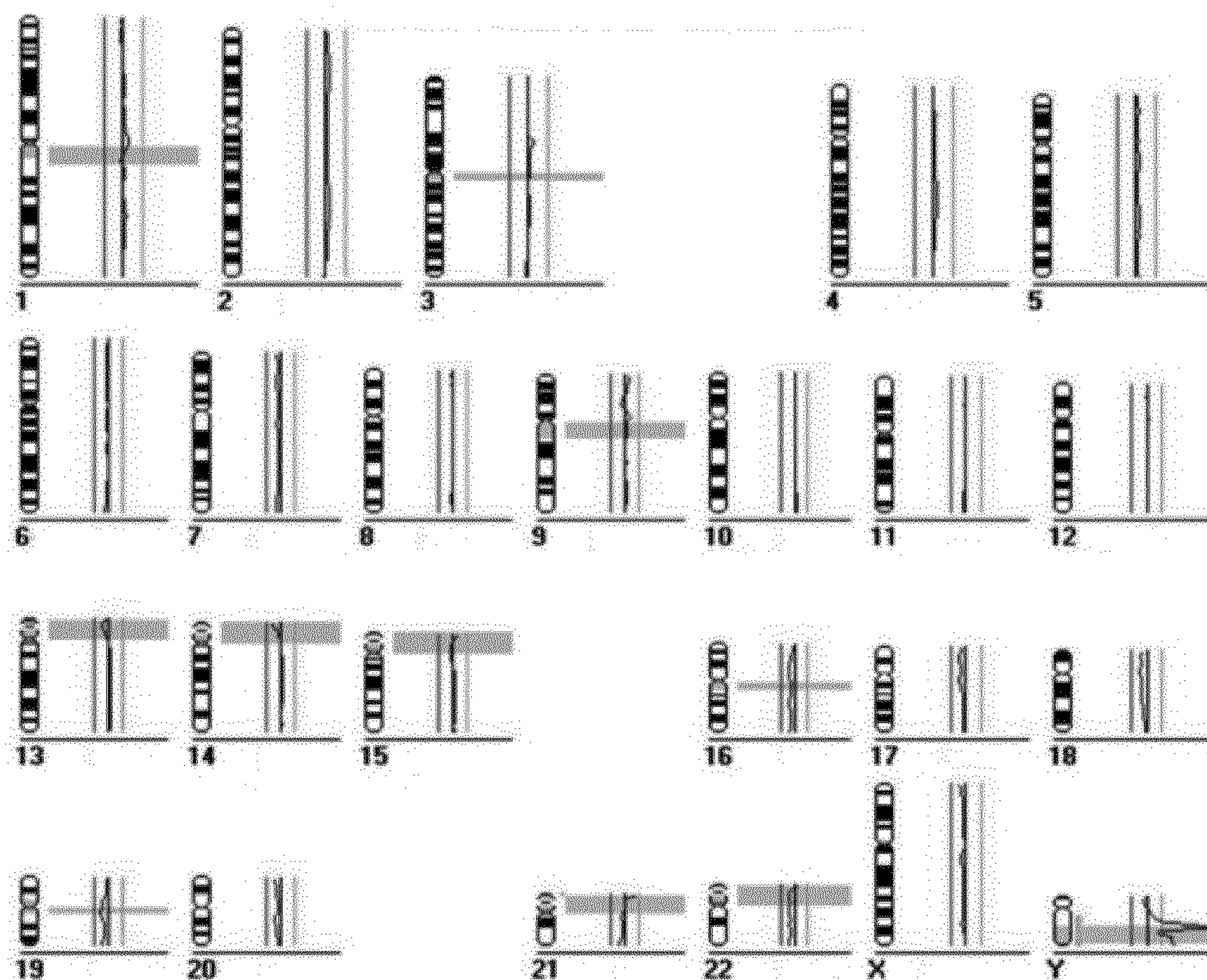
20/51

Figure 8 cont.

3214 LKN1**3425 KMN1**

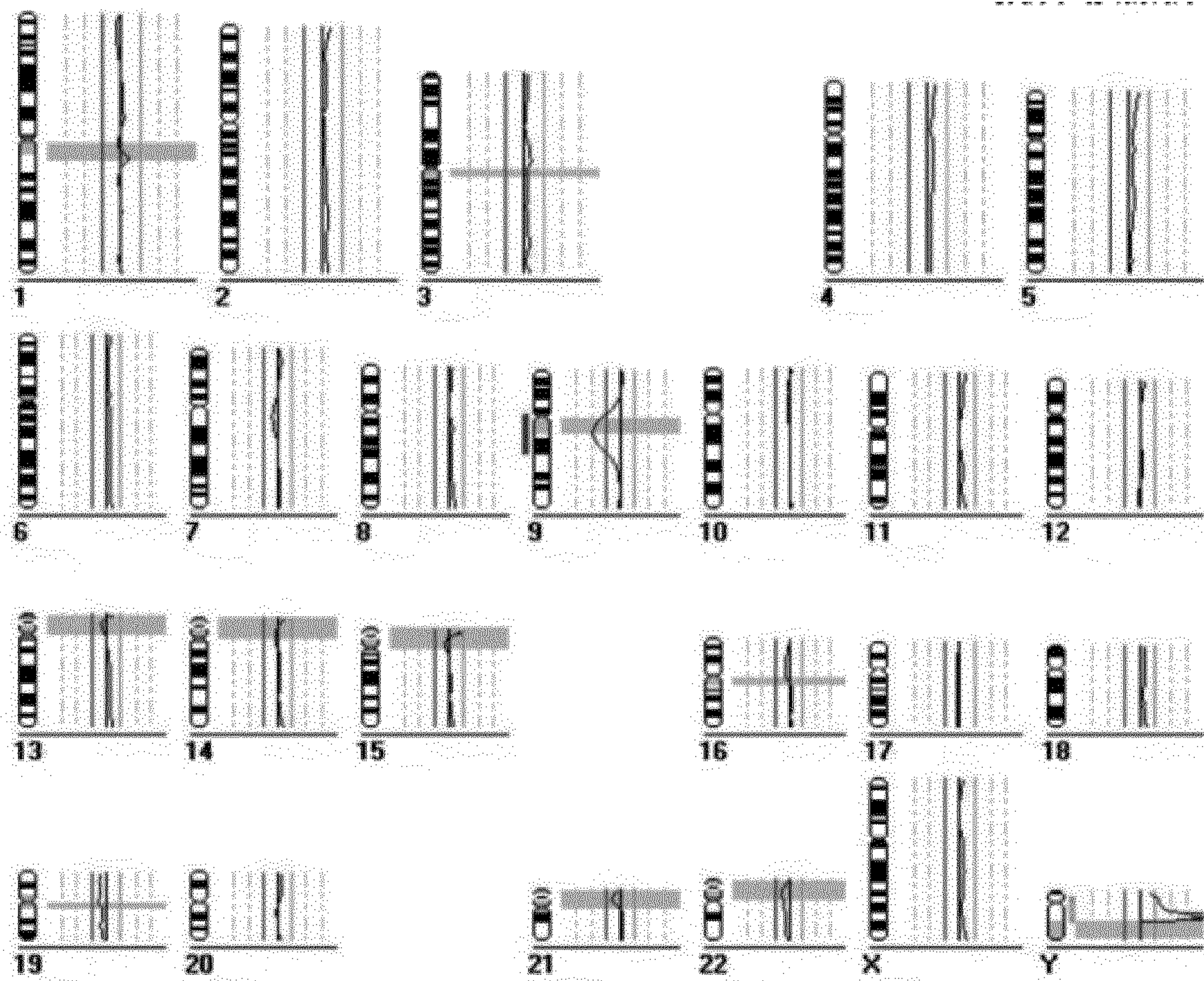
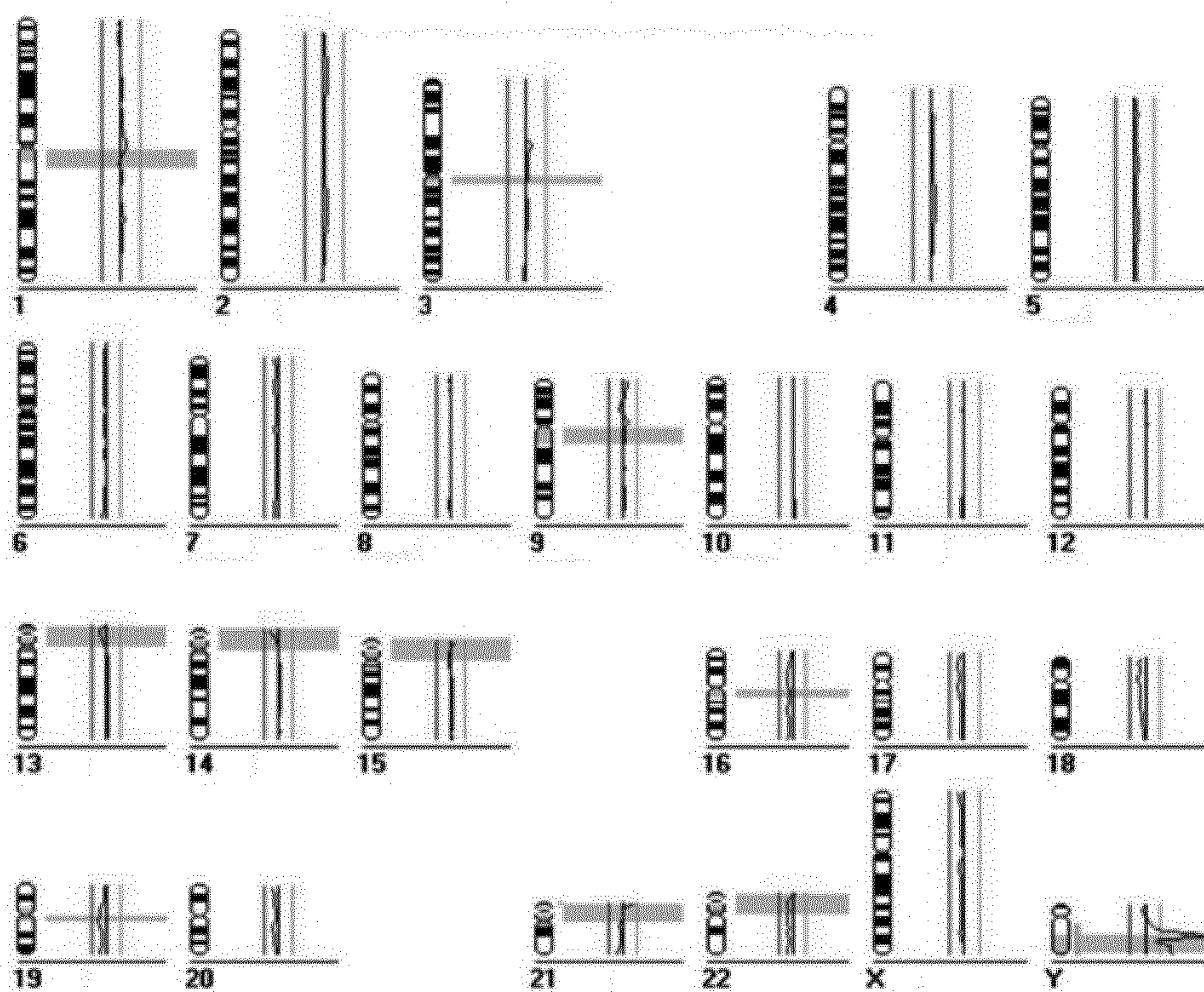
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Figure 8 cont.

3596 KMN1**778 NZ1**

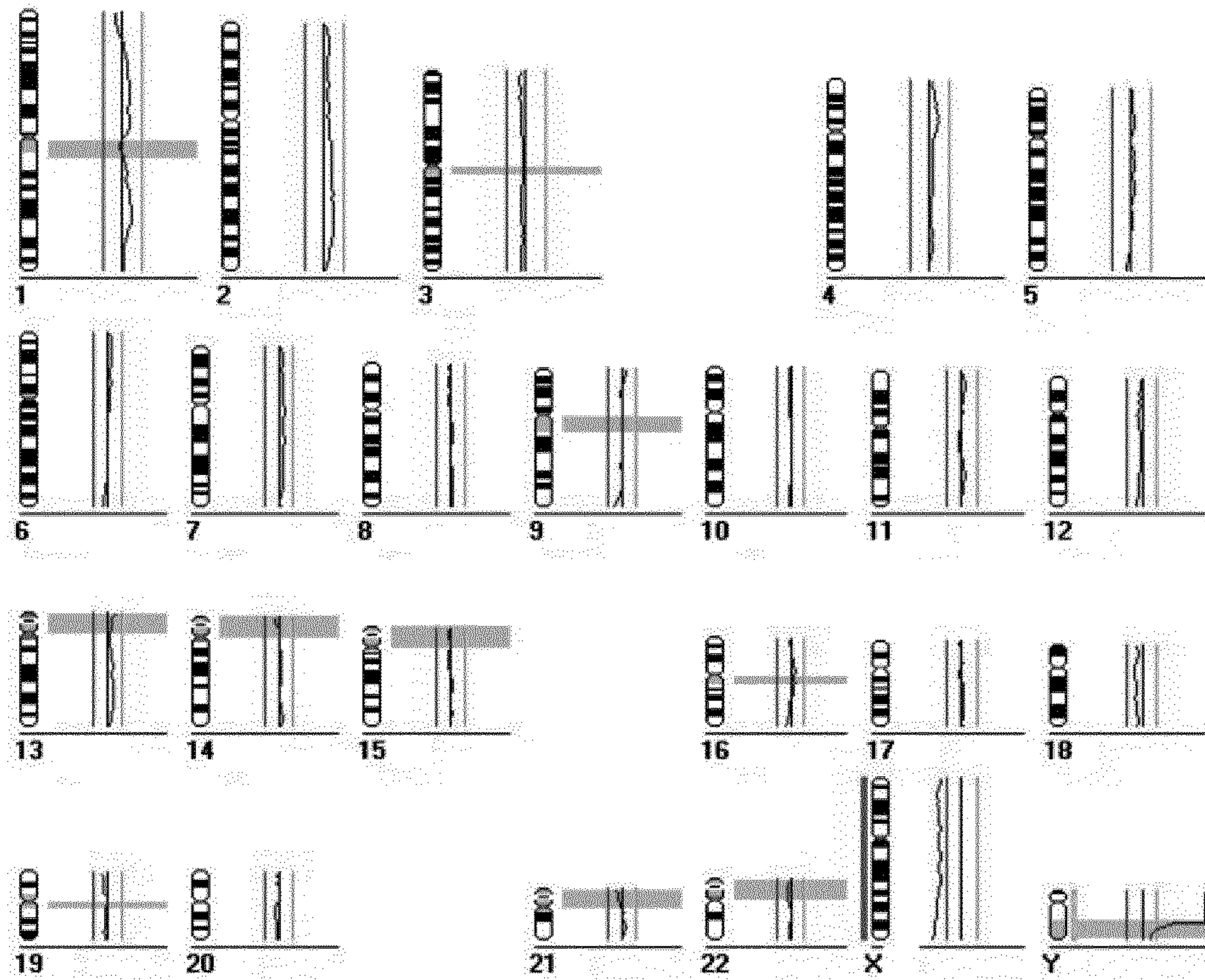
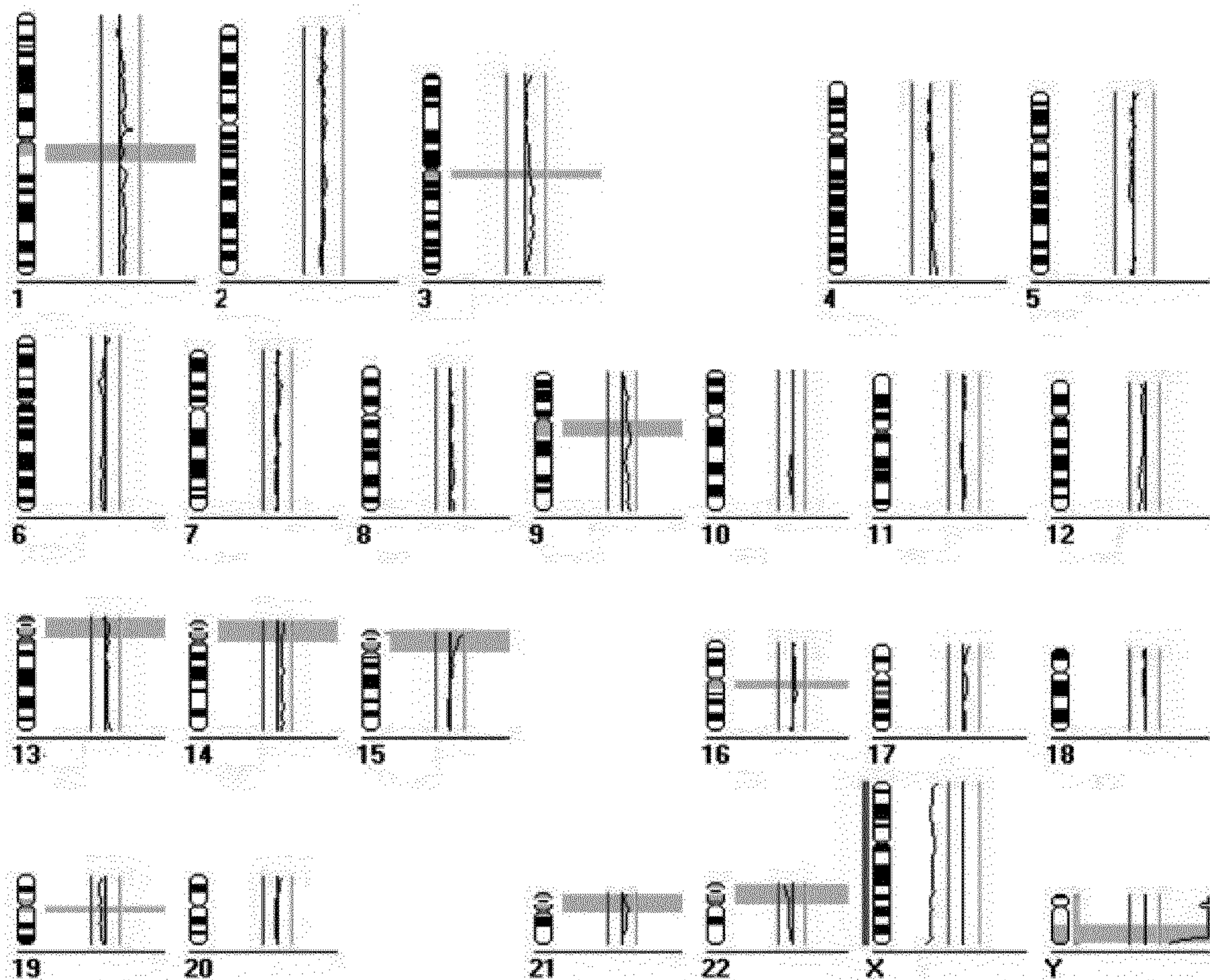
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Figure 8 cont.

3641 KMN2**778 NZ2**

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Figure 8 cont.

1032 NZ1**1032 NZ2**

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Figure 8 cont.

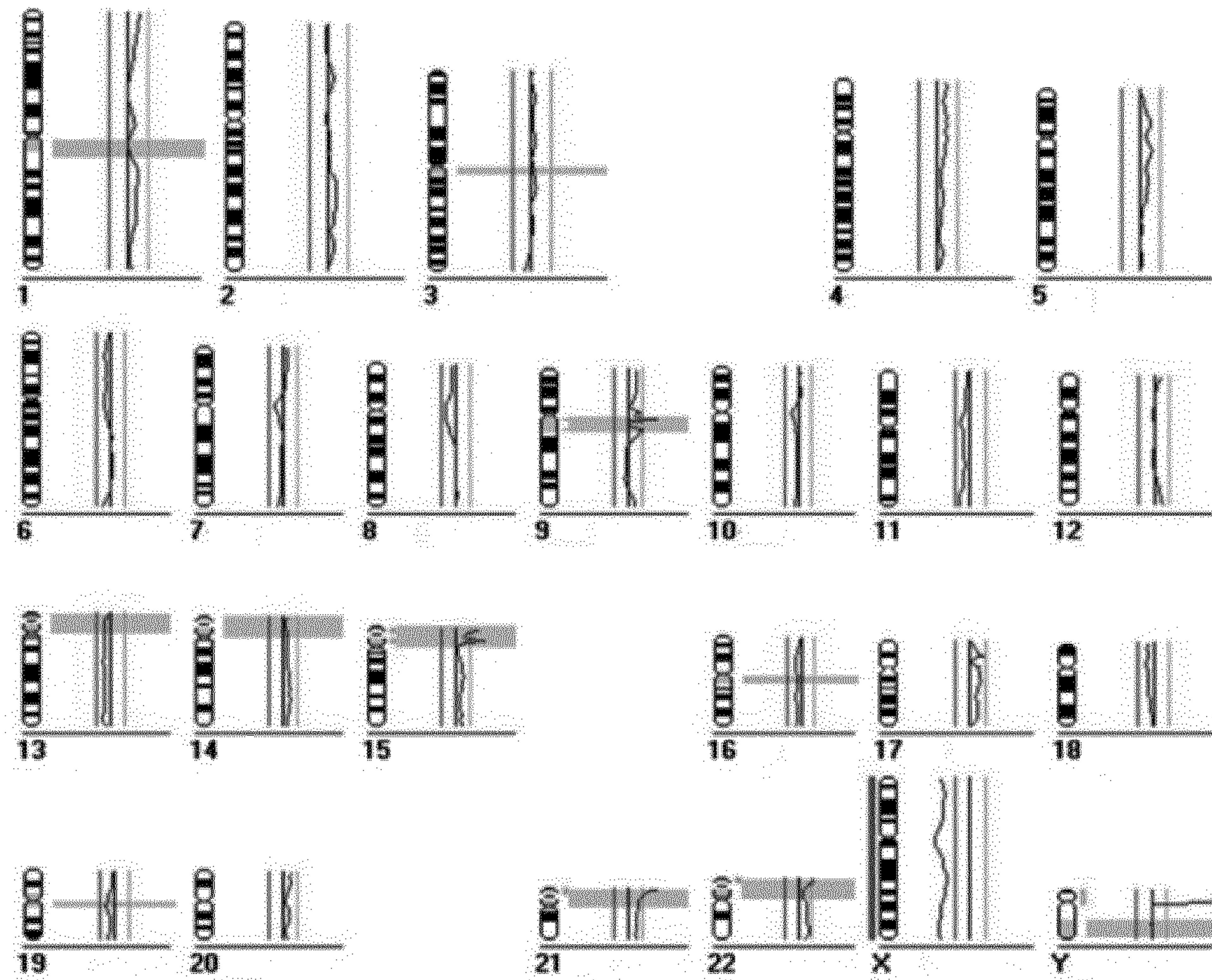
1077 NZ2

Figure 8 cont.

LK 16 N2

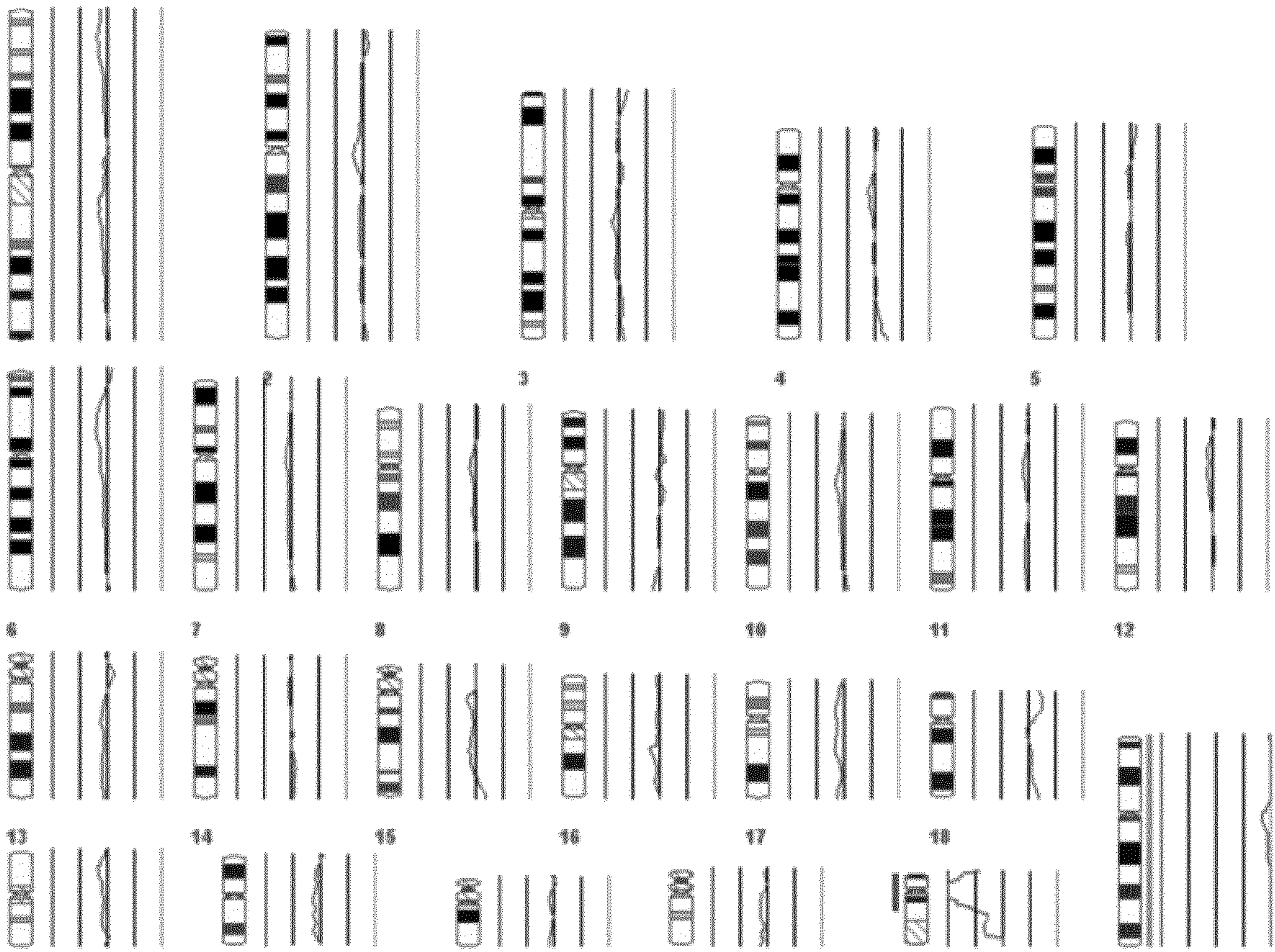


Figure 8 cont.

LK 16 N1

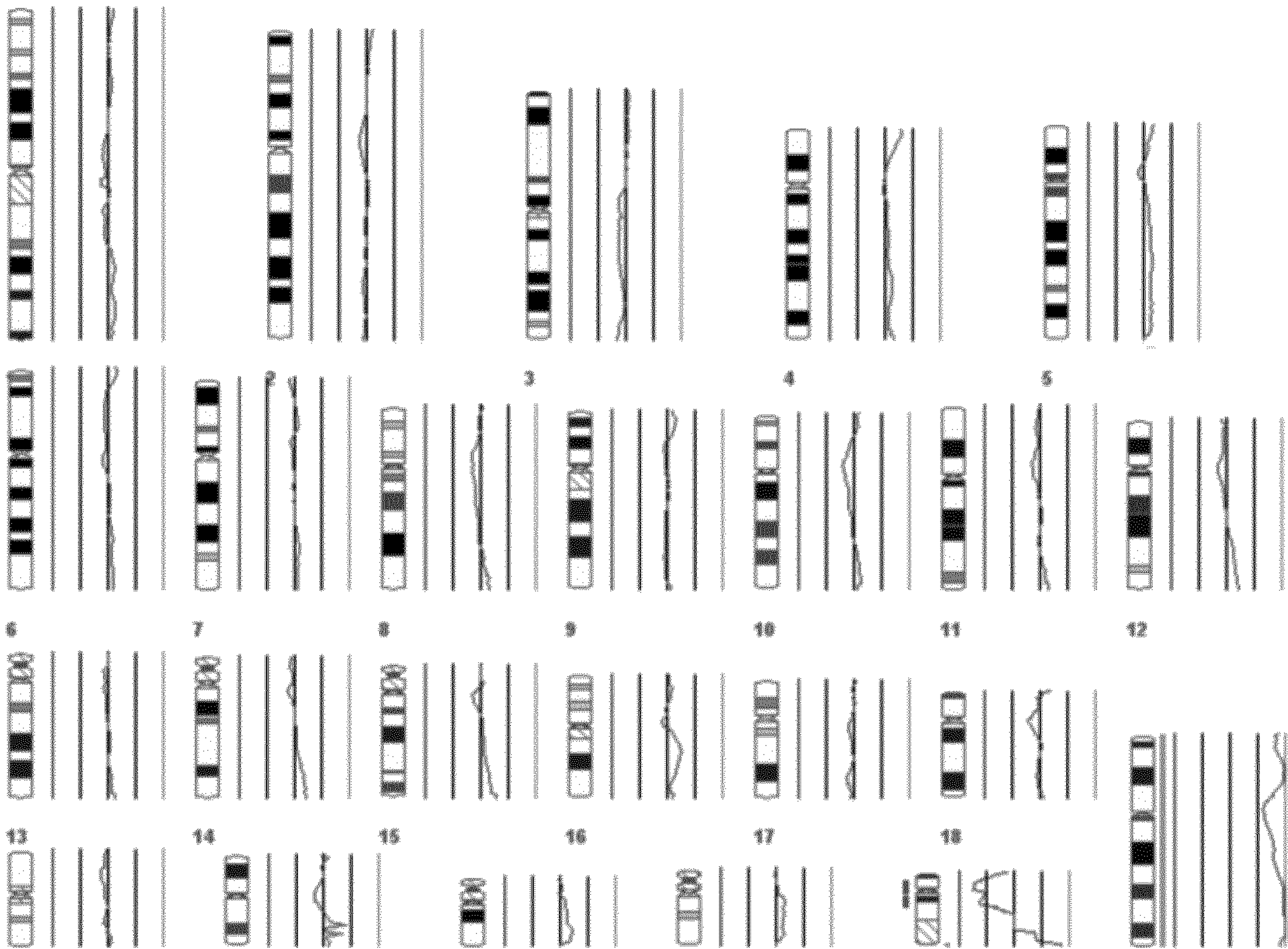


Figure 8 cont.
LK 16 N3

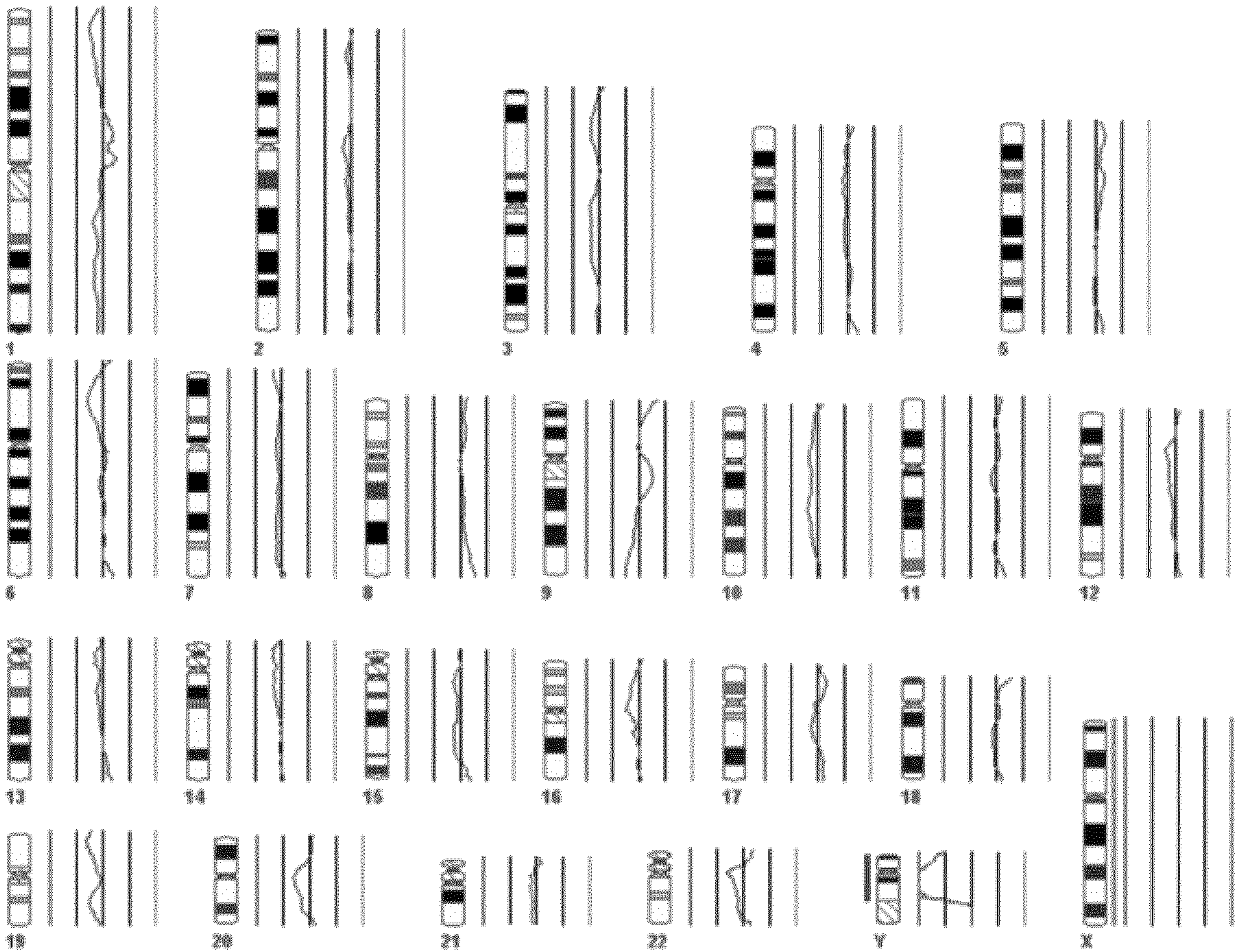


Figure 8 cont.

RPC 94 N

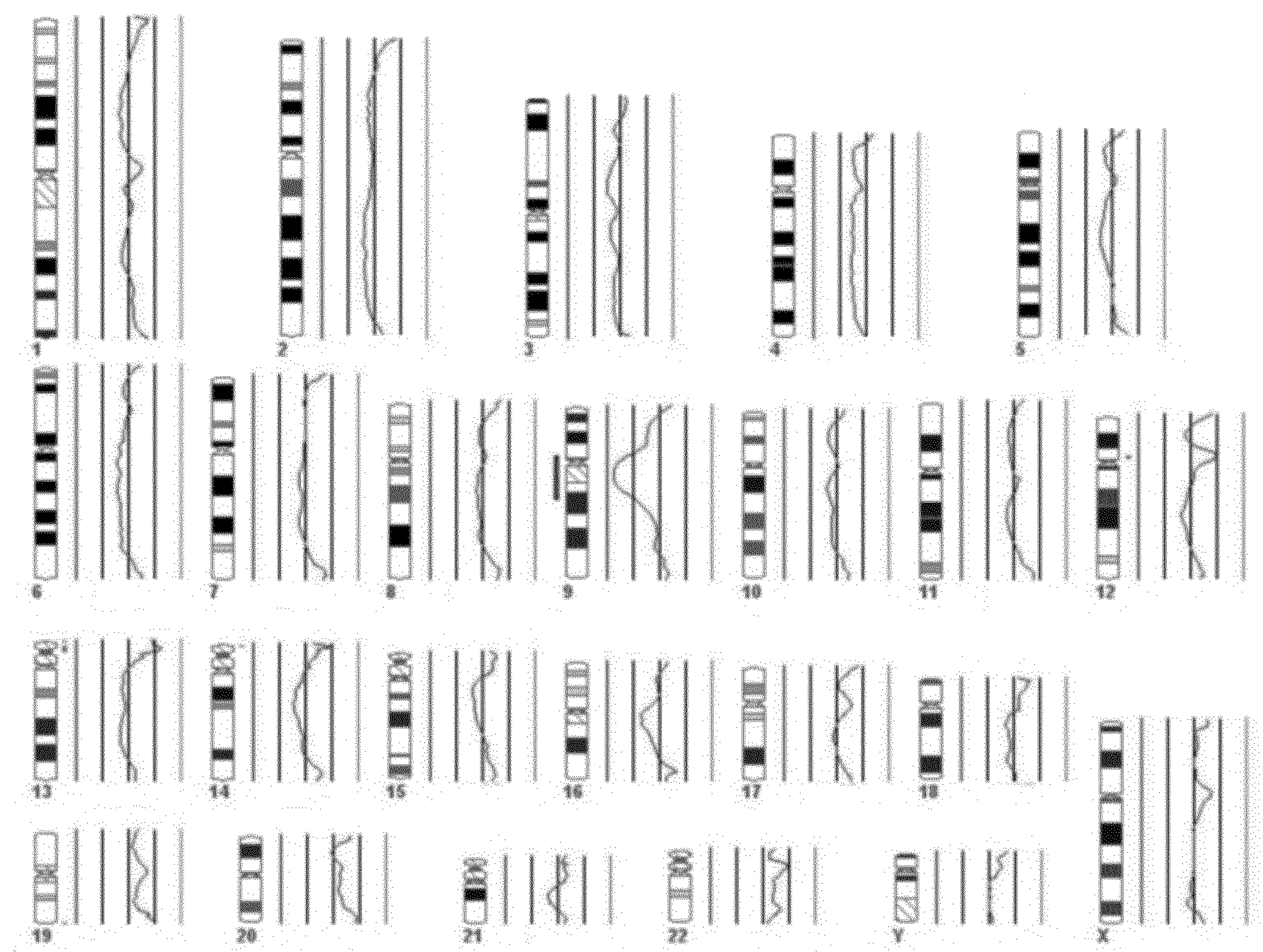


Figure 8 cont.

114 N

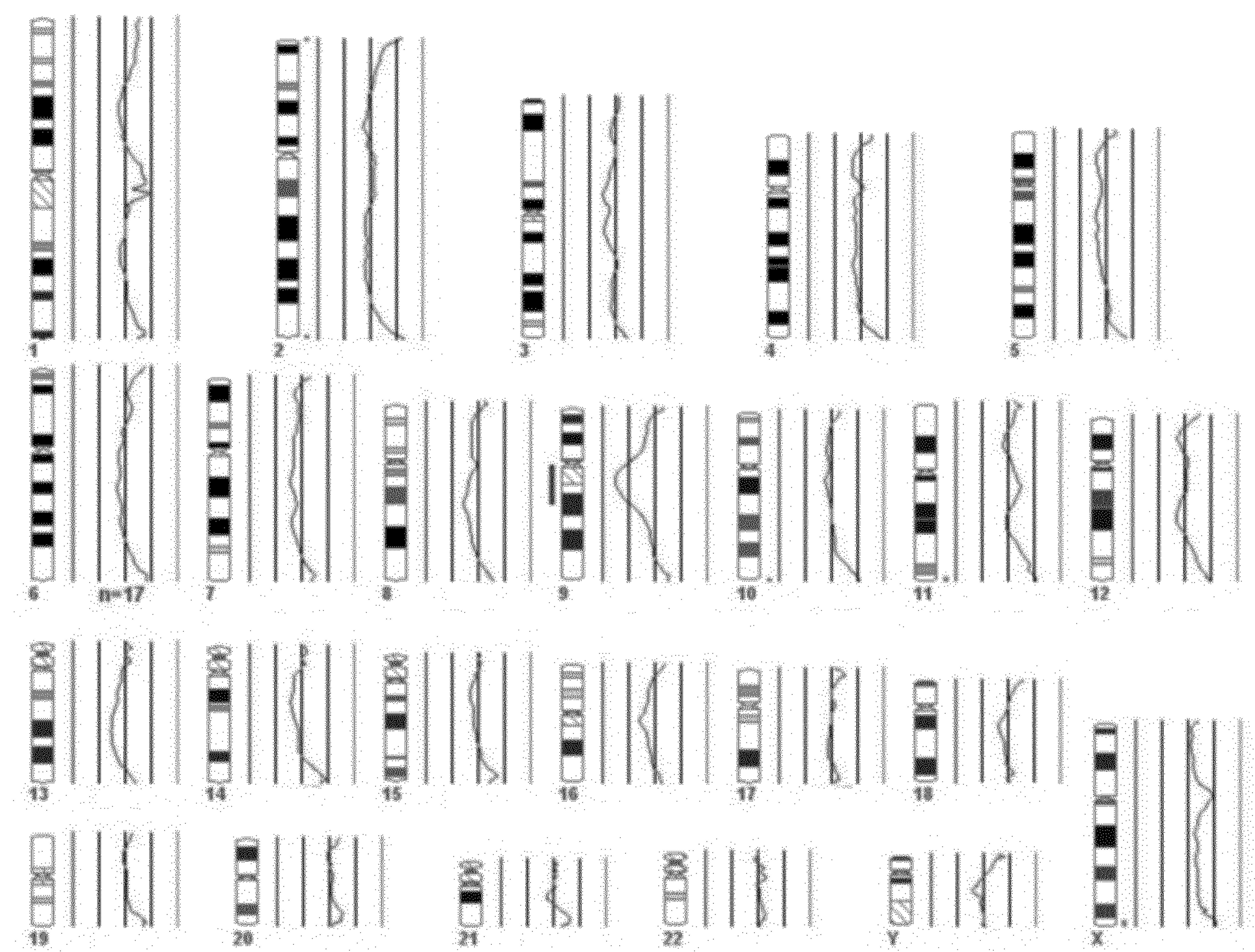


Figure 8 cont.
856-3b

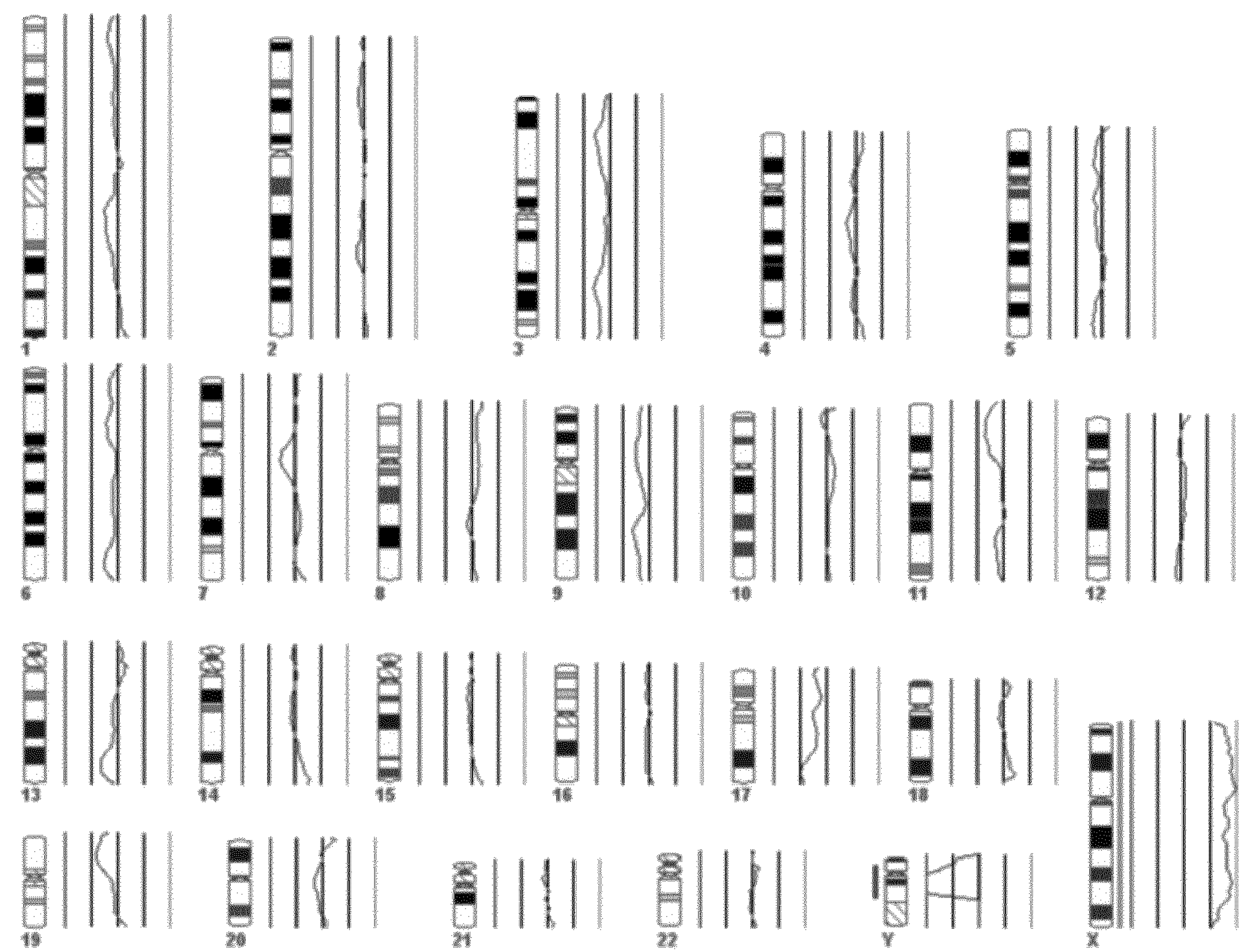


Figure 8 cont.

983-9SMb16

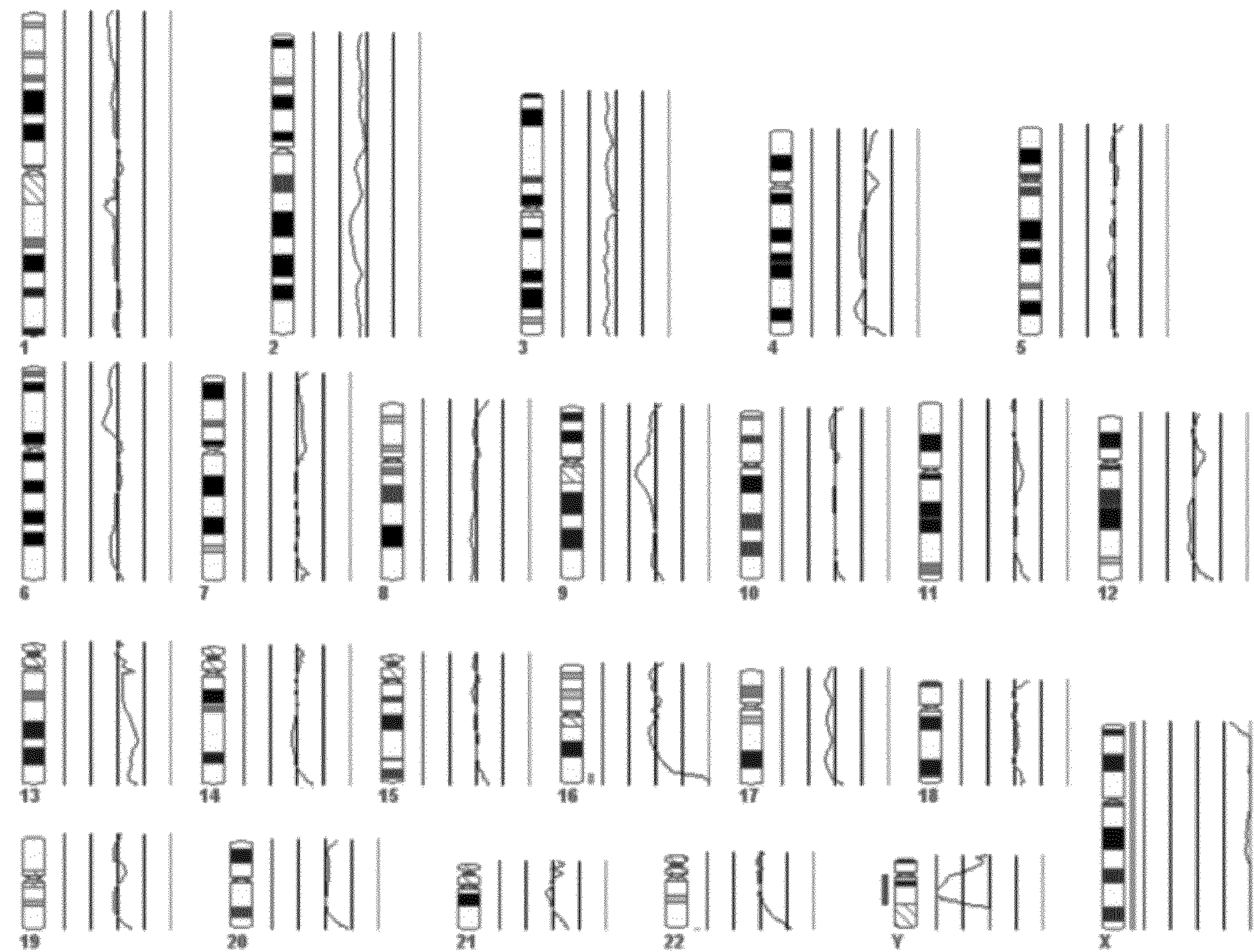


Figure 8 cont.

741-9SMb

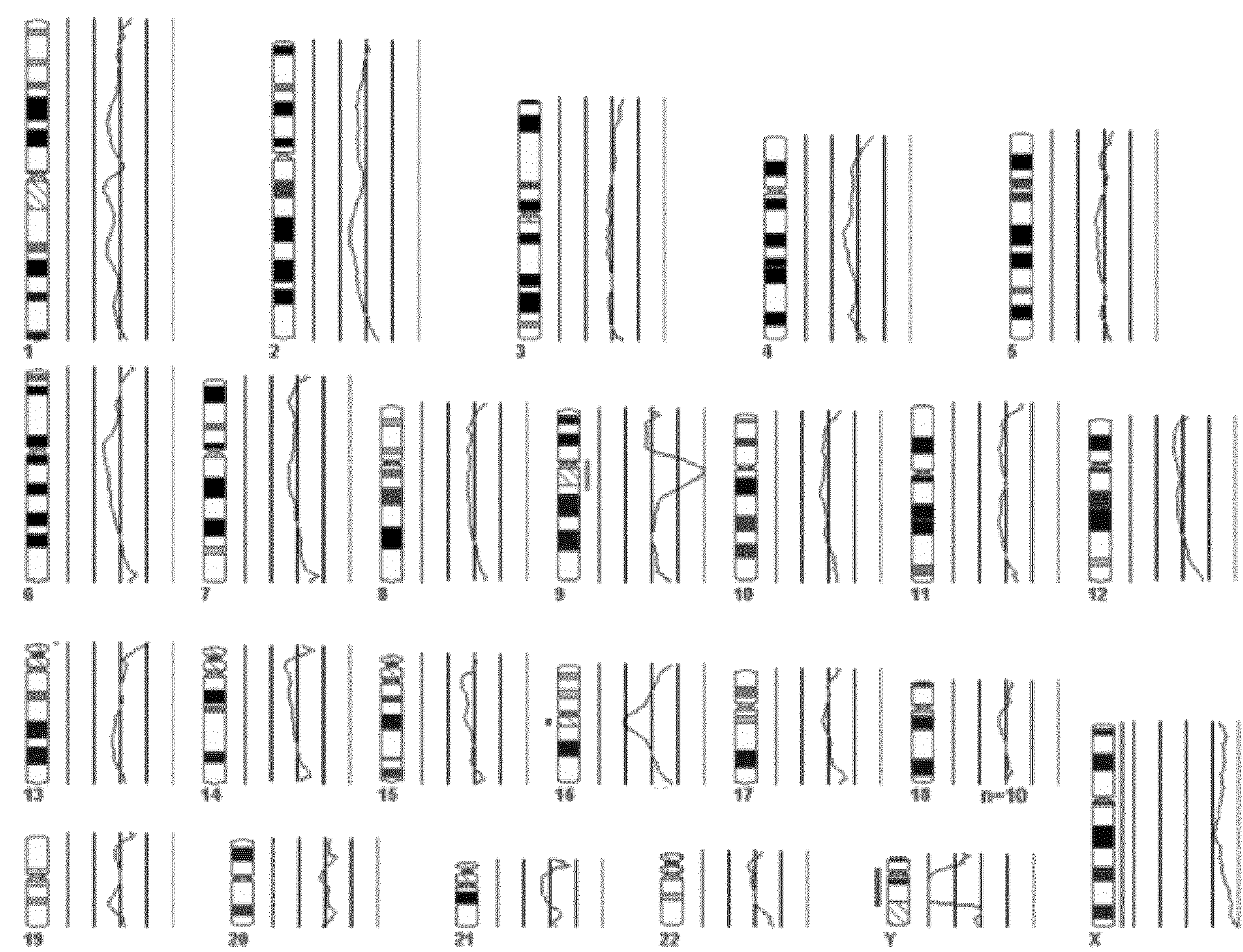


Figure 8 cont.

PBL SC 1

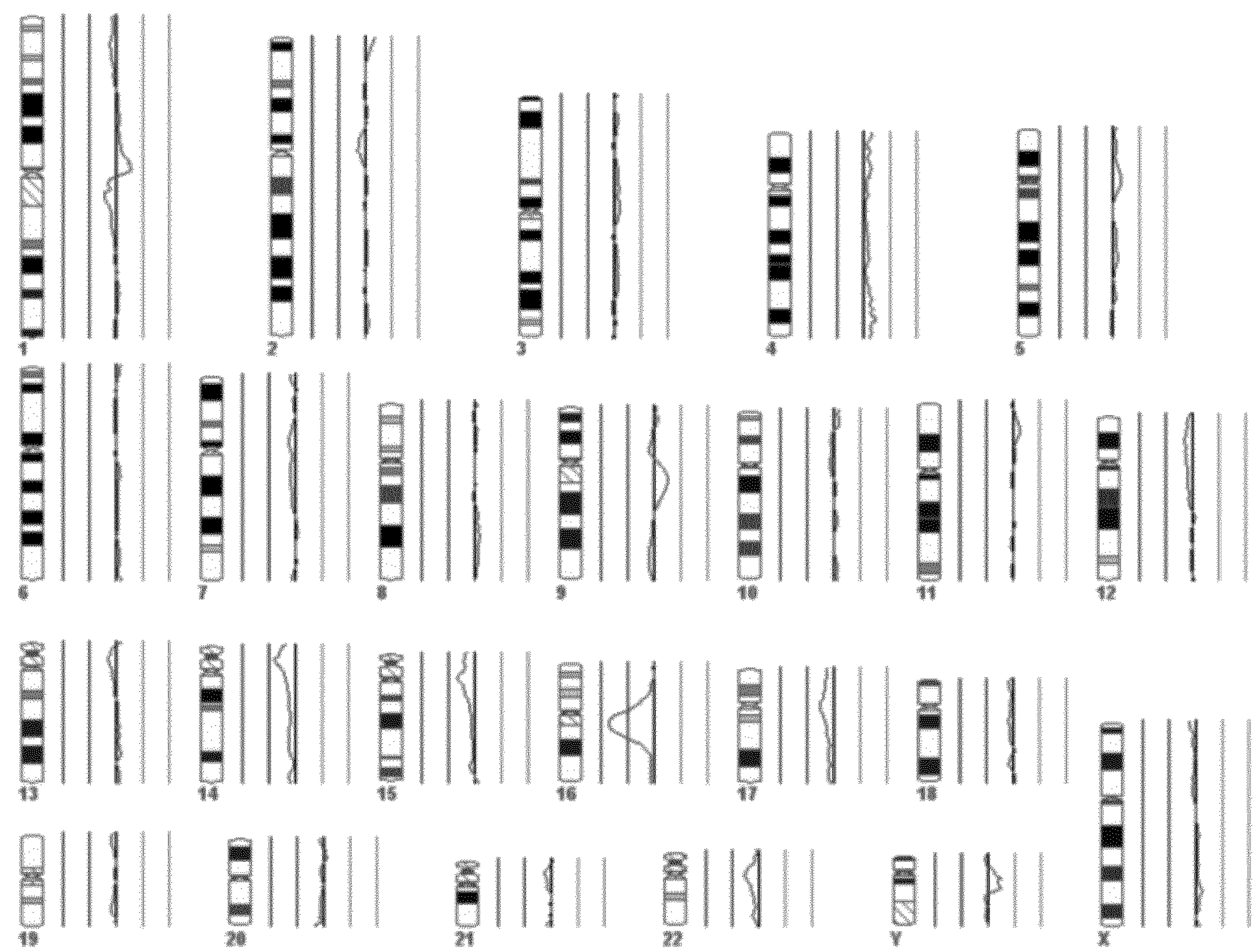


Figure 8 cont.

PBL SC 2

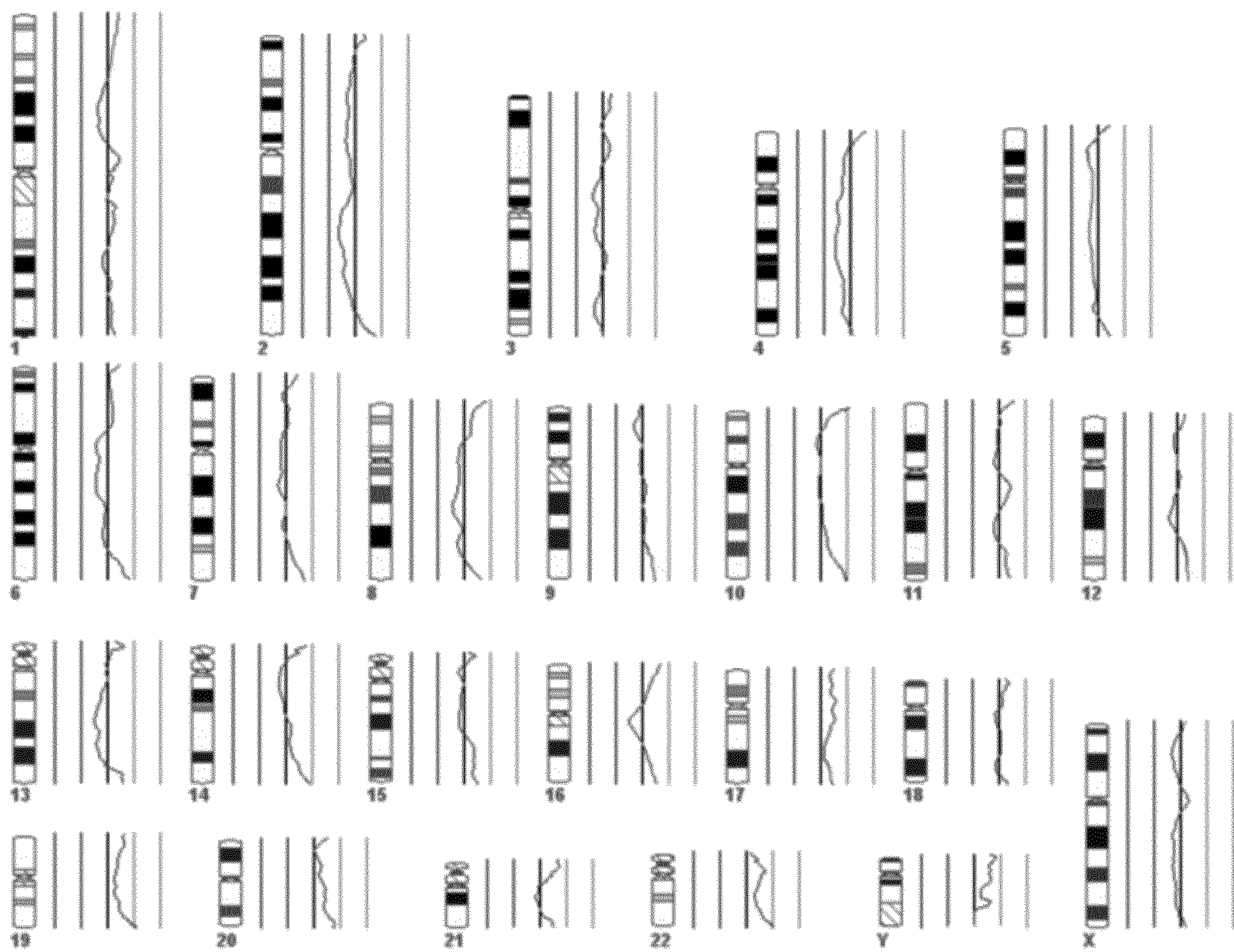


Figure 8 cont.

PBL SC 3

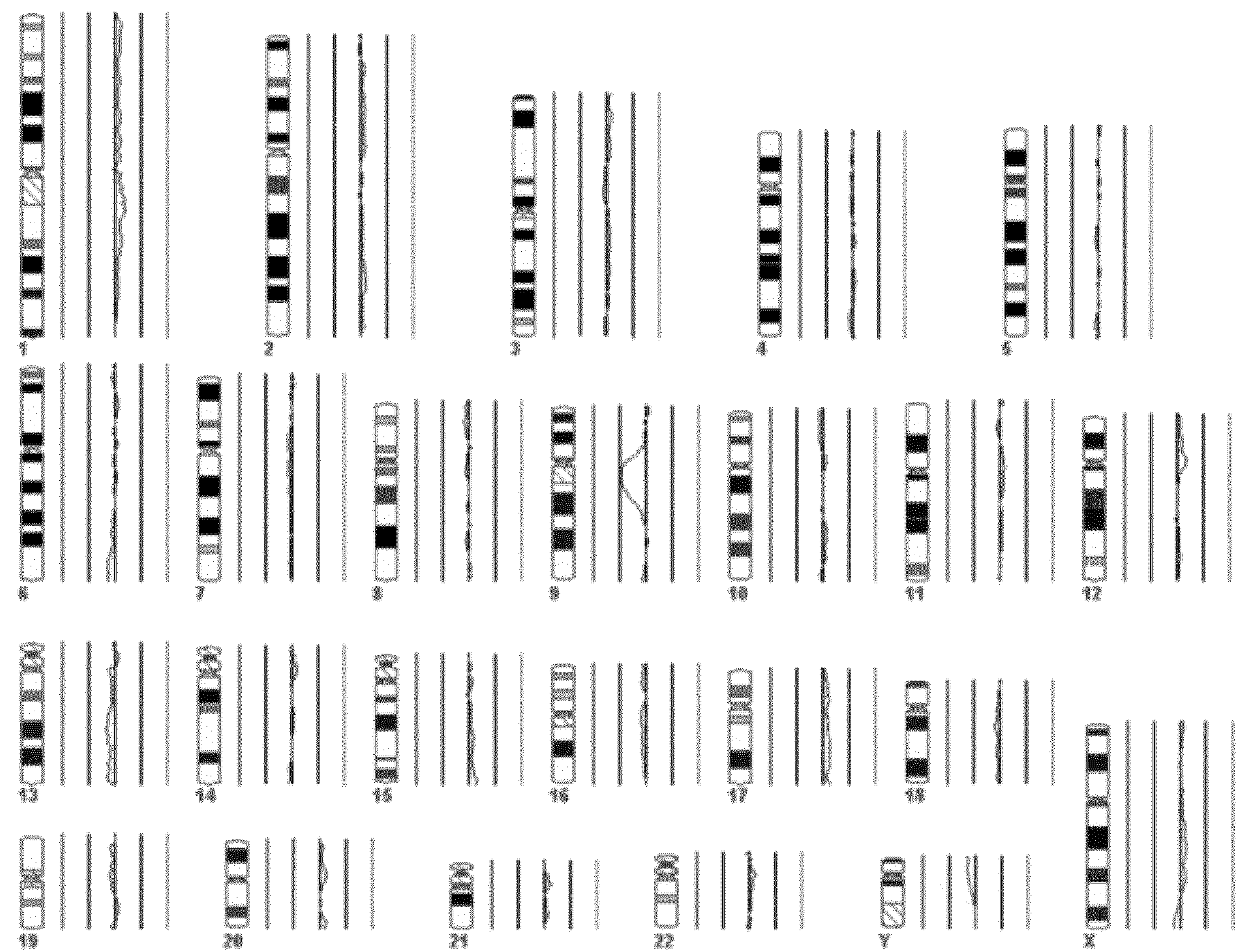


Figure 8 cont.

PBL SC 4

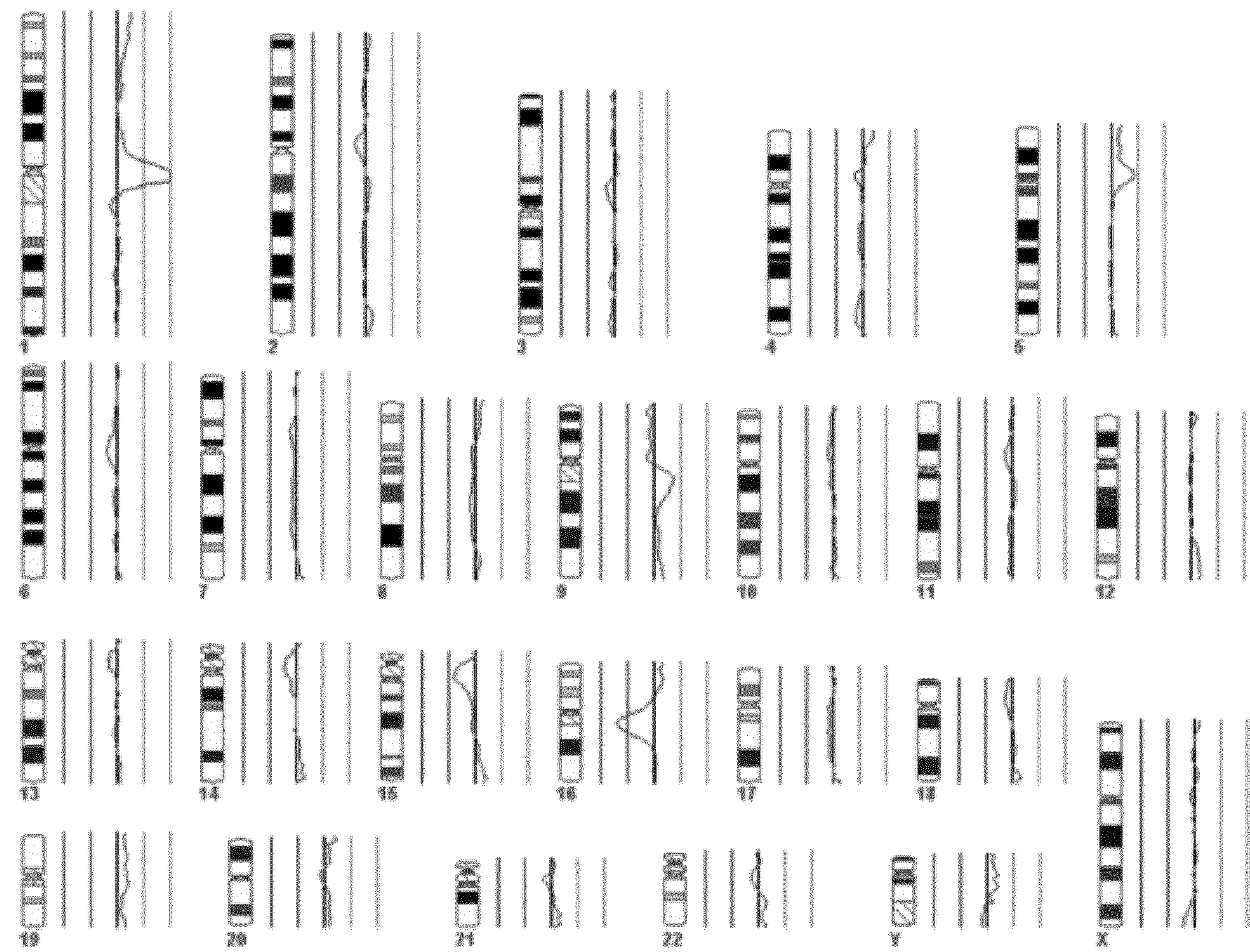


Figure 8 cont.

PBL SC 5

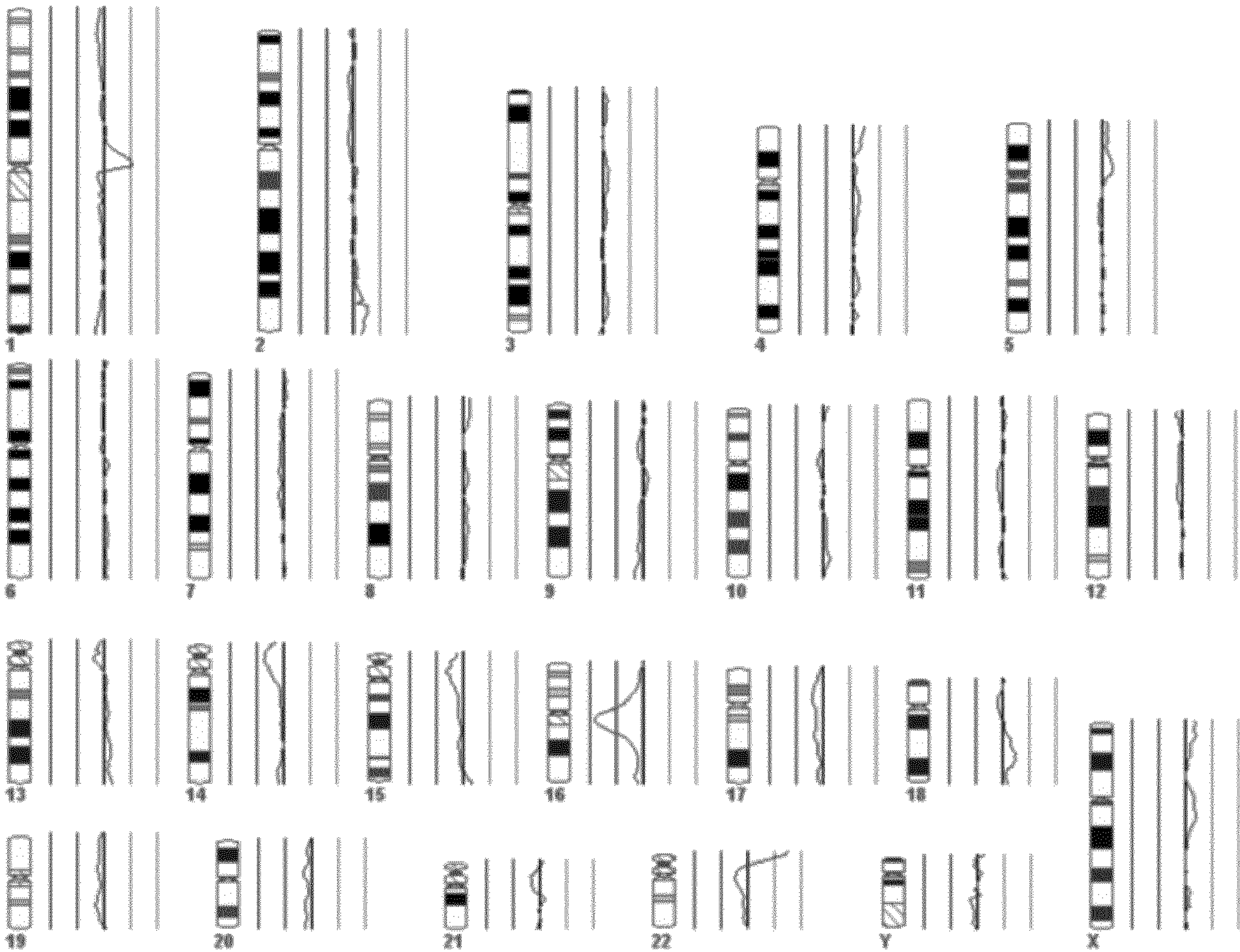
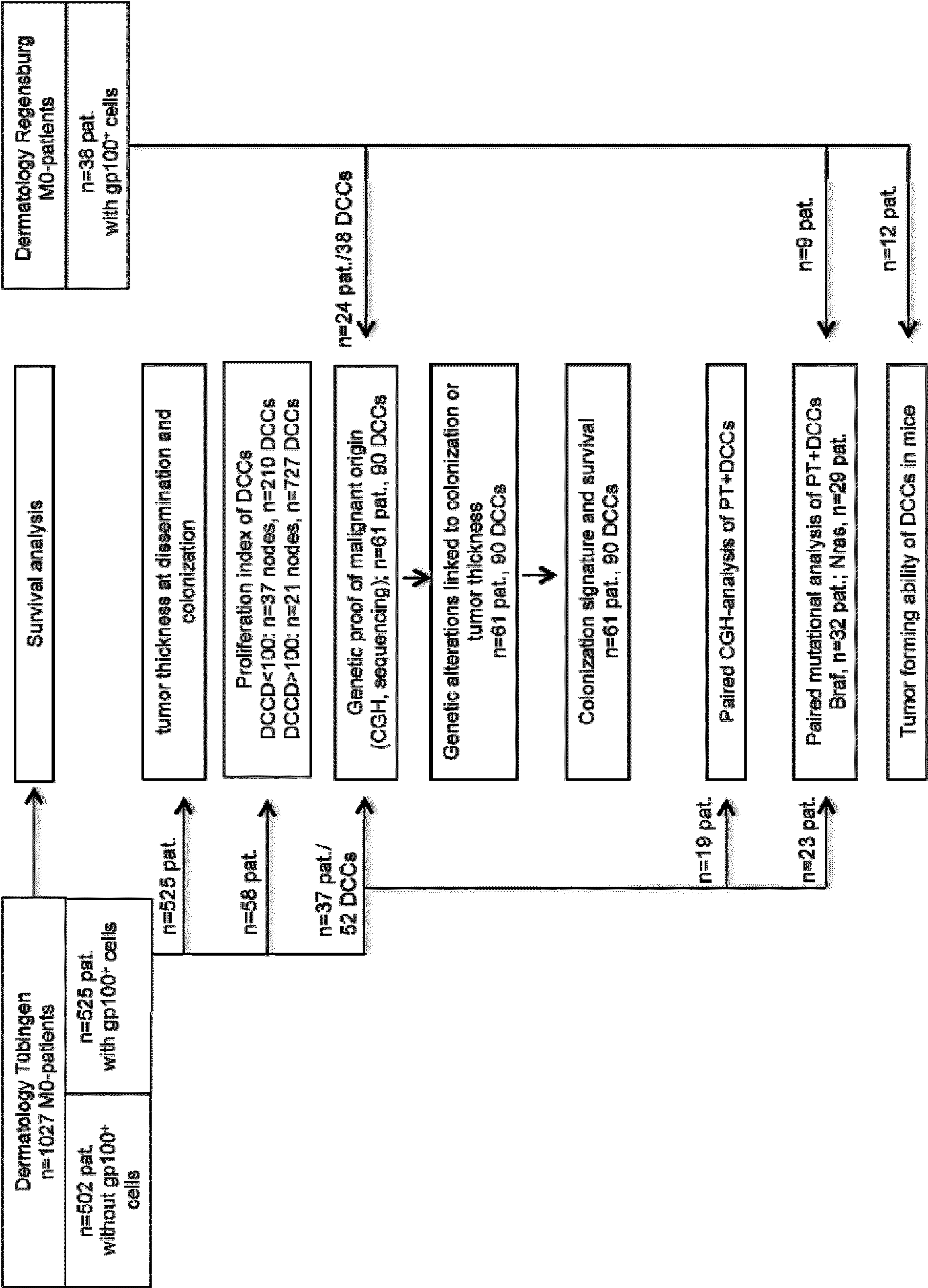


Figure 9



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Figure 10

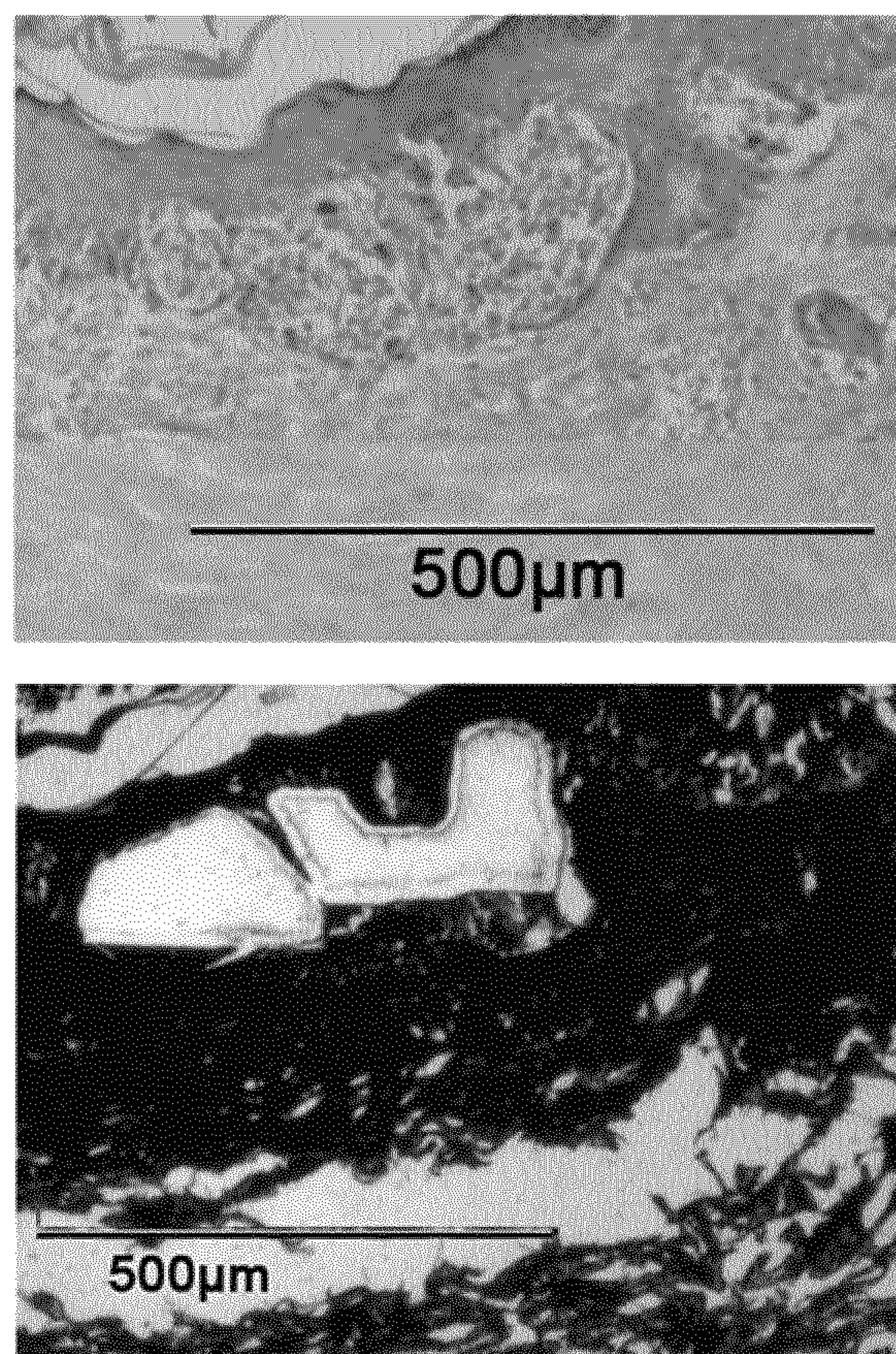


Figure 11

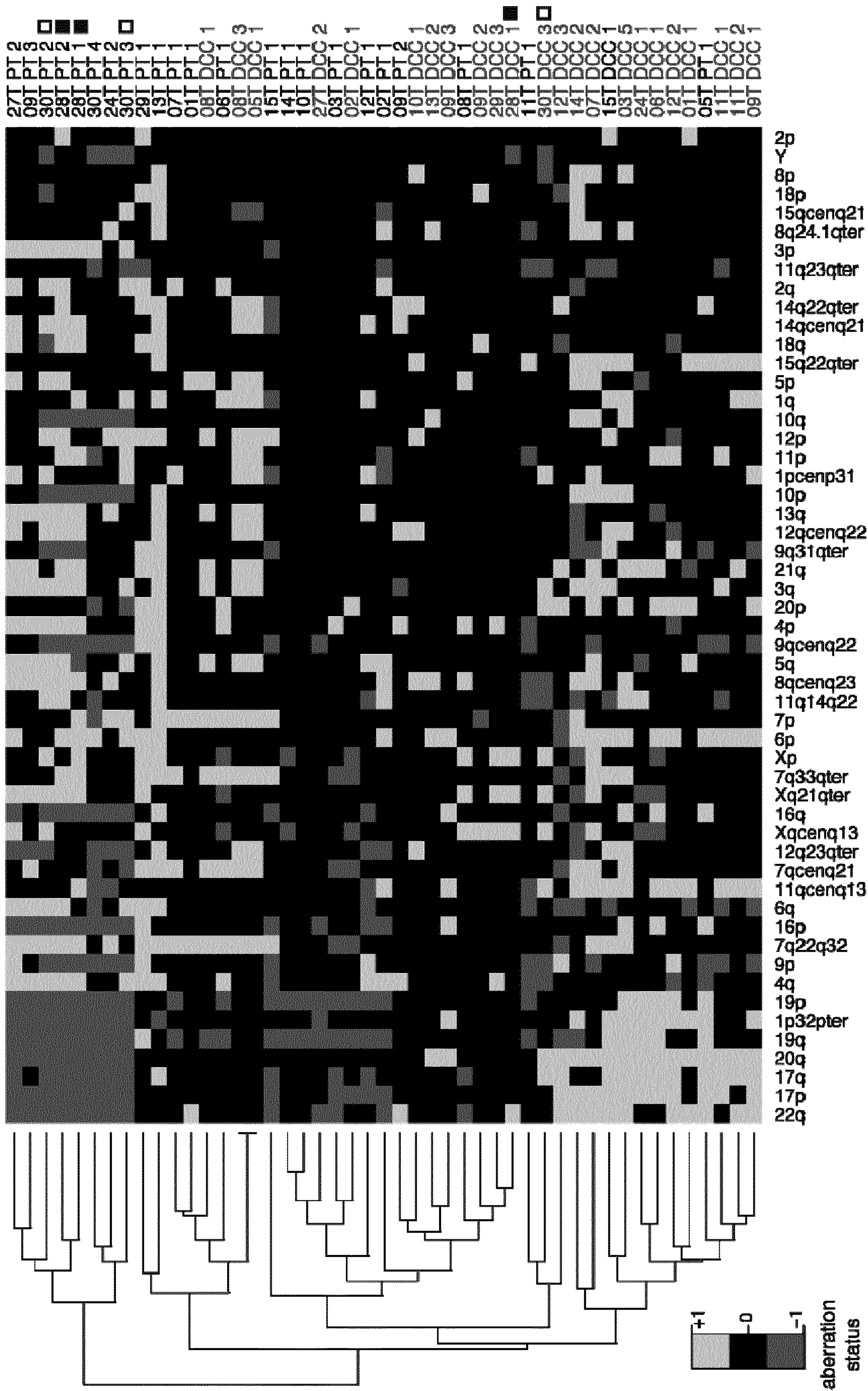


Figure 12

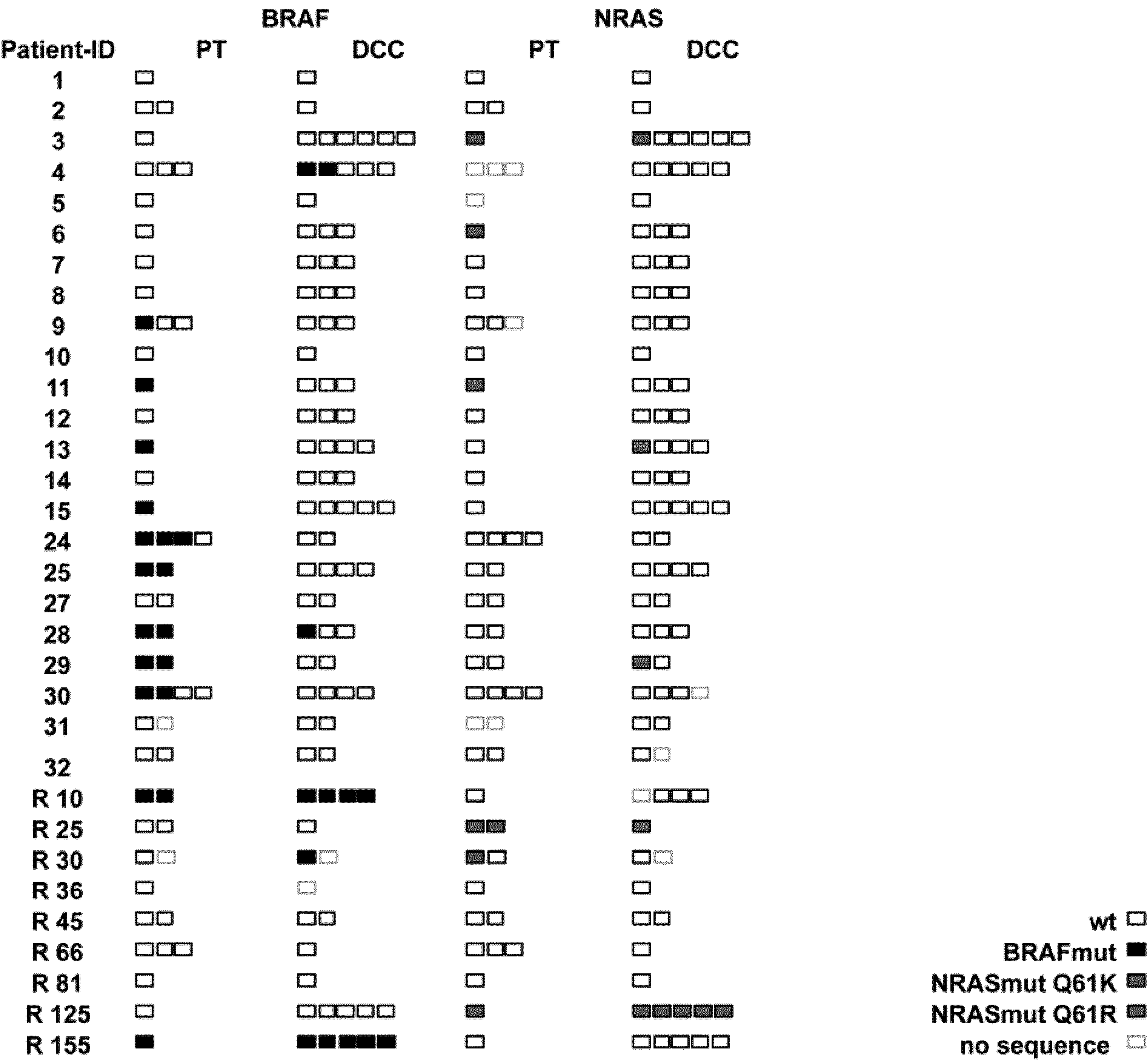


Figure 13

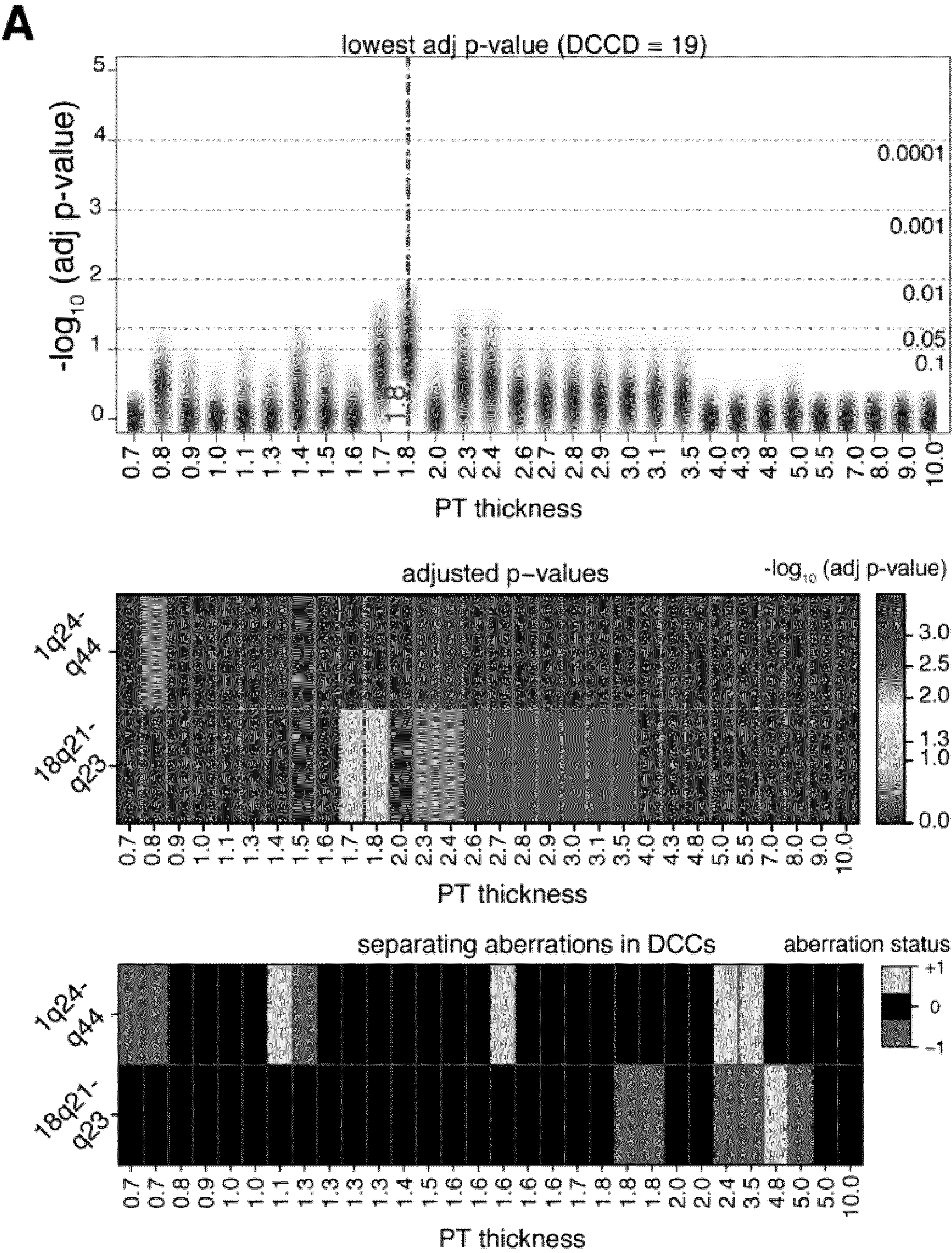


Figure 13 cont.

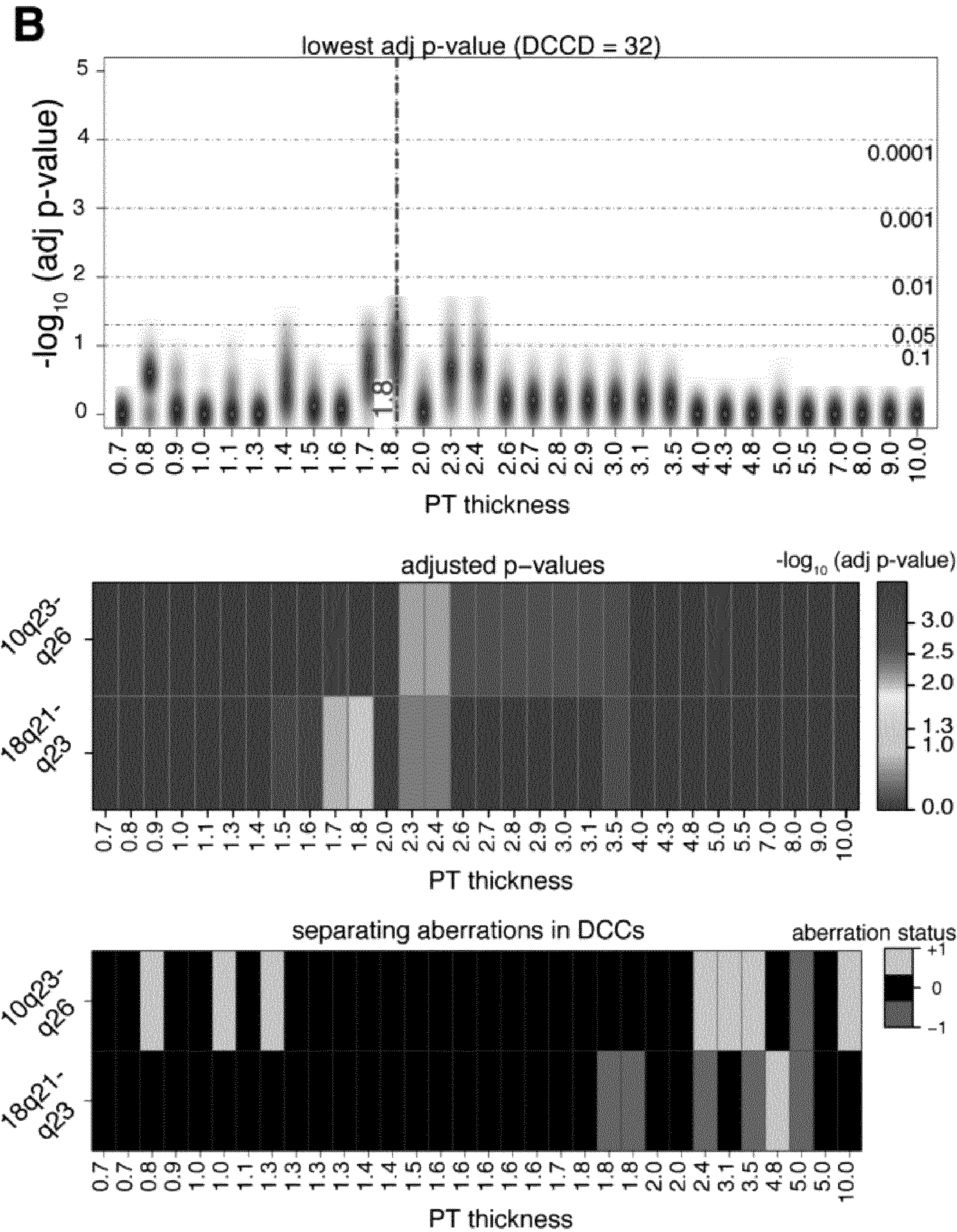


Figure 13 cont.

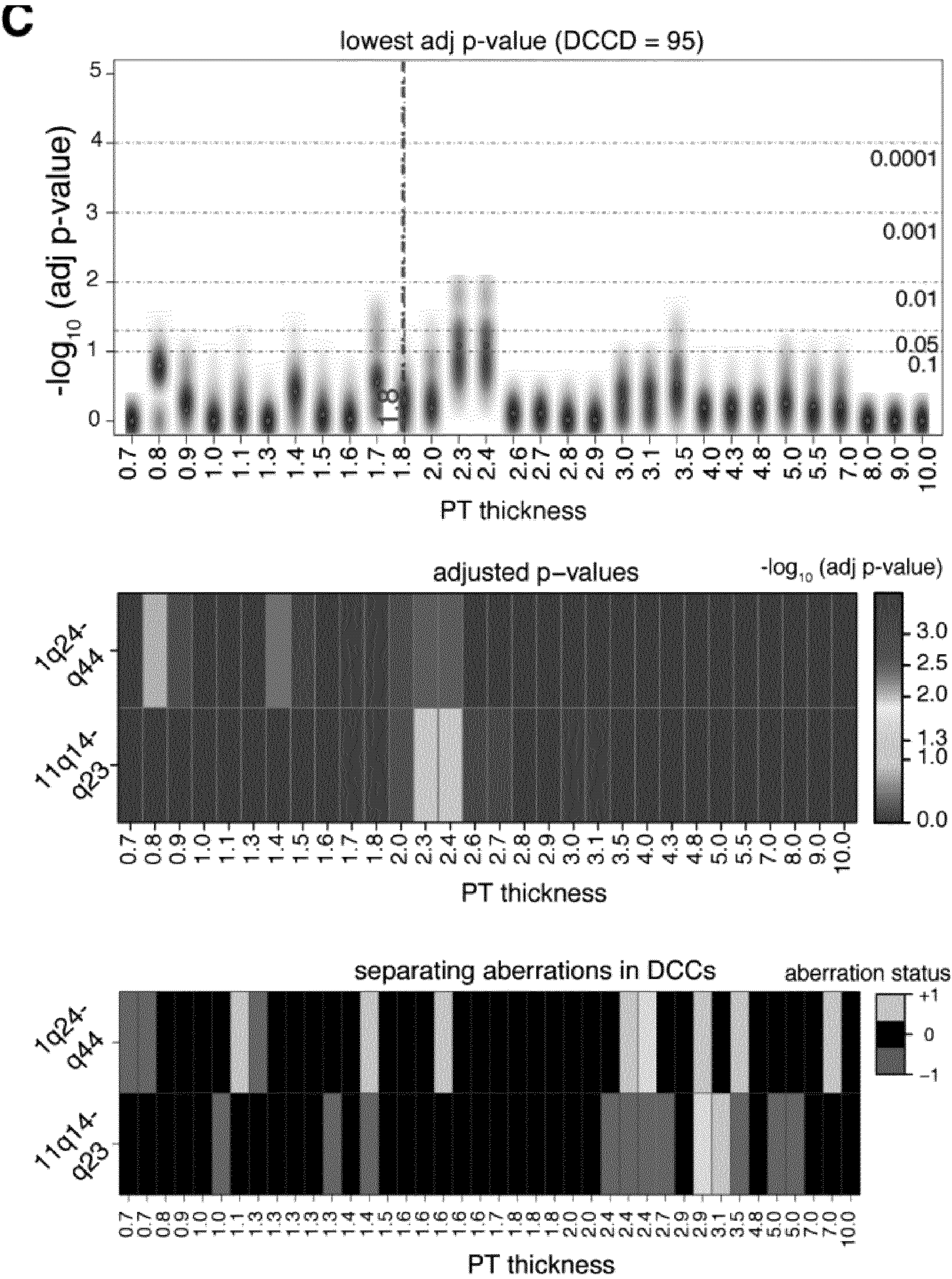


Figure 14

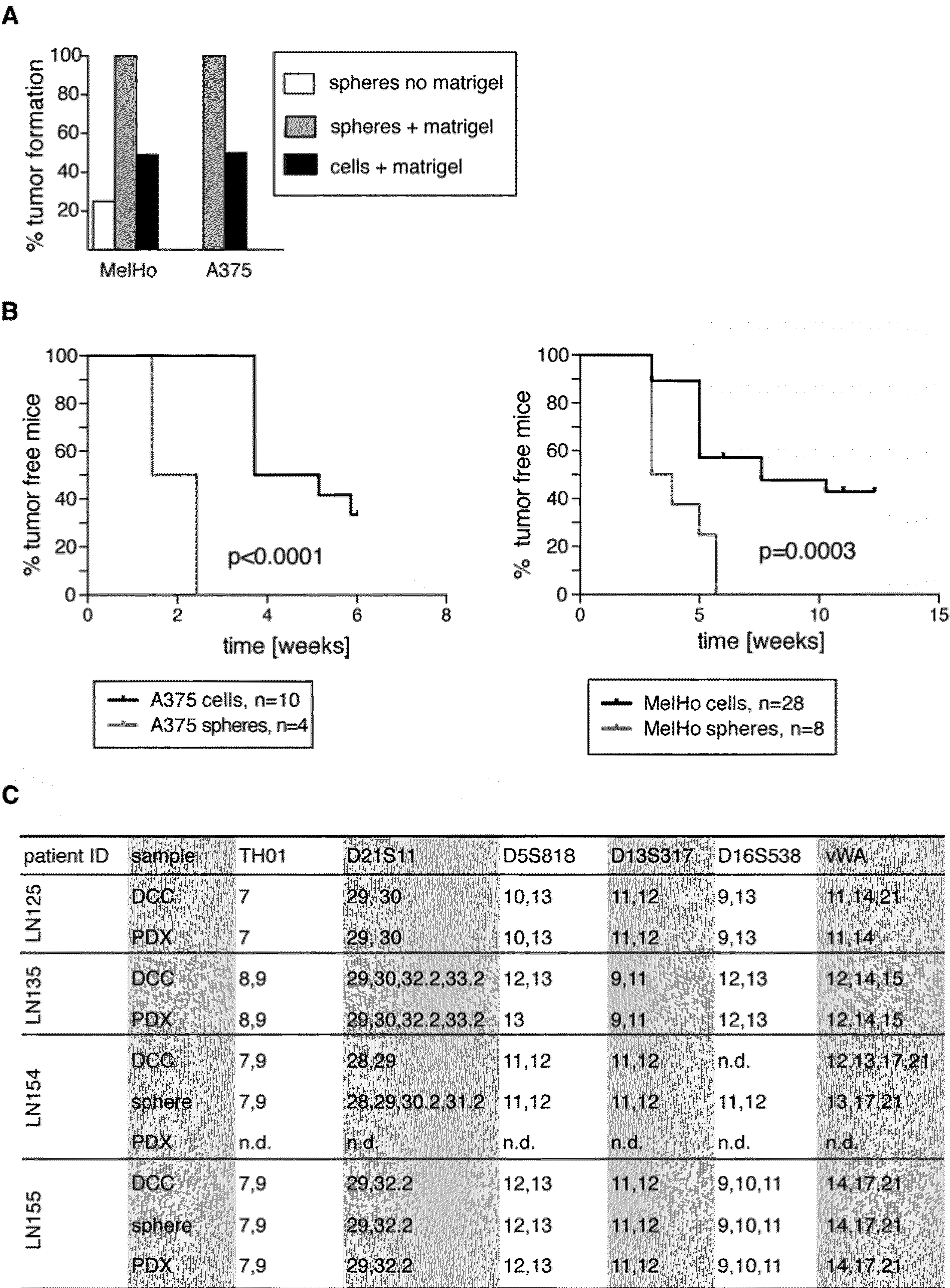


Table 1

Baseline characteristics of melanoma patients.

	number of patients	percentage [%]	median	range	interquartile range
gender					
female	25	41			
male	36	59			
age [years]			61	20 - 78	47.5 - 70
Breslow`s thickness [mm]			2.35	0.6 - 10.0	1.3 - 4.08
Ulceration					
no	39	63.9			
yes	19	31.2			
not specified	3	4.9			
localization					
extremities	32	52.5			
trunk or head	29	47.5			
nodal status					
histopathology					
negative	37	60.7			
positive	24	39.3			
DCCD			32	1 - 800000	3 - 177
clinical stage					
IA	4	6.5			
IB	15	24.6			
IIA	7	11.5			
IIB	5	8.2			
IIC	2	3.3			
IIIA	11	18.0			
IIIB	13	21.3			
IIIC	4	6.5			

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Figure 15

A

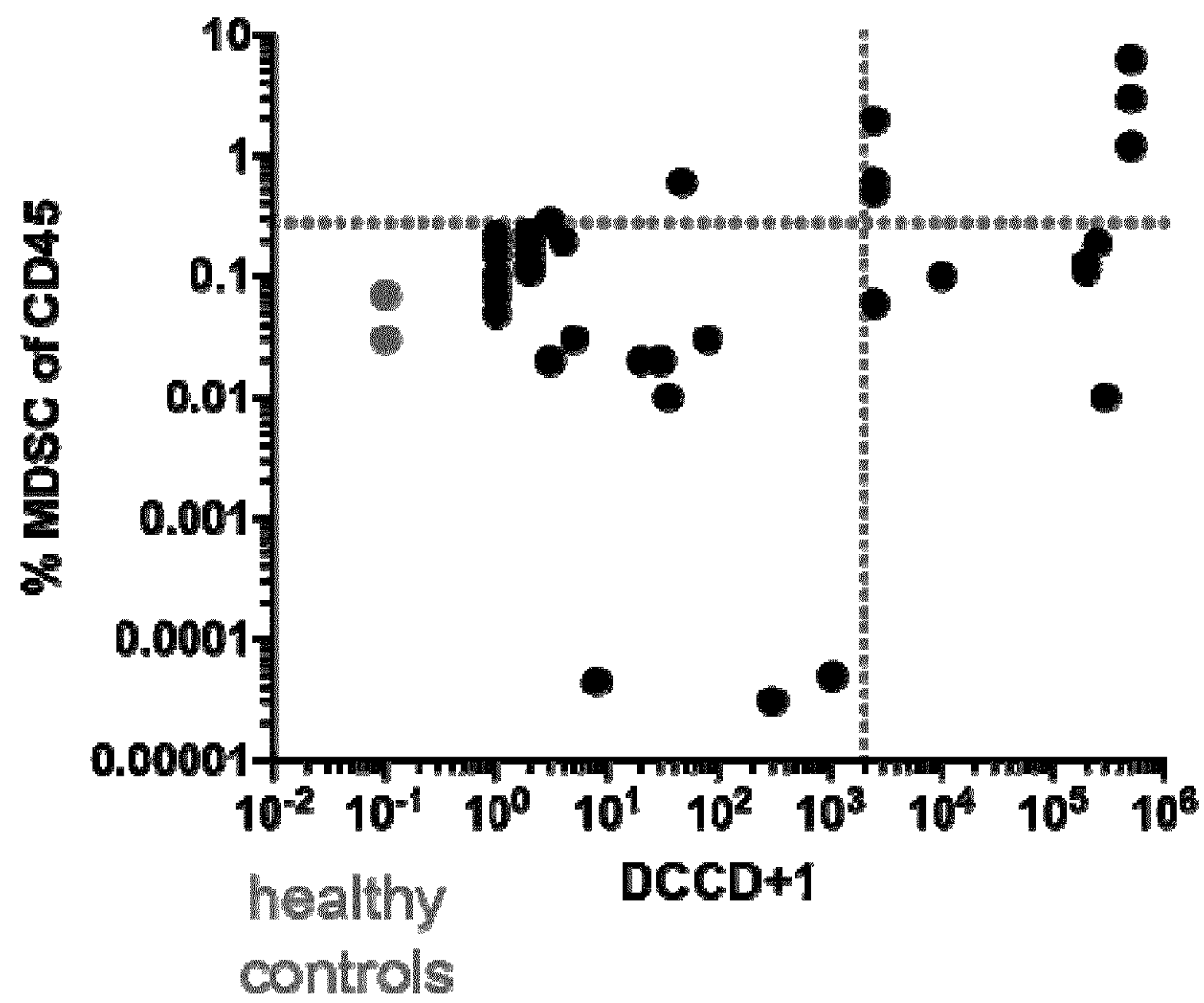


Figure 15

B

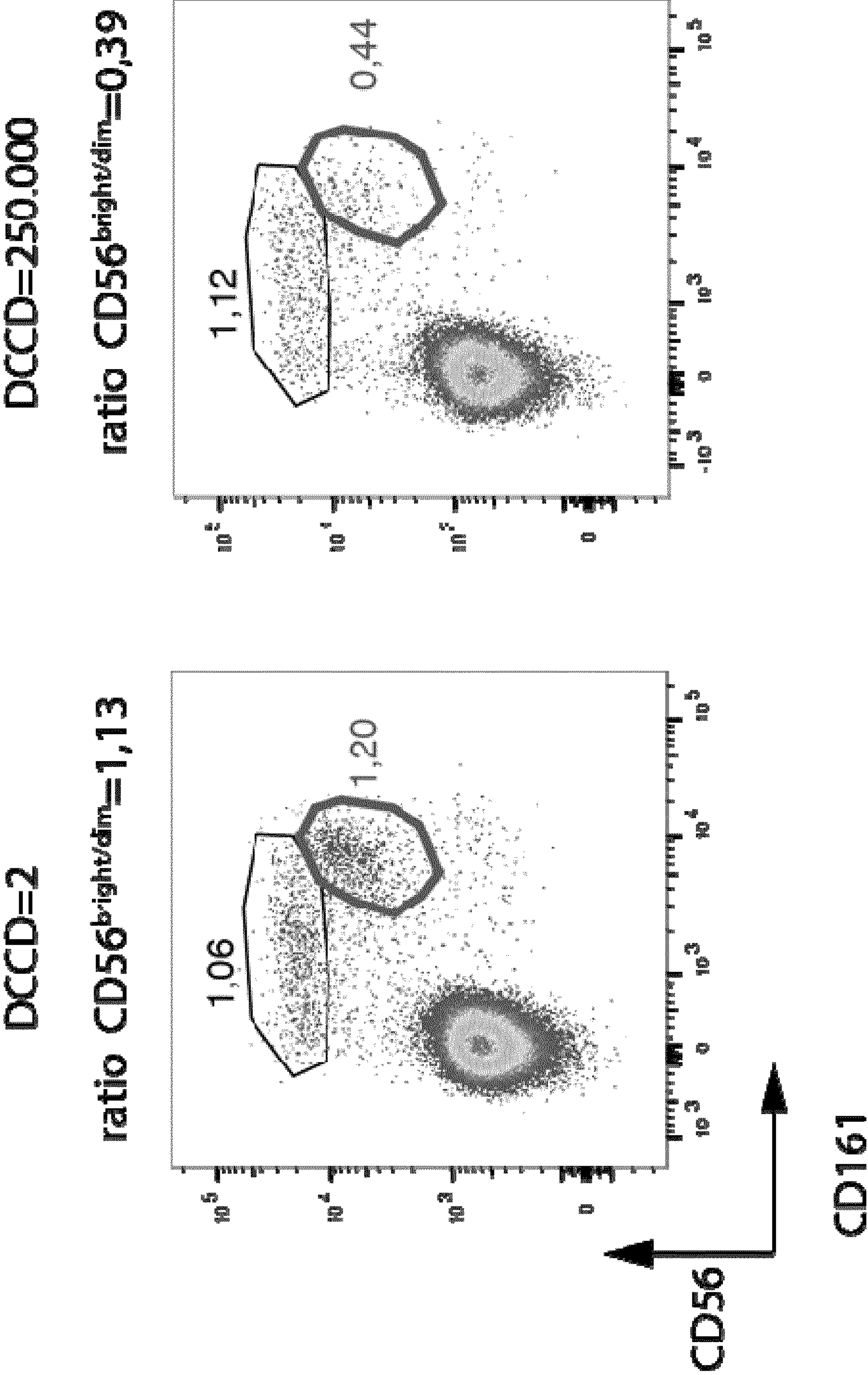


Figure 15

B cont.

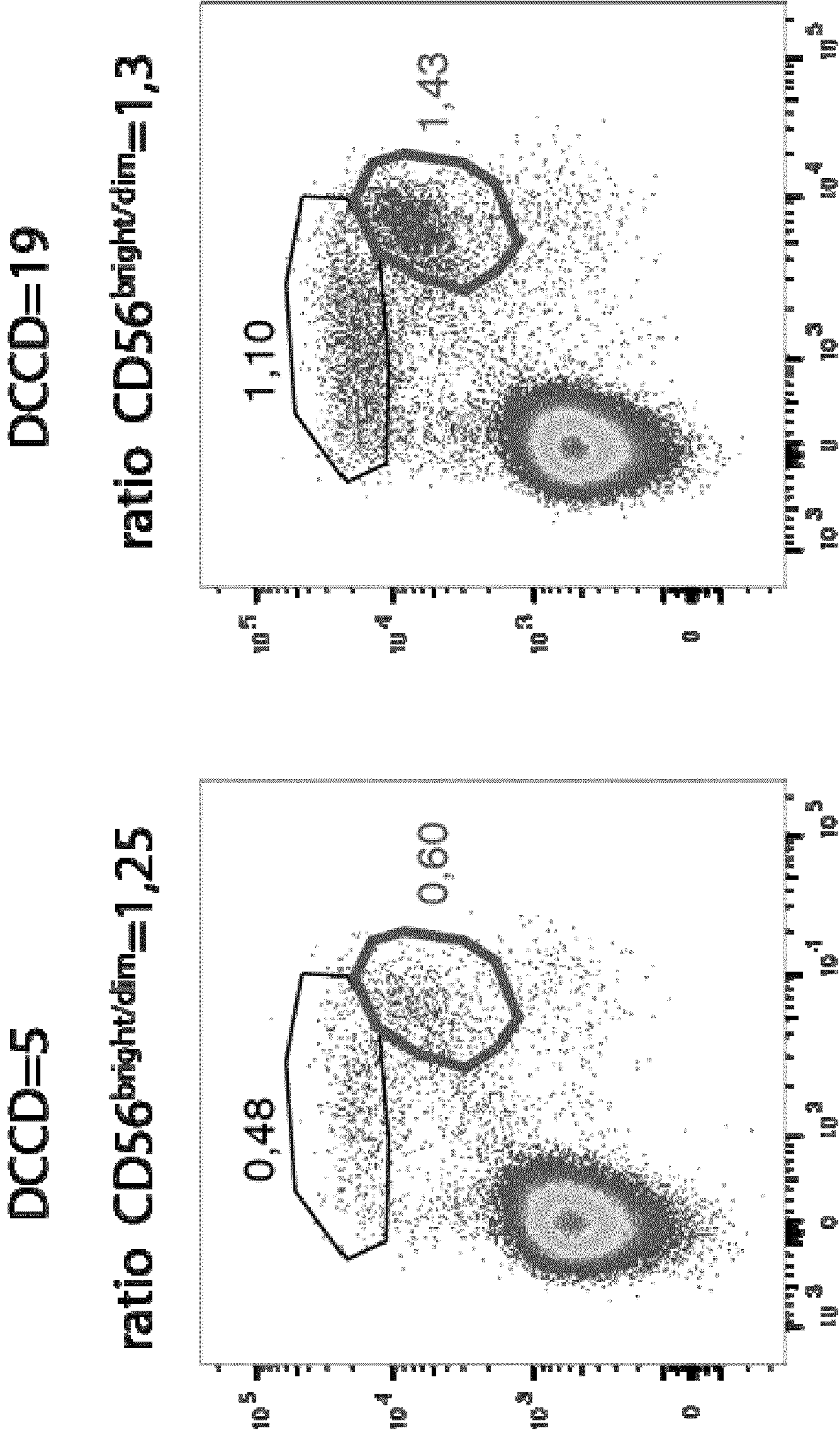


Figure 16

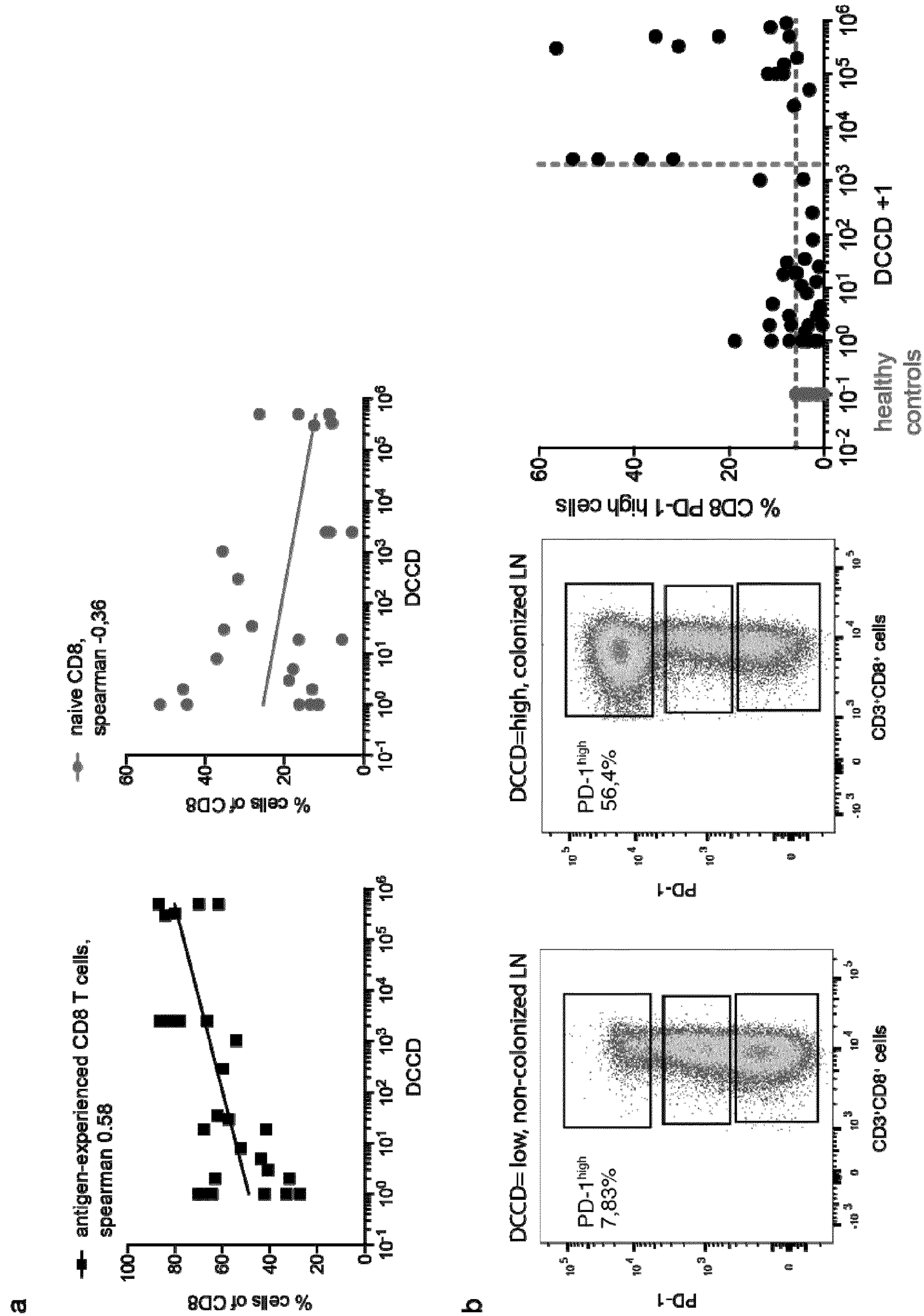


Figure 16 cont.

