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(72) Inventeurs/Inventors:
WISCHHUSEN, JORG, DE;
SCHAFFER, TINA, DE;
WEIDE, BENJAMIN, DE
(73) Propriétaire/Owner:
JULIUS-MAXIMILIANS-UNIVERSITÄT WÜRZBURG, DE
(74) Agent: NORTON ROSE FULBRIGHT CANADA
LLP/S.E.N.C.R.L., S.R.L.

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(57) Abrégé/Abstract:

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- (71) **Applicant:** JULIUS-MAXIMILIANS-UNIVERSITÄT
WÜRZBURG [DE/DE]; Sanderring 2, 97070 Würzburg
(DE).
- (72) **Inventors:** WISCHHUSEN, Jörg; Leutfresserweg 14a,
97082 Würzburg (DE). SCHÄFER, Tina; Sterenstrasse 10,
97074 Würzburg (DE). WEIDE, Benjamin; Mohlstr. 11,
72074 Tübingen (DE).
- (74) **Agents:** POLZ LEO, HOFFMANN EITLE PATENT-
UND RECHTSANWÄLTE PARTMBB et al.; Arabellas-
traße 30, 81925 München (DE).
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GDF-15 as a diagnostic marker for melanoma

FIELD OF THE INVENTION

The present invention relates to methods for predicting the probability of survival of a human melanoma cancer patient, and to apparatuses and kits which can be used in these methods.

BACKGROUND

The serum level of lactate dehydrogenase (sLDH) is the most widely used prognostic biomarker in melanoma and has been incorporated in the AJCC staging system for melanoma patients with distant metastases since 2001 (Balch, CM et al., J Clin Oncol / 19 / 3635-48. 2001). sLDH had been identified as an independent prognostic marker for patients with unresectable disease by different research groups (Sirott, MN et al., Cancer / 72 / 3091-8. 1993; Eton, O et al., J Clin Oncol / 16 / 1103-11. 1998; Manola, J et al., J Clin Oncol / 18 / 3782-93. 2000). Results from a comprehensive meta-analysis based on a large pool of clinical studies (31,857 patients with various solid tumors) confirmed the consistent effect of elevated LDH on OS (HR = 1.48, 95%CI = 1.43 to 1.53) across all disease subgroups and stages, with particular relevance for metastatic tumors. While the exact mechanism underlying tumor promotion by LDH remains unknown and may also be related to hypoxia and metabolic reprogramming via a Warburg effect, LDH also reflects high tumor burden (Zhang, J., Yao, Y.-H., Li, B.-G., Yang, Q., Zhang, P.-Y., and Wang, H.-T. (2015). Prognostic value of pretreatment serum lactate dehydrogenase level in patients with solid tumors: a systematic review and meta-analysis. Scientific Reports 5, 9800). Still, there is a need for improved prognostic biomarkers for melanoma patients.

Serum concentrations of S100B (sS100B) are widely used mainly in Europe to screen patients without evidence of disease to detect recurrences early (Pflugfelder, A et al., J Dtsch Dermatol Ges / 11 Suppl 6 / 1-116, 1-26. 2013). A meta-analysis by Mocellin et al. summarized the evidence on the suitability of sS100B to predict patients' survival. Twenty-two series enrolling 3393 patients comprising all stages were included in this analysis. Serum S100B positivity was associated with significantly poorer survival in melanoma patients of all stages especially in the subgroup of stage I to III patients independent from other prognostic factors (Mocellin, S et al., Int J Cancer / 123 / 2370-6. 2008). In prior studies, it was demonstrated that sS100B and sLDH had independent impact on prognosis of patients with distant metastases and the combined analysis of both markers might be used to select patients for complete metastasectomy (Weide, B et al., PLoS One / 8 / e81624. 2013; Weide, B et al., Br J Cancer / 107 / 422-8. 2012). However, despite this large evidence, no worldwide consensus exists on its implementation in the routine clinical setting in melanoma patients.

Growth and Differentiation Factor-15 (GDF-15, also known as Macrophage Inhibitor Cytokine-1 (MIC-1), Placental TGF- β (PTGF β), Placental Bone Morphogenetic Protein (PLAB), Nonsteroidal Anti-inflammatory

Drug-Activated Gene (NAG1) or Prostate-Derived Factor (PDF) is over-expressed in tumor cells of several types of solid cancers (Mimeault, M et al., Br J Cancer / 108 / 1079-91. 2013; Bock, AJ et al., Int J Gynecol Cancer / 20 / 1448-55. 2010; Zhang, L et al., Oral Oncol / 45 / 627-32. 2009). GDF-15 is induced by a number of tumor suppressor pathways including p53, GSK-3 β , and EGR-1 (Wang, X et al., Biochem Pharmacol / 85 / 597-606. 2013) and there is also evidence that GDF-15 itself can exert tumor suppressive effects, as shown in nude mouse xenograft models (Martinez, JM et al., J Pharmacol Exp Ther / 318 / 899-906. 2006; Eling, TE et al., J Biochem Mol Biol / 39 / 649-55. 2006) and in transgenic mice (Baek, SJ et al., Mol Pharmacol / 59 / 901-8. 2001). With regard to tumor cells both pro- and anti-apoptotic effects have been described for GDF-15 (Mimeault, M et al., Br J Cancer / 108 / 1079-91. 2013; Baek, SJ et al., Mol Pharmacol / 59 / 901-8. 2001; Zimmers, TA et al., J Cancer Res Clin Oncol / 136 / 571-6. 2010; Jones, MF et al., Cell Death Differ / 2015) and a multitude of possible signaling pathways has been suggested (Mimeault, M and Batra SK, J Cell Physiol / 224 / 626-35. 2010). Further complexity was added recently when the unprocessed pro-protein was shown to go into the nucleus where it altered TGF-beta dependent SMAD signaling and thereby transcription patterns (Min, KW et al., Oncogene / 2015). In vivo, constitutive GDF-15 overexpression reduced tumor formation but increased metastasis in an animal model for prostate cancer (Husaini, Y et al., PLoS One / 7 / e43833. 2012). GDF-15 was further shown to induce cancer cachexia (Johnen, H et al., Nat Med / 13 / 1333-40. 2007). Similarly, patent applications WO 2005/099746 and WO 2009/021293 relate to an anti-human-GDF-15 antibody (Mab26) capable of antagonizing effects of human GDF-15 (hGDF-15) on tumor-induced weight loss *in vivo* in mice. WO 2014/049087 and PCT/EP2015/056654 relate to monoclonal antibodies to hGDF-15 and medical uses thereof.

Clinically, a high GDF-15 serum level (sGDF-15) was found to correlate with the presence of bone metastases and poor prognosis in prostate cancer (Selander, KS et al., Cancer Epidemiol Biomarkers Prev / 16 / 532-7. 2007). In colorectal cancer, patients with high plasma levels showed shorter time to recurrence and reduced overall survival (Wallin, U et al., Br J Cancer / 104 / 1619-27. 2011). The allelic H6D polymorphism in the GDF-15 gene was further identified as independent predictor of metastasis at the time of diagnosis (Brown, DA, Clin Cancer Res / 9 / 2642-50. 2003). The association between high sGDF-15 and poor outcome was further shown for thyroid, pancreatic, gastric, ovarian and other cancers (Mimeault, M and Batra SK, J Cell Physiol / 224 / 626-35. 2010; Bauskin, AR et al., Cancer Res / 66 / 4983-6. 2006; Brown, DA et al., Clin Cancer Res / 15 / 6658-64. 2009; Blanco-Calvo, M et al., Future Oncol / 10 / 1187-202. 2014; Staff, AC et al., Gynecol Oncol / 118 / 237-43. 2010). Similar findings have also been reported for the level of GDF-15 tissue expression as assessed by immunohistochemistry (Wallin, U et al., Br J Cancer / 104 / 1619-27. 2011). In melanoma, GDF-15 expression was found to increase from benign nevi over primary melanoma to melanoma metastases (Mauerer, A et al., Exp Dermatol / 20 / 502-7. 2011; Boyle, GM et al., J Invest Dermatol / 129 / 383-91. 2009). Serum concentrations of GDF-15 were indicative for metastasis in patients with uveal melanoma (Suesskind, D et al., Graefes Arch Clin Exp Ophthalmol / 250 / 887-95. 2012) and correlated with stage in patients with cutaneous melanoma (Kluger, HM et al., ClinCancer Res / 17 / 2417-25. 2011). Riker et

al. compared gene expression in melanoma metastasis and primary tumor, and identified GDF-15 as the only soluble factor among the top 5 genes correlating with metastasis (Riker, AI et al., BMC Med Genomics / 1 / 13. 2008). Boyle et al. (Boyle, GM et al., J Invest Dermatol / 129 / 383-91. 2009) found by immunohistochemistry that 15 of 22 primary melanomas expressed low levels of GDF-15 whereas 16 of 16 melanoma metastasis showed strong expression. Furthermore, knock-down of GDF-15 in three melanoma cell lines results in decreased tumorigenicity in the same study. Before that, Talantov et al. (Talantov, D et al., Clin Cancer Res / 11 / 7234-42. 2005) had already identified GDF-15 in melanoma metastases, but not in nevi and normal lymph nodes. Similar findings were reported in a study of Mauere et al. who found GDF-15 to be preferentially expressed in metastatic tumors and in some primary melanomas, but not in melanocytic nevi (Mauere, A et al., Exp Dermatol / 20 / 502-7. 2011). However, a direct role of GDF-15 in metastasis has only been shown in prostate cancer where constitutive overexpression of GDF-15 slowed cancer development but increased metastases (Husaini, Y et al., PLoS One / 7 / e43833. 2012). Clinical relevance of GDF-15 serum levels in melanoma patients was reported in two studies. GDF-15 serum concentrations were associated with metastasis in a cohort of 188 patients with metastatic (n=170) or non-metastatic (n=18) uveal melanoma (Suesskind, D et al., Graefes Arch Clin Exp Ophthalmol / 250 / 887-95. 2012). Finally, Kluger et al. reported a correlation between plasma levels of GDF-15 and stage in 216 patients with cutaneous melanoma (Kluger, HM et al., ClinCancer Res / 17 / 2417-25. 2011). In contrast to these findings, however, some studies have suggested an anti-tumorigenic role of GDF-15 (see, for instance, Liu T et al: "Macrophage inhibitory cytokine 1 reduces cell adhesion and induces apoptosis in prostate cancer cells." Cancer Res., vol. 63, no. 16, 1 August 2003, pp.5034-5040).

Thus, from the above-mentioned studies, and in view of the complex functional role of human GDF-15 (hGDF-15) in various cancers and its different effects on primary tumors and metastases in prostate cancer, it remained, however, unknown whether hGDF-15 could be used as a prognostic clinical marker for patient survival in melanoma.

Thus, there is a need in the art for prognostic biomarkers for melanoma, and in particular for improved prognostic biomarkers in melanoma, and for methods which allow to predict patient survival in melanoma more reliably.

DESCRIPTION OF THE INVENTION

The present invention meets the above needs and solves the above problems in the art by providing the embodiments described below:

In particular, in order to solve the above problems, the present inventors set out to investigate the impact of serum GDF-15 levels on overall survival (OS) of melanoma patients. In the course of these studies, the present inventors have surprisingly found that the probability of survival in melanoma patients significantly

decreases with increasing hGDF-15 levels in the patient sera and vice versa. For instance, the inventors have shown that a high serum level of hGDF-15 is a potent biomarker for poor overall survival in tumor-free stage III and unresectable stage IV melanoma patients.

Thus, according to the invention, the probability of survival in melanoma patients inversely correlates with hGDF-15 levels.

Moreover, in the studies of the inventors, Cox regression analysis revealed that the knowledge of hGDF-15 serum levels adds independent prognostic information, e.g. if considered in combination with the M-category, and is superior to the established biomarker LDH in patients with distant metastasis.

Therefore, according to the invention, hGDF-15 levels can be used as a biomarker for the prediction of survival. This biomarker is advantageous, e.g. because it has a prognostic value that is independent of known biomarkers such as LDH. This means that if hGDF-15 levels are used for the prediction of melanoma patient survival according to the invention, they may, in a preferred aspect of the invention, be combined with additional biomarkers.

According to the invention, the combination of hGDF-15 levels as a biomarker with additional biomarkers such as LDH or S100B provides an improved prediction of survival, which is improved even when compared to the use of hGDF-15 levels alone.

Additionally, the use of hGDF-15 level as a biomarker is also advantageous because it allows to provide a prediction of survival that includes sub-groups of melanoma patients such as macroscopically tumor-free stage III patients, for which S100B represents the only available predictive and diagnostic marker.

Thus, the present invention provides improved means to predict survival of melanoma patients by providing the preferred embodiments described below:

1. A method for predicting the probability of survival of a human melanoma patient, wherein the method comprises the steps of:
 - a) determining the level of hGDF-15 in a human blood sample obtained from said patient; and
 - b) predicting said probability of survival based on the determined level of hGDF-15 in said human blood sample; wherein a decreased level of hGDF-15 in said human blood sample indicates an increased probability of survival.
2. The method according to item 1, wherein step b) comprises comparing said level of hGDF-15 determined in step a) with a hGDF-15 threshold level, wherein said probability is predicted based on the comparison of said level of hGDF-15 determined in step a) with said hGDF-15 threshold level; and

wherein a level of hGDF-15 in said human blood sample which is decreased compared to said hGDF-15 threshold level indicates that the probability of survival is increased compared to a probability of survival at or above said hGDF-15 threshold level.

3. The method according to item 1 or 2, wherein the human blood sample is a human serum sample.
4. The method according to item 3, wherein the hGDF-15 threshold level is a level selected from the range of between 1.1 ng/ml and 2.2 ng/ml, wherein the hGDF-15 threshold level is preferably a hGDF-15 level selected from the range of between 1.2 ng/ml and 2.0 ng/ml, wherein the hGDF-15 hGDF-15 threshold level is more preferably a hGDF-15 level selected from the range of between 1.3 ng/ml and 1.8 ng/ml, and wherein the hGDF-15 threshold level is still more preferably a hGDF-15 level selected from the range of between 1.4 ng/ml and 1.6 ng/ml.
5. The method according to item 4, wherein the hGDF-15 threshold level is 1.5 ng/ml.
6. The method according to item 3, wherein the hGDF-15 threshold level is a level selected from the range of between 3.3 ng/ml and 4.3 ng/ml, wherein the hGDF-15 threshold level is preferably a level selected from the range of between 3.6 ng/ml and 4.0 ng/ml, and wherein the hGDF-15 threshold level is more preferably 3.8.
7. The method according to any one of the preceding items,
wherein step a) further comprises determining the level of lactate dehydrogenase in said human blood sample, and
wherein in step b), said probability of survival is also predicted based on the determined level of lactate dehydrogenase in said human blood sample; and wherein a decreased level of lactate dehydrogenase in said human blood sample indicates an increased probability of survival.
8. The method according to item 7, wherein step b) comprises comparing said level of lactate dehydrogenase determined in step a) with a lactate dehydrogenase threshold level, wherein said probability is also predicted based on the comparison of said level of lactate dehydrogenase determined in step a) with said lactate dehydrogenase threshold level; and wherein a level of lactate dehydrogenase in said human blood sample which is decreased compared to said lactate dehydrogenase threshold level or is at said lactate dehydrogenase threshold level indicates that the probability of survival is increased compared to a probability of survival above said lactate dehydrogenase threshold level.
9. The method according to any one of the preceding items,
wherein step a) further comprises determining the level of S100B in said human blood sample, and
wherein in step b), said probability of survival is also predicted based on the determined level of S100B in said human blood sample; and wherein a decreased level of S100B in said human blood sample indicates an increased probability of survival.
10. The method according to item 9, wherein step b) comprises comparing said level of S100B determined in step a) with a S100B threshold level, wherein said probability is predicted based on the comparison of said level of S100B determined in step a) with said S100B threshold level; and wherein a level of S100B in said human blood sample which is decreased compared to said S100B threshold level or is

at said S100B threshold level indicates that the probability of survival is increased compared to a probability of survival above said S100B threshold level.

11. The method according to any of the preceding items, wherein in step b), said probability of survival is also predicted based on the age of said patient; and wherein an increased age indicates a decreased probability of survival.
12. The method according to item 11, wherein step b) comprises comparing said age of said patient to a threshold age, wherein said probability is predicted based on the comparison of said age of said patient with said threshold age; and wherein an age of said patient which is equal to or increased compared to said threshold age indicates that the probability of survival is decreased compared to a probability of survival below said threshold age.
13. The method according to item 12, wherein said threshold age is selected from the range of 60 to 65 years.
14. The method according to item 13, wherein said threshold age is 63 years.
15. The method according to any one of the preceding items, wherein in step b), said probability of survival is also predicted based on metastasis; and wherein the presence of metastases in visceral organs other than lung indicates that the probability of survival is decreased as compared to the probability of survival when metastases are absent from visceral organs other than lung.
16. The method according to any one of the preceding items, wherein the human melanoma patient is not a tumor-free melanoma stage IV patient.
17. The method according to any one of the preceding items, wherein the human melanoma patient is a tumor-free stage III melanoma patient or an unresectable stage IV melanoma patient.
18. The method according to any one of items 1-16, wherein the human melanoma patient is a stage IV melanoma patient.
19. The method according to any one of items 1-16, wherein the human melanoma patient is a stage III melanoma patient.
20. The method according to item 17, wherein the human melanoma patient is a tumor-free stage III melanoma patient.
21. The method according to item 17, wherein the human melanoma patient is an unresectable stage IV melanoma patient.
22. The method according to any of the preceding items, wherein step a) comprises determining the level of hGDF-15 by using one or more antibodies capable of binding to hGDF-15 or an antigen-binding portion thereof.

23. The method according to item 22, wherein the one or more antibodies capable of binding to hGDF-15 or the antigen-binding portion thereof form a complex with hGDF-15.
24. The method according to item 22 or 23, wherein the one or more antibodies comprise at least one polyclonal antibody.
25. The method according to item 22, 23 or 24, wherein the one or more antibodies or the antigen-binding portion comprise at least one monoclonal antibody or an antigen-binding portion thereof.
26. The method according to item 25, wherein the binding is binding to a conformational or discontinuous epitope on hGDF-15, and wherein the conformational or discontinuous epitope is comprised by the amino acid sequences of SEQ ID No: 25 and SEQ ID No: 26.
27. The method according to item 25 or 26, wherein the antibody or antigen-binding portion thereof comprises a heavy chain variable domain which comprises a CDR1 region comprising the amino acid sequence of SEQ ID NO: 3, a CDR2 region comprising the amino acid sequence of SEQ ID NO: 4 and a CDR3 region comprising the amino acid sequence of SEQ ID NO: 5, and wherein the antibody or antigen-binding portion thereof comprises a light chain variable domain which comprises a CDR1 region comprising the amino acid sequence of SEQ ID NO: 6, a CDR2 region comprising the amino acid sequence ser-ala-ser and a CDR3 region comprising the amino acid sequence of SEQ ID NO: 7.
28. The method according to any one of claims 1 to 27, wherein in step a), the level of hGDF-15 is determined by capturing hGDF-15 with an antibody or antigen-binding fragment thereof according to any one of claims 25 to 27 and by detecting hGDF-15 with a polyclonal antibody, or by detecting hGDF-15 with a monoclonal antibody or antigen-binding fragment thereof which binds to a different epitope than the antibody which captures hGDF-15.
29. The method according to any one of the preceding items, wherein the method is an *in vitro* method.
30. The method according to any one of the preceding items, wherein in step a), the level of hGDF-15 in the human blood sample is determined by an enzyme linked immunosorbent assay.
31. The method according to any one of items 1-29, wherein in step a), the level of hGDF-15 in the human blood sample is determined by an electrochemiluminescence assay.
32. The method according to item 31, wherein the electrochemiluminescence assay is a sandwich detection method comprising a step of forming a detection complex between
 - (A) streptavidin-coated beads;
 - (B) a biotinylated first antibody or antigen-binding portion thereof capable of binding to hGDF-15;
 - (C) hGDF-15 from the sample; and
 - (D) a ruthenium complex-labelled second antibody or antigen-binding portion thereof capable of binding to hGDF-15;

wherein said detection complex has the structure (A)-(B)-(C)-(D), and wherein the biotinylated first antibody or antigen-binding portion thereof binds to a first hGDF-15 epitope and the ruthenium complex-labelled second antibody or antigen-binding portion thereof binds to a second hGDF-15 epitope which is different from said first hGDF-15 epitope,

wherein the method further comprises a step of detecting the detection complex by measuring electrochemiluminescence,

and wherein the level of hGDF-15 in the human blood sample is determined based on the electrochemiluminescence measurement.

33. An apparatus configured to perform the method according to any one of items 1-32.
34. The apparatus according to item 25, wherein the apparatus is an electrochemiluminescence analyzer configured to perform the method according to item 31 or item 32.
35. A detection kit comprising:
 - (i) streptavidin-coated beads;
 - (ii) a biotinylated first antibody or antigen-binding portion thereof capable of binding to hGDF-15;
 - (iii) recombinant hGDF-15, preferably in form of a buffered solution comprising recombinant hGDF-15, the buffered solution having a pH in the range of 4 to 7;
 - (iv) a ruthenium complex-labelled second antibody or antigen-binding portion thereof capable of binding to hGDF-15; and optionally
 - (v) instructions for use, preferably instructions for use in a method according to items 1-32; and preferably
 - (vi) a container containing said recombinant hGDF-15, wherein the surface of the container which is in contact with recombinant hGDF-15 is coated with a non-adhesive material.

wherein the biotinylated first antibody or antigen-binding portion thereof is capable of binding to a first hGDF-15 epitope and the ruthenium complex-labelled second antibody or antigen-binding portion thereof is capable of binding to a second hGDF-15 epitope which is different from said first hGDF-15 epitope.

36. The detection kit according to item 35, wherein one of the first antibody or antigen-binding portion thereof capable of binding to hGDF-15 and second antibody or antigen-binding portion thereof capable of binding to hGDF-15 is an antibody or antigen-binding portion thereof according to any one of items 26 to 28.
37. Use of a detection kit of any one of items 35 to 36 in an in vitro method for the prediction of the probability of survival of a human melanoma patient.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Overall survival of distinct melanoma patient populations according to GDF-15 serum levels. Kaplan-Meier curves are shown for overall survival of 468 tumor-free stage III (A), 206 unresectable stage IV (B) and 87 tumor-free stage IV (C) patients with either normal (<1.5 ng/mL) or elevated (≥ 1.5 ng/mL) GDF-15 levels. Censoring is indicated by vertical lines; p-values were calculated by log rank statistics. In Figures 1A and 1B, the upper curves are those for normal hGDF-15 levels, and the lower curves are those for elevated hGDF-15 levels.

Figure 2: Combinations of S100B and GDF-15 serum levels and their correlation with overall survival of stage III patients. Using a Cox regression model, S100B and hGDF-15 levels were shown to independently predict prognosis of tumor-free stage III patients. Kaplan-Meier curves of overall survival for patients with different biomarker combinations are presented for 466 patients. Censoring is indicated by vertical lines. In Figure 2, the highest curve is the curve for normal hGDF-15 levels and normal S100B levels, the 2nd highest curve is the curve for elevated hGDF-15 levels, the 2nd lowest curve is the curve for elevated S100B levels, and the lowest curve is the one for elevated hGDF-15 levels and elevated S100B levels.

Figure 3: Overall survival of unresectable stage IV patients according to combinations of serum levels of LDH and GDF-15, and the pattern of distant metastasis. The independent prognostic impact of GDF-15 serum levels on overall survival is illustrated for M-categories M1a/b (A) as well as for M1c patients (B). Broken lines indicate all patients of the given M-category. Continuous lines represent sub-groups with low (blue) or high (red) GDF-15 levels, respectively. Differences in OS between patients with high or low GDF-15 levels were significant for M1a/b and for M1c patients (log-rank p-values 0.047 and 0.003, respectively). In (C), overall survival is displayed according to the number of unfavorable values considering all 3 independent prognostic factors according to model 1 of Cox regression analysis (LDH levels, pattern of visceral metastasis, and GDF-15 levels). The order of curves (i.e. the order from the highest curve to the lowest curve) in the legend contained in panels (A) to (C) of the figure reflects the order of curves in the respective panels.

Figure 4: Overall survival correlates with GDF-15 serum levels in melanoma patients. 762 patients were randomly assigned to two cohorts. In the identification cohort (254 patients), different cut-off values were tested by Kaplan-Meier analysis and log rank tests to obtain the best possible discrimination between patients with high and low GDF-15 serum levels. Overall survival of patients of the identification cohort according to the optimized cut-off point (<1.5 ng/mL vs. ≥ 1.5 ng/mL) is shown in (A). Differences in overall survival were confirmed in 508 patients of the validation cohort (B). Censoring is indicated by vertical lines; p-values were calculated by log rank statistics. In Figures 4A and 4B, the upper curves are those for normal hGDF-15 levels, and the lower curves are those for elevated hGDF-15 levels.

Figure 5: Overall survival according to S100B serum levels. Kaplan-Meier curves are shown for overall survival of 466 tumor-free stage III (A), 193 unresectable stage IV (B) and 83 tumor-free stage IV (C) patients. Patients were categorized based on S100B serum levels (normal vs. elevated). Censoring is indicated by vertical lines; p-values were calculated by log rank statistics. In Figures 5A to 5C, the upper curves are those for normal S100B levels, and the lower curves are those for elevated S100B levels.

Figure 6: Overall survival of stage III patients according to the number of unfavorable values considering serum levels of GDF-15, S100B, age, and sub-stage. Model 2 of Cox regression analysis (Table 2) revealed an independent negative prognostic impact for GDF-15 levels >1.5 ng/mL, for elevated S100B levels, for age <63 years, and for sub-stage IIIC. Patients were now stratified according to the number of unfavorable values among those four factors. The resulting Kaplan-Meier curves of overall survival are presented and censoring is indicated by vertical lines. The highest curve is the curve, wherein all factors are favorable. The 2nd highest curve is the curve, wherein one factor is unfavorable. The 3rd highest curve is the curve, wherein two factors are unfavorable. The 2nd lowest curve is the curve, wherein three factors are unfavorable. The lowest curve is the curve, wherein all factors are unfavorable.

Figure 7: Overall survival of unresectable stage IV patients according to the number of unfavorable values considering serum levels of GDF-15, S100B, the pattern of distant metastasis, and age. Model 2 of Cox regression analysis (Table 3) revealed an independent negative prognostic impact for GDF-15 levels >1.5 ng/mL, for elevated S100B levels, for the metastatic involvement of visceral organs other than lung, and for age of 63 years or older. Patients were thus stratified according to the number of unfavorable factors. The resulting Kaplan-Meier curves for overall survival are shown and censoring is indicated by vertical lines. The highest curve is the curve, wherein all factors are favorable. The 2nd highest curve is the curve, wherein one factor is unfavorable. The 3rd highest curve is the curve, wherein two factors are unfavorable. The 2nd lowest curve is the curve, wherein three factors are unfavorable. The lowest curve is the curve, wherein all factors are unfavorable.

Figure 8: Overall survival subsequent to serum sampling of stage III patients according to combinations of different factors. A nomogram (a) was developed for tumor-free stage III patients using the nomogram function of R considering the relative impact of single independent factors according to multivariate analysis (sGDF-15, sS100B, pattern of loco-regional metastasis). A risk score ranging between 0 and 266 points was calculated for 466 stage III patients with complete data. In (b), Kaplan-Meier curves of overall survival subsequent to serum sampling is displayed for different risk score categories. Censoring is indicated by vertical lines.

Figure 9: Overall survival subsequent to serum sampling of unresectable stage IV patients according to combinations of different factors. GDF-15 serum levels have independent impact on overall survival of unresectable stage IV patients in addition to the M category. This is illustrated by the significant differences in OS according to sGDF-15 in both, M1a/b patients (a), and M1c patients (b). A nomogram (c) was developed for unresectable stage IV patients using the nomogram function of R considering the relative impact of single independent factors according to multivariate analysis (sGDF-15, sS100B, CNS involvement, and number of involved distant sites). A risk score ranging between 0 and 334 points was calculated for 193 unresectable stage IV patients with complete data. In (d), Kaplan-Meier curves of overall survival subsequent to serum sampling is displayed for different risk score categories. Censoring is indicated by vertical lines.

Figure 10: Correlations of sGDF-15 with stage/disease status and sLDH. Serum GDF-15 levels are shown for tumor-free stage III (n=468), tumor-free stage IV patients (n=87) and unresectable stage IV patients (n=206) (a). In unresectable stage IV patients, sGDF-15 is presented for according to the number of distant metastases (b) or stratified according to sLDH (c). Red bars indicate median levels of GDF-15; ** p < 0.01; *** p < 0.001 using Mann Whitney tests.

Figure 11: Overall survival subsequent to serum sampling correlates with GDF-15 serum levels in melanoma patients. 761 patients were randomly assigned to two cohorts. In the identification cohort (254 patients), different cut-off values were tested by Kaplan-Meier analysis and log rank tests to obtain the best possible discrimination between patients with high and low GDF-15 serum levels. Overall survival subsequent to serum sampling of patients of the identification cohort according to the optimized cut-off point (<1.5ng/mL vs. ≥1.5ng/mL) is shown in (a). Differences in overall survival subsequent to serum sampling were confirmed in 507 patients of the validation cohort (b). Censoring is indicated by vertical lines; p-values were calculated by log rank statistics.

Figure 12: Association of sGDF-15, sS100B and sLDH with OS according to time-point of serum sampling in tumor-free stage III patients. Overall survival of tumor-free stage III patients according to sGDF-15 (left), sS100B (middle) and sLDH (right) according to the time point of last recurrence before serum sampling. Patients were categorized as being tumor-free for less than 6 months (a-c), for between 6 months and 2 years (d-f) or for more than 2 years (g-i) since detection of last metastasis. Censoring is indicated by vertical lines; p-values were calculated by log rank statistics.

Figure 13: Association of sGDF-15, sS100B and sLDH with OS according to time-point of serum sampling in unresectable stage IV patients. Overall survival of unresectable stage IV patients according to sGDF-15 (left), S100B (middle) and LDH (right) according to the time span since diagnosis of stage IV disease. The first distant metastasis was detected within 6 months (a-c), between 6 months and 2 years (d-f) and more than 2

years (g-i) before serum sampling. Censoring is indicated by vertical lines; p-values were calculated by log rank statistics.

Figure 14: Overall survival subsequent to serum sampling according to S100B serum levels. Kaplan-Meier curves are shown for overall survival subsequent to serum sampling of 466 tumor-free stage III (a), 83 tumor-free stage IV (b) and 193 unresectable stage IV (c) patients. Patients were categorized based on S100B serum levels (normal vs. elevated). Censoring is indicated by vertical lines; p-values were calculated by log rank statistics.

DETAILED DESCRIPTION OF THE INVENTION

Definitions and General Techniques

Unless otherwise defined below, the terms used in the present invention shall be understood in accordance with their common meaning known to the person skilled in the art.

The term "antibody" as used herein refers to any functional antibody that is capable of specific binding to the antigen of interest, as generally outlined in chapter 7 of Paul, W.E. (Ed.): *Fundamental Immunology* 2nd Ed. Raven Press, Ltd., New York 1989. Without particular limitation, the term "antibody" encompasses antibodies from any appropriate source species, including chicken and mammalian such as mouse, goat, non-human primate and human. Preferably, the antibody is a humanized antibody. The antibody is preferably a monoclonal antibody which can be prepared by methods well-known in the art. The term "antibody" encompasses an IgG-1, -2, -3, or -4, IgE, IgA, IgM, or IgD isotype antibody. The term "antibody" encompasses monomeric antibodies (such as IgD, IgE, IgG) or oligomeric antibodies (such as IgA or IgM). The term "antibody" also encompasses – without particular limitations – isolated antibodies and modified antibodies such as genetically engineered antibodies, e.g. chimeric antibodies.

The nomenclature of the domains of antibodies follows the terms as known in the art. Each monomer of an antibody comprises two heavy chains and two light chains, as generally known in the art. Of these, each heavy and light chain comprises a variable domain (termed V_H for the heavy chain and V_L for the light chain) which is important for antigen binding. These heavy and light chain variable domains comprise (in an N-terminal to C-terminal order) the regions FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4 (FR, framework region; CDR, complementarity determining region which is also known as hypervariable region). The identification and assignment of the above-mentioned antibody regions within the antibody sequence is generally in accordance with Kabat et al. (*Sequences of proteins of immunological interest*, U.S. Dept. of Health and Human Services, Public Health Service, National Institutes of Health, Bethesda, Md. 1983), or Chothia et al. (*Conformations of immunoglobulin hypervariable regions*. *Nature*. 1989 Dec 21-28;342(6252):877-83.), or may be performed by using the IMGT/V-QUEST software described in Giudicelli et

al. (IMGT/V-QUEST, an integrated software program for immunoglobulin and T cell receptor V-J and V-D-J rearrangement analysis. *Nucleic Acids Res.* 2004 Jul 1;32(Web Server issue):W435-40.). Preferably, the antibody regions indicated above are identified and assigned by using the IMGT/V-QUEST software.

A "monoclonal antibody" is an antibody from an essentially homogenous population of antibodies, wherein the antibodies are substantially identical in sequence (i.e. identical except for minor fraction of antibodies containing naturally occurring sequence modifications such as amino acid modifications at their N- and C-termini). Unlike polyclonal antibodies which contain a mixture of different antibodies directed to either a single epitope or to numerous different epitopes, monoclonal antibodies are directed to the same epitope and are therefore highly specific. The term "monoclonal antibody" includes (but is not limited to) antibodies which are obtained from a monoclonal cell population derived from a single cell clone, as for instance the antibodies generated by the hybridoma method described in Köhler and Milstein (*Nature*, 1975 Aug 7;256(5517):495-7) or Harlow and Lane ("*Antibodies: A Laboratory Manual*" Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York 1988). A monoclonal antibody may also be obtained from other suitable methods, including phage display techniques such as those described in Clackson et al. (*Nature*, 1991 Aug 15;352(6336):624-8) or Marks et al. (*J Mol Biol.* 1991 Dec 5;222(3):581-97). A monoclonal antibody may be an antibody that has been optimized for antigen-binding properties such as decreased K_d values, optimized association and dissociation kinetics by methods known in the art. For instance, K_d values may be optimized by display methods including phage display, resulting in affinity-matured monoclonal antibodies. The term "monoclonal antibody" is not limited to antibody sequences from particular species of origin or from one single species of origin. Thus, the meaning of the term "monoclonal antibody" encompasses chimeric monoclonal antibodies such as humanized monoclonal antibodies.

"Humanized antibodies" are antibodies which contain human sequences and a minor portion of non-human sequences which confer binding specificity to an antigen of interest (e.g. human GDF-15). Typically, humanized antibodies are generated by replacing hypervariable region sequences from a human acceptor antibody by hypervariable region sequences from a non-human donor antibody (e.g. a mouse, rabbit, rat donor antibody) that binds to an antigen of interest (e.g. human GDF-15). In some cases, framework region sequences of the acceptor antibody may also be replaced by the corresponding sequences of the donor antibody. In addition to the sequences derived from the donor and acceptor antibodies, a "humanized antibody" may either contain other (additional or substitute) residues or sequences or not. Such other residues or sequences may serve to further improve antibody properties such as binding properties (e.g. to decrease K_d values) and/or immunogenic properties (e.g. to decrease antigenicity in humans). Non-limiting examples for methods to generate humanized antibodies are known in the art, e.g. from Riechmann et al. (*Nature*, 1988 Mar 24; 332(6162):323-7) or Jones et al. (*Nature*, 1986 May 29-Jun 4; 321(6069):522-5).

The term **"human antibody"** relates to an antibody containing human variable and constant domain sequences. This definition encompasses antibodies having human sequences bearing single amino acid substitutions or modifications which may serve to further improve antibody properties such as binding properties (e.g. to decrease K_d values) and/or immunogenic properties (e.g. to decrease antigenicity in humans). The term **"human antibody"** excludes humanized antibodies where a portion of non-human sequences confers binding specificity to an antigen of interest.

An **"antigen-binding portion"** of an antibody as used herein refers to a portion of an antibody that retains the capability of the antibody to specifically bind to the antigen (e.g. hGDF-15, PD-1, PD-L1 or CTLA4). This capability can, for instance, be determined by determining the capability of the antigen-binding portion to compete with the antibody for specific binding to the antigen by methods known in the art. The antigen-binding portion may contain one or more fragments of the antibody. Without particular limitation, the antigen-binding portion can be produced by any suitable method known in the art, including recombinant DNA methods and preparation by chemical or enzymatic fragmentation of antibodies. Antigen-binding portions may be Fab fragments, F(ab') fragments, F(ab')₂ fragments, single chain antibodies (scFv), single-domain antibodies, diabodies or any other portion(s) of the antibody that retain the capability of the antibody to specifically bind to the antigen.

An **"antibody"** (e.g. a monoclonal antibody) or an **"antigen-binding portion"** may have been derivatized or be linked to a different molecule. For example, molecules that may be linked to the antibody are other proteins (e.g. other antibodies), a molecular label (e.g. a fluorescent, luminescent, colored or radioactive molecule), a pharmaceutical and/or a toxic agent. The antibody or antigen-binding portion may be linked directly (e.g. in form of a fusion between two proteins), or via a linker molecule (e.g. any suitable type of chemical linker known in the art).

As used herein, the terms **"binding"** or **"bind"** refer to specific binding to the antigen of interest (e.g. human GDF-15). Preferably, the K_d value is less than 100 nM, more preferably less than 50 nM, still more preferably less than 10 nM, still more preferably less than 5 nM and most preferably less than 2 nM.

The term **"epitope"** as used herein refers to a small portion of an antigen that forms the binding site for an antibody.

In the context of the present invention, for the purposes of characterizing the binding properties of antibodies, binding or competitive binding of antibodies or their antigen-binding portions to the antigen of interest (e.g. human GDF-15) is preferably measured by using surface plasmon resonance measurements as a reference standard assay, as described below.

The terms " K_D " or " K_D value" relate to the equilibrium dissociation constant as known in the art. In the context of the present invention, these terms relate to the equilibrium dissociation constant of an antibody with respect to a particular antigen of interest (e.g. human GDF-15). The equilibrium dissociation constant is a measure of the propensity of a complex (e.g. an antigen-antibody complex) to reversibly dissociate into its components (e.g. the antigen and the antibody). For the antibodies according to the invention, K_D values (such as those for the antigen human GDF-15) are preferably determined by using surface plasmon resonance measurements as described below.

An "isolated antibody" as used herein is an antibody that has been identified and separated from the majority of components (by weight) of its source environment, e.g. from the components of a hybridoma cell culture or a different cell culture that was used for its production (e.g. producer cells such as CHO cells that recombinantly express the antibody). The separation is performed such that it sufficiently removes components that may otherwise interfere with the suitability of the antibody for the desired applications (e.g. with a therapeutic use of the anti-human GDF-15 antibody according to the invention). Methods for preparing isolated antibodies are known in the art and include Protein A chromatography, anion exchange chromatography, cation exchange chromatography, virus retentive filtration and ultrafiltration. Preferably, the isolated antibody preparation is at least 70 % pure (w/w), more preferably at least 80 % pure (w/w), still more preferably at least 90 % pure (w/w), still more preferably at least 95 % pure (w/w), and most preferably at least 99 % pure (w/w), as measured by using the Lowry protein assay.

A "diabody" as used herein is a small bivalent antigen-binding antibody portion which comprises a heavy chain variable domain linked to a light chain variable domain on the same polypeptide chain linked by a peptide linker that is too short to allow pairing between the two domains on the same chain. This results in pairing with the complementary domains of another chain and in the assembly of a dimeric molecule with two antigen binding sites. Diabodies may be bivalent and monospecific (such as diabodies with two antigen binding sites for human GDF-15), or may be bivalent and bispecific (e.g. diabodies with two antigen binding sites, one being a binding site for human GDF-15, and the other one being a binding site for a different antigen). A detailed description of diabodies can be found in Holliger P et al. ("Diabodies": small bivalent and bispecific antibody fragments." *Proc Natl Acad Sci U S A*. 1993 Jul 15;90(14):6444-8.).

A "single-domain antibody" (which is also referred to as "NanobodyTM") as used herein is an antibody fragment consisting of a single monomeric variable antibody domain. Structures of and methods for producing single-domain antibodies are known from the art, e.g. from Holt LJ et al. ("Domain antibodies: proteins for therapy." *Trends Biotechnol*. 2003 Nov;21(11):484-90.), Saerens D et al. ("Single-domain antibodies as building blocks for novel therapeutics." *Curr Opin Pharmacol*. 2008 Oct;8(5):600-8. Epub 2008 Aug 22.), and Arbabi Ghahroudi M et al. ("Selection and identification of single domain antibody fragments from camel heavy-chain antibodies." *FEBS Lett*. 1997 Sep 15;414(3):521-6.).

The terms "significant", "significantly", etc. as used herein refer to a statistically significant difference between values as assessed by appropriate methods known in the art, and as assessed by the methods referred to herein.

In accordance with the present invention, each occurrence of the term "comprising" may optionally be substituted with the term "consisting of".

The terms "cancer" and "cancer cell" is used herein in accordance with their common meaning in the art (see for instance Weinberg R. et al.: The Biology of Cancer. Garland Science: New York 2006. 850p).

The cancers, for which a prediction of a clinical outcome, in particular a prediction of patient survival according to the present invention is provided, is melanoma. As used herein, the term "melanoma" is used in accordance with its general meaning known in the art. Melanomas are classified according to the AJCC staging system for melanoma patients with distant metastases since 2001 (Balch, CM et al., J Clin Oncol / 19 / 3635-48. 2001). The melanoma stages referred to herein refer to this staging system. In a preferred aspect of the present invention in accordance with all of the embodiments of the present invention, the melanoma is not a uveal melanoma.

The melanoma patients, for which a prediction of survival according to the invention is provided, may be subject to a treatment of the melanoma. As used herein, terms such as "treatment of cancer" or "treating cancer" or "treatment of melanoma" or "treating melanoma" refer to a therapeutic treatment. As used herein, such treatments do not only include treatments of the melanoma itself but also palliative treatments. Such palliative treatments are known in the art and include, for instance, treatments which only improve the symptoms of the melanoma disease.

As referred to herein, a treatment of cancer can be a first-line therapy, a second-line therapy or a third-line therapy or a therapy that is beyond third-line therapy. The meaning of these terms is known in the art and in accordance with the terminology that is commonly used by the US National Cancer Institute.

A treatment of cancer does not exclude that additional or secondary therapeutic benefits also occur in patients. For example, an additional or secondary benefit may be an influence on cancer-induced weight loss.

As referred to herein, a "tumor-free" melanoma patient is a patient in which no primary tumor and no metastasis can be detected according to clinical standard methods known in the art. This, however, does not

exclude that tumors (or micrometastases) exist in the patient, which are below the detection limit, or that tumor cells exist, which may form a new tumor.

Blood samples:

As referred to herein, the term "blood sample" includes, without limitation, whole blood, serum and plasma samples. It also includes other sample types such as blood fractions other than serum and plasma. Such samples and fractions are known in the art.

Blood samples which are used for the methods according to the invention can be any types of blood samples which contain hGDF-15. Suitable types of blood samples containing hGDF-15 are known in the art and include serum and plasma samples. Alternatively, further types of blood samples which contain hGDF-15 can also be readily identified by the skilled person, e.g. by measuring whether hGDF-15 is contained in these samples, and which levels of hGDF-15 are contained in these samples, by using the methods disclosed herein.

Clinical outcomes:

According to the invention, levels of hGDF-15 in human blood samples can be used to predict the probability of survival of a human melanoma patient.

Survival of patient groups can be analysed by methods known in the art, e.g. by Kaplan-Meier curves.

Appropriate time periods for the assessment of survival are known in the art and will be chosen by the skilled person with due regard to factors such as the respective stage of the melanoma.

For example, survival, preferably short-term survival, may, for instance, be predicted for time points of 6 months, 12 months and/or 18 months after the time point when the prediction was made. Alternatively, survival, preferably long-term survival, may, for instance, be assessed at a time point of 2 years, 3 years, 5 years and/or 10 years after the time point when the prediction was made.

Predicting the probability of a positive clinical outcome according to the invention

For predicting the probability of a positive clinical outcome (e.g. survival) according to the invention, e.g. based on hGDF-15 levels, the methods for predicting, which are defined above in the preferred embodiments, are preferably used.

In order to practice the methods of the invention, statistical methods known in the art can be employed.

For instance, overall survival can be analyzed by Cox regression analysis.

Preferred statistical methods, which can be used according to the invention to generate statistical models of patient data from clinical studies, are disclosed in Example 1. It is understood that the statistical methods disclosed in Example 1 are not limited to the particular features of Example 1 such as the melanoma stage, the particular threshold levels chosen and the particular statistical values obtained in the Example. Rather, these methods disclosed in Example 1 can generally be used in connection with any embodiment of the present invention.

hGDF-15 levels

In an advantageous aspect of the invention, there is an inverse relationship between hGDF-15 levels and the probability of a positive clinical outcome, in particular the probability of survival, in human melanoma patients. Thus, according to the invention, a decreased level of hGDF-15 indicates an increased probability of survival in human melanoma patients.

Thus, as used herein, terms such as "wherein a decreased level of hGDF-15 in said human blood sample indicates an increased probability of survival" mean that the level of hGDF-15 in said human blood sample and the probability of survival follow an inverse relationship. Thus, the higher the level of hGDF-15 in said human blood sample is, the lower is the probability of survival.

For instance, in connection with the methods for predicting according to the invention defined herein, hGDF-15 threshold levels can be used.

According to the invention, the inverse relationship between hGDF-15 levels and the probability of survival applies to any threshold value, and hence the invention is not limited to particular threshold values.

Preferable hGDF-15 threshold levels are hGDF-15 serum levels as defined above in the preferred embodiments.

Alternatively, hGDF-15 threshold levels according to the present invention can be obtained, and/or further adjusted, by using the above-mentioned statistical methods, e.g. the methods of Example 1.

An hGDF-15 threshold level may be a single hGDF-15 threshold level. The invention also encompasses the use of more than one hGDF-15 threshold level, e.g. 2, 3, 4, 5, 6, 7, 8, 9, 10 or more hGDF-15 threshold levels.

For each single hGDF-15 threshold level of the one or more hGDF-15 threshold levels, a corresponding probability of survival can be predicted at a given time point.

hGDF-15 levels in blood samples can be measured by any methods known in the art. For instance, a preferred method of measuring hGDF-15 levels in blood samples including serum levels is a measurement of hGDF-15 levels by Enzyme-Linked Immunosorbent Assay (ELISA) by using antibodies to hGDF-15. Such ELISA methods are exemplified in Example 1, but can also include bead-based methods like the Luminex technology and others. Alternatively, hGDF-15 levels in blood samples including serum levels may be determined by known electrochemiluminescence immunoassays using antibodies to hGDF-15. For instance, the Roche Elecsys® technology can be used for such electrochemiluminescence immunoassays. Other possible methods would include antibody-based detection from bodily fluids after separation of proteins in an electrical field.

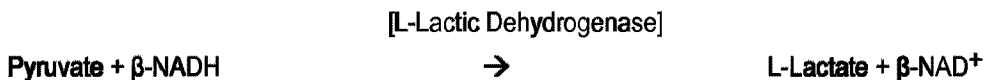
The median hGDF-15 serum level of healthy human control individuals is < 0.8 ng/ml. The expected range is between 0.2 ng/ml and 1.2 ng/ml in healthy human controls (Reference: Tanno T et al.: "Growth differentiation factor 15 in erythroid health and disease." *Curr Opin Hematol.* 2010 May; 17(3): 184–190.).

According to the invention, preferable hGDF-15 threshold levels are hGDF-15 serum levels as defined above in the preferred embodiments.

It is understood that for these hGDF-15 serum levels, and based on the disclosure of the invention provided herein, corresponding hGDF-15 levels in other blood samples can be routinely obtained by the skilled person (e.g. by comparing the relative level of hGDF-15 in serum with the respective level in other blood samples). Thus, the present invention also encompasses preferred hGDF-15 levels in plasma, whole blood and other blood samples, which correspond to each of the preferred hGDF-15 serum levels and ranges indicated above.

Lactate dehydrogenase levels

Lactate dehydrogenase levels in blood samples can be measured by any methods known in the art. Lactate dehydrogenase (LDH) levels are typically measured in enzymatic units (U). One unit will reduce 1.0 μ mole of pyruvate to L-lactate per minute at pH 7.5 at 37°C.



Lactate and NAD⁺ are converted to pyruvate and NADH by the action of LDH. NADH strongly absorbs light at 340 nm, whereas NAD⁺ does not. The rate of increase in absorbance at 340 nm is directly proportional to the LDH activity in the sample. Thus, LDH units are preferably determined by measuring absorbance at 340 nm.

Various clinically accepted diagnostic tests are available for the measurement of LDH levels. In accordance with the present invention, tests which can be applied to melanoma will be selected based on known clinical standards. Isoform-specific tests for LDH can be performed according to methods known in the art.

In a further advantageous aspect of the invention, there is also an inverse relationship between lactate dehydrogenase (LDH) levels and the probability of a positive clinical outcome, in particular the probability of survival, in human melanoma patients. Thus, in an embodiment according to the invention, a decreased level of lactate dehydrogenase indicates an increased probability of survival in melanoma patients.

Thus, as used herein, terms such as "wherein a decreased level of lactate dehydrogenase in said human blood sample indicates an increased probability of survival" mean that the level of lactate dehydrogenase in said human blood sample and the probability of survival follow an inverse relationship. Thus, the higher the level of lactate dehydrogenase in said human blood sample is, the lower is the probability of survival.

For instance, in connection with the methods for predicting according to the invention defined herein, lactate dehydrogenase threshold levels can be used.

According to the invention, the inverse relationship between lactate dehydrogenase levels and the probability of survival applies to any threshold value, and hence the invention is not limited to particular threshold values.

Alternatively, lactate dehydrogenase threshold levels according to the present invention can be obtained, and/or further adjusted, by using the above-mentioned statistical methods, e.g. the methods of Example 1.

A lactate dehydrogenase threshold level may be a single lactate dehydrogenase threshold level. The invention also encompasses the use of more than one lactate dehydrogenase threshold level, e.g. 2, 3, 4, 5, 6, 7, 8, 9, 10 or more lactate dehydrogenase threshold levels.

For each single lactate dehydrogenase threshold level of the one or more lactate dehydrogenase threshold levels, a corresponding probability of survival can be predicted.

According to the invention, preferable lactate dehydrogenase threshold levels are lactate dehydrogenase serum levels as defined above in the preferred embodiments.

In a very preferred embodiment, the lactate dehydrogenase threshold level is a clinically accepted threshold level which distinguishes between normal and elevated LDH levels in patients. Such very preferred clinically accepted threshold levels are known in the art, and will be chosen by the skilled person with regard to the particular specifications of the LDH test.

It is understood that for these lactate dehydrogenase serum levels, and based on the disclosure of the invention provided herein, corresponding lactate dehydrogenase levels in other blood samples can be routinely obtained by the skilled person (e.g. by comparing the relative level of lactate dehydrogenase in serum with the respective level in other blood samples). Thus, the present invention also encompasses preferred lactate dehydrogenase levels in plasma, whole blood and other blood samples, which correspond to each of the preferred lactate dehydrogenase serum levels and ranges indicated above.

S100B levels

In a further advantageous aspect of the invention, there is also an inverse relationship between S100B levels and the probability of a positive clinical outcome, in particular the probability of survival, in human melanoma patients. Thus, in an embodiment according to the invention, a decreased level of S100B indicates an increased probability of survival in melanoma patients.

Thus, as used herein, terms such as "wherein a decreased level of S100B in said human blood sample indicates an increased probability of survival" mean that the level of S100B in said human blood sample and the probability of survival follow an inverse relationship. Thus, the higher the level of S100B in said human blood sample is, the lower is the probability of survival.

For instance, in connection with the methods for predicting according to the invention defined herein, S100B threshold levels can be used.

According to the invention, the inverse relationship between S100B levels and the probability of survival applies to any threshold value, and hence the invention is not limited to particular threshold values.

S100B threshold levels according to the present invention can, for instance, be obtained, and/or further adjusted, by using the above-mentioned statistical methods, e.g. the methods of Example 1.

An S100B threshold level may be a single S100B threshold level. The invention also encompasses the use of more than one S100B threshold level, e.g. 2, 3, 4, 5, 6, 7, 8, 9, 10 or more S100B threshold levels.

In a very preferred embodiment, the S100B threshold level is a clinically accepted threshold level which distinguishes between normal and elevated S100B levels in patients. Such very preferred clinically accepted threshold levels are known in the art, and will be chosen by the skilled person with regard to the particular specifications of the S100B test.

For each single S100B threshold level of the one or more S100B threshold levels, a corresponding probability of survival can be predicted.

S100B levels in blood samples can be measured by any methods known in the art. Such methods include antibody-based assays. A preferred method of measuring S100B levels in blood samples is a measurement of S100B serum levels by electrochemoluminescence assays, e.g. by using an Elecsys S100 electrochemoluminescence immunoassay. Further non-limiting examples of methods to measure S100B levels are given in Gonçalves et al.: "Biological and methodological features of the measurement of S100B, a putative marker of brain injury." *Clinical Biochemistry* 41 (2008) 755–763).

Antibodies capable of binding to hGDF-15 which can be used in accordance with the invention

The methods, apparatuses and kits of the invention may use one or more antibodies capable of binding to hGDF-15 or an antigen-binding portion thereof, as defined above.

It was previously shown that human GDF-15 protein can be advantageously targeted by a monoclonal antibody (WO2014/049087), and that such antibody has advantageous properties including a high binding affinity to human GDF-15, as demonstrated by an equilibrium dissociation constant of about 790pM for recombinant human GDF-15 (see Reference Example 1). Thus, in a preferred embodiment, the invention uses an antibody capable of binding to hGDF-15, or an antigen-binding portion thereof. Preferably, the antibody is a monoclonal antibody capable of binding to hGDF-15, or an antigen-binding portion thereof.

Thus, in a more preferred embodiment, the antibody capable of binding to hGDF-15 or antigen-binding portion thereof in accordance with the invention is a monoclonal antibody capable of binding to human GDF-15, or an antigen-binding portion thereof, wherein the heavy chain variable domain comprises a CDR3 region comprising the amino acid sequence of SEQ ID NO: 5 or an amino acid sequence at least 90% identical thereto, and wherein the light chain variable domain comprises a CDR3 region comprising the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence at least 85% identical thereto. In this embodiment, preferably, the antibody or antigen-binding portion thereof comprises a heavy chain variable domain which comprises a CDR1 region comprising the amino acid sequence of SEQ ID NO: 3 and a CDR2 region comprising the amino acid sequence of SEQ ID NO: 4, and the antibody or antigen-binding portion thereof comprises a light chain variable domain which comprises a CDR1 region comprising the amino acid sequence of SEQ ID NO: 6, and a CDR2 region comprising the amino acid sequence ser-ala-ser.

Thus, in a still more preferred embodiment, the antibody capable of binding to hGDF-15 or antigen-binding portion thereof in accordance with the invention is a monoclonal antibody capable of binding to human GDF-15, or an antigen-binding portion thereof, wherein the antibody or antigen-binding portion thereof comprises a

heavy chain variable domain which comprises a CDR1 region comprising the amino acid sequence of SEQ ID NO: 3, a CDR2 region comprising the amino acid sequence of SEQ ID NO: 4 and a CDR3 region comprising the amino acid sequence of SEQ ID NO: 5, and wherein the antibody or antigen-binding portion thereof comprises a light chain variable domain which comprises a CDR1 region comprising the amino acid sequence of SEQ ID NO: 6, a CDR2 region comprising the amino acid sequence ser-ala-ser and a CDR3 region comprising the amino acid sequence of SEQ ID NO: 7.

In another embodiment in accordance with the above embodiments of the monoclonal antibody capable of binding to human GDF-15, or an antigen-binding portion thereof, the heavy chain variable domain comprises a region comprising an FR1, a CDR1, an FR2, a CDR2 and an FR3 region and comprising the amino acid sequence of SEQ ID NO: 1 or a sequence 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical thereto, and the light chain variable domain comprises a region comprising an FR1, a CDR1, an FR2, a CDR2 and an FR3 region and comprising the amino acid sequence of SEQ ID NO: 2 or a sequence 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical thereto.

In another embodiment in accordance with the above embodiments of the monoclonal antibody capable of binding to human GDF-15, or an antigen-binding portion thereof, the heavy chain variable domain comprises a CDR1 region comprising the amino acid sequence of SEQ ID NO: 3 and a CDR2 region comprising the amino acid sequence of SEQ ID NO: 4, and the light chain variable domain comprises a CDR1 region comprising the amino acid sequence of SEQ ID NO: 6 and a CDR2 region comprising the amino acid sequence of SEQ ID NO: 7. In a preferred aspect of this embodiment, the antibody may have CDR3 sequences as defined in any of the embodiments of the invention described above.

In another embodiment in accordance with the monoclonal antibody capable of binding to human GDF-15, or an antigen-binding portion thereof, the antigen-binding portion is a single-domain antibody (also referred to as "NanobodyTM"). In one aspect of this embodiment, the single-domain antibody comprises the CDR1, CDR2, and CDR3 amino acid sequences of SEQ ID NO: 3, SEQ ID NO: 4, and SEQ ID NO: 5, respectively. In another aspect of this embodiment, the single-domain antibody comprises the CDR1, CDR2, and CDR3 amino acid sequences of SEQ ID NO: 6, ser-ala-ser, and SEQ ID NO: 7, respectively. In a preferred aspect of this embodiment, the single-domain antibody is a humanized antibody.

Preferably, the antibodies capable of binding to human GDF-15 or the antigen-binding portions thereof have an equilibrium dissociation constant for human GDF-15 that is equal to or less than 100 nM, less than 20 nM, preferably less than 10 nM, more preferably less than 5 nM and most preferably between 0.1 nM and 2 nM.

In another embodiment in accordance with the above embodiments of the monoclonal antibody capable of binding to human GDF-15, or an antigen-binding portion thereof, the antibody capable of binding to human

GDF-15 or the antigen-binding portion thereof binds to the same human GDF-15 epitope as the antibody to human GDF-15 obtainable from the cell line B1-23 deposited with the Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH (DSMZ) under the accession No. DSM ACC3142. As described herein, antibody binding to human GDF-15 in accordance with the present invention is preferably assessed by surface plasmon resonance measurements as a reference standard method, in accordance with the procedures described in Reference Example 1. Binding to the same epitope on human GDF-15 can be assessed similarly by surface plasmon resonance competitive binding experiments of the antibody to human GDF-15 obtainable from the cell line B1-23 and the antibody that is expected to bind to the same human GDF-15 epitope as the antibody to human GDF-15 obtainable from the cell line B1-23.

In a very preferred embodiment, the antibody capable of binding to human GDF-15 or the antigen-binding portion thereof is a monoclonal antibody capable of binding to human GDF-15, or an antigen-binding portion thereof, wherein the binding is binding to a conformational or discontinuous epitope on human GDF-15 comprised by the amino acid sequences of SEQ ID No: 25 and SEQ ID No: 26. In a preferred aspect of this embodiment, the antibody or antigen-binding portion thereof is an antibody or antigen-binding portion thereof as defined by the sequences of any one of the above embodiments.

In a further embodiment in accordance with the above embodiments, antibodies including the antibody capable of binding to human GDF-15 or the antigen-binding portion thereof can be modified, e.g. by a tag or a label.

A tag can, for instance, be a biotin tag or an amino acid tag. Non-limiting examples of such acid tag tags include Polyhistidin (His-) tags, FLAG-tag, Hemagglutinin (HA) tag, glycoprotein D (gD) tag, and c-myc tag. Tags may be used for various purposes. For instance, tags may be used to assist purification of the antibody capable of binding to human GDF-15 or the antigen-binding portion thereof. Preferably, such tags are present at the C-terminus or N-terminus of the antibody capable of binding to human GDF-15 or the antigen-binding portion thereof.

As used herein, the term "label" relates to any molecule or group of molecules which can facilitate detection of the antibody. For instance, labels may be enzymatic such as horseradish peroxidase (HRP), alkaline phosphatase (AP) or glucose oxidase. Enzymatically labelled antibodies may, for instance, be employed in enzyme-linked immunosorbent assays. Labels may also be radioactive isotopes, DNA sequences (which may, for instance, be used to detect the antibodies by polymerase chain reaction (PCR)), fluorogenic reporters and electrochemiluminescent groups (e.g. ruthenium complexes). As an alternative to labelling, antibodies used according to the invention, in particular an antibody capable of binding to human GDF-15 or the antigen-binding portion thereof, can be detected directly, e.g. by surface plasmon resonance measurements.

Methods and Techniques

Generally, unless otherwise defined herein, the methods used in the present invention (e.g. cloning methods or methods relating to antibodies) are performed in accordance with procedures known in the art, e.g. the procedures described in Sambrook et al. ("Molecular Cloning: A Laboratory Manual.", 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York 1989), Ausubel et al. ("Current Protocols in Molecular Biology." Greene Publishing Associates and Wiley Interscience; New York 1992), and Harlow and Lane ("Antibodies: A Laboratory Manual" Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York 1988).

Binding of antibodies to their respective target proteins can be assessed by methods known in the art. The binding of monoclonal antibodies to their respective targets is preferably assessed by surface plasmon resonance measurements. These measurements are preferably carried out by using a Biorad ProteOn XPR36 system and Biorad GLC sensor chips, as exemplified for anti-human GDF-15 mAb-B1-23 in Reference Example 1.

Sequence Alignments of sequences according to the invention are performed by using the BLAST algorithm (see Altschul et al.(1990) "Basic local alignment search tool." Journal of Molecular Biology 215. p. 403-410.; Altschul et al.: (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389-3402). Preferably, the following parameters are used: Max target sequences 10; Word size 3; BLOSUM 62 matrix; gap costs: existence 11, extension 1; conditional compositional score matrix adjustment. Thus, when used in connection with sequences, terms such as "identity" or "identical" refer to the identity value obtained by using the BLAST algorithm.

Monoclonal antibodies according to the invention can be produced by any method known in the art, including but not limited to the methods referred to in Siegel DL ("Recombinant monoclonal antibody technology." Transfus Clin Biol. 2002 Jan;9(1):15-22). In one embodiment, an antibody according to the invention is produced by the hybridoma cell line B1-23 deposited with the Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH (DSMZ) under the accession No. DSM ACC3142 under the Budapest treaty. The deposit was filed on September 29, 2011.

Levels of human GDF-15 (hGDF-15) can be measured by any method known in the art, including measurements of hGDF-15 protein levels by methods including (but not limited to) mass spectrometry for proteins or peptides derived from human GDF-15, Western Blotting using antibodies specific to human GDF-15, strip tests using antibodies specific to human GDF-15, or immunocytochemistry using antibodies specific to human GDF-15. A preferred method of measuring hGDF-15 serum levels is a measurement of hGDF-15 serum levels by Enzyme-Linked Immunosorbent Assay (ELISA) by using antibodies to GDF-15. Such ELISA

methods are exemplified in Example 1. Alternatively, hGDF-15 serum levels may be determined by known electrochemiluminescence immunoassays using antibodies to hGDF-15. For instance, the Roche Elecsys® technology can be used for such electrochemiluminescence immunoassays.

Apparatuses of the invention

The invention also relates to the apparatuses defined above.

An apparatus of the invention can be any apparatus which is configured to perform the methods of the invention.

As used herein, the term "configured to perform" means that the apparatus is specifically configured for the recited method steps. For instance, an apparatus configured to perform a method which uses a particular threshold level will be specifically configured to use that particular threshold.

In a preferred embodiment, the apparatus is an electrochemiluminescence analyzer such as Cobas® analyzer. In this embodiment, if LDH is measured, this may, for instance, be measured on an additional apparatus, which is not an electrochemiluminescence analyzer, and which is configured to perform LDH measurements such as enzymatic tests. Thus, in a preferred aspect of this embodiment, the electrochemiluminescence analyzer of the invention is configured to perform the methods of the invention except for the measurements of LDH levels.

Kits of the invention

The invention also relates to the kits defined above.

The recombinant hGDF-15 contained in the kits may be present in a form which can conveniently be used for calibration purposes. For instance, it may be present in the form of stock solutions which cover several concentrations in the range of 0 to 15 ng/ml, e.g. at least one concentration in the range of 0-1 ng/ml, at least one concentration in the range of 1-3 ng/ml, at least one concentration in the range of 3-6 ng/ml, and preferably at least one further concentration in the range of 6-10 ng/ml, and more preferably further comprising at least one further concentration in the range of 10-15 ng/ml.

Sequences

The amino acid sequences referred to in the present application are as follows (in an N-terminal to C-terminal order; represented in the one-letter amino acid code):

SEQ ID No: 1 (Region of the Heavy Chain Variable Domain comprising an FR1, a CDR1, an FR2, a CDR2 and an FR3 region from the Polypeptide Sequence of monoclonal anti-human GDF-15 mAb-B1-23):

QVKLQQSGPGILQSSQTLSTCSFSGFSLSTSGMGVSWIRQPSGKGLEWLAHIYWDDDKRYNPTLKSRLTISK
DPSRNQVFLKITSVDTADTATYYC

SEQ ID No: 2 (Region of the Light Chain Variable Domain comprising an FR1, a CDR1, an FR2, a CDR2 and an FR3 region from the Polypeptide Sequence of monoclonal anti-human GDF-15 mAb-B1-23):

DIVLTQSPKFMSTSVGDRVSVTCKASQNVGTNVAWFLQKPGQSPKALIYSASYRYSGVPDRFTGSGSGTDFT
LTISNVQSEDLAEYFC

SEQ ID No: 3 (Heavy Chain CDR1 Region Peptide Sequence of monoclonal anti-human GDF-15 mAb-B1-23):

GFSLSTSGMG

SEQ ID No: 4 (Heavy Chain CDR2 Region Peptide Sequence of monoclonal anti-human GDF-15 mAb-B1-23):

IYWDDDK

SEQ ID No: 5 (Heavy Chain CDR3 Region Peptide Sequence of monoclonal anti-human GDF-15 mAb-B1-23):

ARSSYGAMDY

SEQ ID No: 6 (Light Chain CDR1 Region Peptide Sequence of monoclonal anti-human GDF-15 mAb-B1-23):

QNVGTN

Light Chain CDR2 Region Peptide Sequence of monoclonal anti-human GDF-15 mAb-B1-23:

SAS

SEQ ID No: 7 (Light Chain CDR3 Region Peptide Sequence of monoclonal anti-human GDF-15 mAb-B1-23):

QQYNNFPYT

SEQ ID No: 8 (recombinant mature human GDF-15 protein):

GSARNGDHCPLGPGRCCRLHTVRASLEDLGWADWVLSPREVQVTCIGACPSQFRAANMHAQIKTSLHRLK
PDTVPAPCCVPASYNPMVLIQKTDGTGVS LQTYDDLLAKDCHCI

SEQ ID No: 9 (human GDF-15 precursor protein):

MPGQELRTVNGSQMLLVLLVLSWLP HGGALSLAEASRASFPGPSELHSEDSRFREL RKRYEDLLTRLRANQS
WEDSNTDLVPAPAVRILTPEVRLGSGGHLHLRISRAALPEGLPEASRLHRLFRLSPTASRSWDVTRPLRRQL
SLARPOAPALHLRLSPPPSQSDQLLAESSSARPOLELHLRPQAARGRRRARARNGDHCPLGPGRCCRLHTV
RASLEDLGWADWVLSPREVQVTCIGACPSQFRAANMHAQIKTSLHRLKPDTVPAPCCVPASYNPMVLIQK
DTGVS LQTYDDLLAKDCHCI

SEQ ID No: 10 (human GDF-15 precursor protein + N-terminal and C-terminal GSGS linker):

GSGSGSGMPGQELRTVNGSQMLLVLLVLSWLP HGGALSLAEASRASFPGPSELHSEDSRFREL RKRYEDLL
TRLRANQSWEDSNTDLVPAPAVRILTPEVRLGSGGHLHLRISRAALPEGLPEASRLHRLFRLSPTASRSWDV
TRPLRRQLSLARPOAPALHLRLSPPPSQSDQLLAESSSARPOLELHLRPQAARGRRRARARNGDHCPLGP
RCCRLHTVRASLEDLGWADWVLSPREVQVTCIGACPSQFRAANMHAQIKTSLHRLKPDTVPAPCCVPASY
NPMVLIQKTDGTGVS LQTYDDLLAKDCHCIGSGSGSG

SEQ ID No: 11 (Flag peptide): DYKDDDDKGG

SEQ ID No: 12 (HA peptide): YPYDVPDYAG

SEQ ID No: 13 (peptide derived from human GDF-15): ELHLRPQAARGRR

SEQ ID No: 14 (peptide derived from human GDF-15): LHLRPQAARGRRR

SEQ ID No: 15 (peptide derived from human GDF-15): HLRPQAARGRRRA

SEQ ID No: 16 (peptide derived from human GDF-15): LRPQAARGRRRAR

SEQ ID No: 17 (peptide derived from human GDF-15): RPQAARGRRRARAR

SEQ ID No: 18 (peptide derived from human GDF-15): PQAARGRRRARAR

SEQ ID No: 19 (peptide derived from human GDF-15): QAARGRRRARARN

SEQ ID No: 20 (peptide derived from human GDF-15): MHAQIKTSLHRLK

SEQ ID No: 25 (GDF-15 peptide comprising part of the GDF-15 Epitope that binds to B1-23):

EVQVTMCIGACPSQFR

SEQ ID No: 26 (GDF-15 peptide comprising part of the GDF-15 Epitope that binds to B1-23):

TDTGVSLQTYDDLAKDCHCI

The nucleic acid sequences referred to in the present application are as follows (in a 5' to 3' order; represented in accordance with the standard nucleic acid code):

SEQ ID No: 21 (DNA nucleotide sequence encoding the amino acid sequence defined in SEQ ID No: 1):

CAAGTGAAGCTGCAGCAGTCAGGCCCTGGGATATTGCAGTCCTCCCAGACCCTCAGTCTGACTTGTCT
TTCTCTGGGTTTTCACTGAGTACTTCTGGTATGGGTGTGAGCTGGATTCGTCAGCCTTCAGGAAAGGGTC
TGGAGTGGCTGGCACACATTTACTGGGATGATGACAAGCGCTATAACCCAACCCTGAAGAGCCGGCTCA
CAATCTCCAAGGATCCCTCCAGAAACCAGGTATTCCTCAAGATCACCAGTGTGGACACTGCAGATACTGC
CACATACTACTGT

SEQ ID No: 22 (DNA nucleotide sequence encoding the amino acid sequence defined in SEQ ID No: 2):

GACATTGTGCTCACCCAGTCTCCAAAATTCATGTCCACATCAGTAGGAGACAGGGTCAGCGTCACCTGCA
AGGCCAGTCAGAAATGTGGGTACTAATGTGGCCTGGTTTCTACAGAAACCAGGGCAATCTCCTAAAGCACT
TATTTACTCGGCATCCTACCGGTACAGTGGAGTCCCTGATCGCTTCACAGGCAGTGGATCTGGGACAGA
TTTCACTCTCACCATCAGCAACGTGCAGTCTGAAGACTTGGCAGAGTATTTCTGT

SEQ ID No: 23 (DNA nucleotide sequence encoding the amino acid sequence defined in SEQ ID No: 5):

GCTCGAAGTTCCTACGGGGCAATGGACTAC

SEQ ID No: 24 (DNA nucleotide sequence encoding the amino acid sequence defined in SEQ ID No: 7):

CAGCAATATAACAACCTTCCGTACACG

Examples

Reference Examples 1 to 3 exemplify an antibody to hGDF-15, which can be used in the methods, kits, and in the apparatuses according to the invention. This hGDF-15 antibody is a monoclonal antibody which is known from WO 2014/049087.

Reference Example 1: Generation and characterization of the GDF-15 Antibody B1-23

The antibody B1-23 was generated in a GDF-15 knock out mouse. Recombinant human GDF-15 (SEQ ID No: 8) was used as the immunogen.

The hybridoma cell line B1-23 producing mAb-B1-23 was deposited by the Julius-Maximilians-Universität Würzburg, Sanderring 2, 97070 Würzburg, Germany, with the Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH (DSMZ) under the accession No. DSM ACC3142, in accordance with the Budapest Treaty.

By means of a commercially available test strip system, B1-23 was identified as an IgG2a (kappa chain) isotype. Using surface plasmon resonance measurements, the dissociation constant (K_d) was determined as follows:

Binding of the monoclonal anti-human-GDF-15 antibody anti-human GDF-15 mAb-B1-23 according to the invention was measured by employing surface plasmon resonance measurements using a Biorad ProteOn XPR36 system and Biorad GLC sensor chips:

For preparing the biosensors recombinant mature human GDF-15 protein was immobilized on flow cells 1 and 2. On one flow cell recombinant GDF-15 derived from Baculovirus-transfected insect cells (HighFive insect cells) and on the other recombinant protein derived from expression in *E. coli* was used. The GLC sensor chip was activated using Sulfo-NHS (N-Hydroxysulfosuccinimide) and EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) (Biorad ProteOn Amine Coupling Kit) according to the manufacturer's recommendation, the sensor surface was subsequently loaded with the proteins up to a density of about 600RU (1RU = 1pg mm⁻²). The non-reacted coupling groups were then quenched by perfusion with 1M ethanolamine pH 8.5 and the biosensor was equilibrated by perfusing the chip with running buffer (10M HEPES, 150mM NaCl, 3.4mM EDTA, 0.005% Tween-20, pH 7.4, referred to as HBS150). As controls two flow cells were used, one empty with no protein coupled and one coupled with a non-physiological protein partner (human Interleukin-5), which was immobilized using the same coupling chemistry and the same coupling density. For interaction measurements anti-human GDF-15 mAb-B1-23 was dissolved in HBS150 and used in six different concentrations as analyte (concentration: 0.4, 0.8, 3, 12, 49 und 98 nM). The analyte was perfused over the biosensor using the one-shot kinetics setup to avoid intermittent

regeneration, all measurements were performed at 25°C and using a flow rate of 100 µl min⁻¹. For processing the bulk face effect and unspecific binding to the sensor matrix was removed by subtracting the SPR data of the empty flow cell (flow cell 3) from all other SPR data. The resulting sensogram was analyzed using the software ProteOn Manager version 3.0. For analysis of the binding kinetics a 1:1 Langmuir-type interaction was assumed. For the association rate constant a value of $5.4 \pm 0.06 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (k_{on}) and for the dissociation rate constant a value of $4.3 \pm 0.03 \times 10^{-4} \text{ s}^{-1}$ (k_{off}) could be determined (values are for the interaction of anti-human GDF-15 mAb-B1-23 with GDF-15 derived from insect cell expression). The equilibrium dissociation constant was calculated using the equation $K_D = k_{\text{off}}/k_{\text{on}}$ to yield a value of about 790 pM. Affinity values for the interaction of GDF-15 derived from E. coli expression and the anti-human GDF-15 mAb-B1-23 differ by less than a factor of 2, rate constants for GDF-15 derived from insect cells and E. coli deviate by about 45% and are thus within the accuracy of SPR measurements and likely do not reflect a real difference in affinity. Under the conditions used the anti-human GDF-15 mAb-B1-23 shows no binding to human interleukin-5 and thus confirms the specificity of the interaction data and the anti-human GDF-15 mAb-B1-23.

The amino acid sequence of recombinant human GDF-15 (as expressed in Baculovirus-transfected insect cells) is:

GSARNGDHCP LGPGRCCRLH TVRASLEDLG WADWVLSPRE VQVTMCIGAC PSQFRAANMH
AQIKTSLHRL KPDTVPAPCC VPASYNPMVL IQKTDGTGVS LQTYDDLLAKD CHCI

(SEQ ID No: 8)

Thus, using surface plasmon resonance measurements, the dissociation constant (K_d) of 790 pM was determined. As a comparison: the therapeutically used antibody Rituximab has a significantly lower affinity ($K_d = 8 \text{ nM}$).

It was previously shown that mAb B1-23 inhibits cancer cell proliferation *in vitro*, and that mAb B1-23 inhibits growth of tumors *in vivo* (WO2014/049087).

Reference Example 2: mAb B1-23 recognizes a conformational or a discontinuous epitope of human GDF-15

Epitope Mapping: Monoclonal mouse antibody GDF-15 against 13mer linear peptides derived from GDF-15

Antigen: GDF-15:

GS GSGSGMPGQELRTVNGSQMLLVLLVLSWLP HGGALSLAEASRASFP GPSELHSEDSRFELRKRYEDLL
TRLRANQSWEDSNTDLVPAPAVRILTPEVRLGSGGHLHLRISR AALPEGLPEASRLHRLFR LSPTASRSWDV
TRPLRRQLSLARPQAPALHLRLSPPPSQSDQLLAESSSARPQLELHLRPQAARGRRRARARNGDHCPLGPG

RCCRLHTVRASLEDLGWADWVLSPREVQVTMCIGACPSQFRAANMHAQIKTSLHRLKPDTPAPCCVPASY
NPMVLIQKTDGTGVSLLQTYDDLAKDCHCIGSGSGSG (322 amino acids with linker)(SEQ ID No: 10)

The protein sequence was translated into 13mer peptides with a shift of one amino acid. The C- and N-termini were elongated by a neutral GSGS linker to avoid truncated peptides (bold letters).

Control Peptides:

Flag: DYKDDDDKGG (SEQ ID No:13), 78 spots; HA: YPYDVPDYAG (SEQ ID No:14), 78 spots (each array copy)

Peptide Chip Identifier:

000264_01 (10/90, Ala2Asp linker)

Staining Conditions:

Standard buffer: PBS, pH 7.4 + 0.05% Tween 20

Blocking buffer: Rockland blocking buffer MB-070

Incubation buffer: Standard buffer with 10% Rockland blocking buffer MB-070

Primary sample: Monoclonal mouse antibody GDF-15 (1 µg/µl): Staining in incubation buffer for 16 h at 4°C at a dilution of 1:100 and slight shaking at 500 rpm

Secondary antibody: Goat anti-mouse IgG (H+L) IRDye680, staining in incubation buffer with a dilution of 1:5000 for 30 min at room temperature (RT)

Control antibodies: Monoclonal anti-HA (12CA5)-LL-Atto 680 (1:1000), monoclonal anti-FLAG(M2)-FluoProbes752 (1:1000); staining in incubation buffer for 1 h at RT

Scanner:

Odyssey Imaging System, LI-COR Biosciences

Settings: offset: 1mm; resolution: 21 µm; intensity green/red: 7/7

Results:

After 30 min pre-swelling in standard buffer and 30 min in blocking buffer, the peptide array with 10, 12 and 15mer B7H3-derived linear peptides was incubated with secondary goat anti-mouse IgG (H+L) IRDye680 antibody only at a dilution of 1:5000 for 1h at room temperature to analyze background interactions of the secondary antibody. The PEPperCHIP® was washed 2x1 min with standard buffer, rinsed with dist. water and dried in a stream of air. Read-out was done with Odyssey Imaging System at a resolution of 21 µm and green/red intensities of 7/7: We observed a weak interaction of arginine-rich peptides (ELHLRPQAARGRR (SEQ ID No:15), LHLRPQAARGRRR (SEQ ID No:16), HLRPQAARGRRRA (SEQ ID No:17), LRPQAARGRRRAR (SEQ ID No:18), RPPQAARGRRRAR (SEQ ID No:19), PQAARGRRRARAR (SEQ ID

No:20) and QAARGRRRARARN (SEQ ID No:21)) that are known as frequent binders, and with the basic peptide MHAQIKTSLHRLK (SEQ ID No:22) due to ionic interactions with the charged antibody dye.

After pre-swelling for 10 min in standard buffer, the peptide microarray was incubated overnight at 4 °C with monoclonal mouse antibody GDF-15 at a dilution of 1:100. Repeated washing in standard buffer (2x1 min) was followed by incubation for 30 min with the secondary antibody at a dilution of 1:5000 at room temperature. After 2x10 sec. washing in standard buffer and short rinsing with dist. water, the PEPperCHIP® was dried in a stream of air. Read-out was done with Odyssey Imaging System at a resolution of 21 µm and green/red intensities of 7/7 before and after staining of control peptides by anti-HA and anti-FLAG(M2) antibodies.

It was shown that none of the linear 13mer peptides derived from GDF-15 interacted with monoclonal mouse antibody GDF-15 even at overregulated intensities. Staining of Flag and HA control peptides that frame the array, however, gave rise to good and homogeneous spot intensities.

Summary:

The Epitope Mapping of monoclonal mouse GDF-15 antibody against GDF-15 did not reveal any linear epitope with the 13mer peptides derived from the antigen. According to this finding it is very likely that monoclonal mouse antibody GDF-15 recognizes a conformational or a discontinuous epitope with low affinity of partial epitopes. Due to the obvious absence of any GDF-15 signal above the background staining of the secondary antibody only, quantification of spot intensities with PepSlide® Analyzer and subsequent peptide annotation were omitted.

Reference Example 3: Structural identification of peptide ligand epitopes by mass spectrometric epitope excision and epitope extraction

The epitope of recombinant human GDF-15 which binds to the antibody B1-23 was identified by means of the epitope excision method and epitope extraction method (Suckau et al. Proc Natl Acad Sci U S A. 1990 December; 87(24): 9848–9852.; R.Stefanescu et al., Eur.J.Mass Spectrom. 13, 69-75 (2007)).

For preparation of the antibody column, the antibody B1-23 was added to NHS-activated 6-aminohexanoic acid coupled sepharose. The sepharose-coupled antibody B1-23 was then loaded into a 0,8 ml microcolumn and washed with blocking and washing buffers.

Epitope extraction experiment:

Recombinant human GDF-15 was digested with trypsin for 2h at 37°C (in solution), resulting in different peptides, according to the trypsin cleavage sites in the protein. After complete digestion, the peptides were

loaded on the affinity column containing the immobilized antibody B1-23. Unbound as well as potentially bound peptides of GDF-15 were used for mass spectrometry analysis. An identification of peptides by means of mass spectrometry was not possible. This was a further indicator that the binding region of GDF-15 in the immune complex B1-23 comprises a discontinuous or conformational epitope. In case of a continuous linear epitope, the digested peptides should bind its interaction partner, unless there was a trypsin cleavage site in the epitope peptide. A discontinuous or conformational epitope could be confirmed by the epitope excision method described in the following part.

Epitope excision experiment:

The immobilized antibody B1-23 on the affinity column was then incubated with recombinant GDF-15 for 2h. The formed immune complex on the affinity column was then incubated with trypsin for 2h at 37°C. The cleavage resulted in different peptides derived from the recombinant GDF-15. The immobilized antibody itself is proteolytically stable. The resulting peptides of the digested GDF-15 protein, which were shielded by the antibody and thus protected from proteolytic cleavage, were eluted under acidic conditions (TFA, pH2), collected and identified by mass spectrometry.

The epitope excision method using MS/MS identification resulted in the following peptides:

Peptide	Position in sequence	Mass	Ion/Charge
EVQVTMCIGACPSQFR (SEQ ID No: 25)	40-55	1769.91	590.50(3+)
TDTGVSLQTYDDLAKDCHCI (SEQ ID No: 26)	94-114	2310,96	771:33(3+)

The part of human GDF-15, which binds the antibody B1-23, comprises a discontinuous or conformational epitope. Mass spectrometry identified 2 peptides in the GDF-15 protein, which are responsible for the formation of the immune complex. These peptides are restricted to the positions 40-55 (EVQVTMCIGACPSQFR) and 94-114 (TDTGVSLQTYDDLAKDCHCI) in the GDF-15 amino acid sequence. Thus, these two peptides comprise an epitope of the GDF-15 protein that binds to the antibody B1-23.

The present invention is illustrated by the following non-limiting Examples:

Example 1:**Patients, Materials and Methods****Patients**

Patients from the Department of Dermatology, University of Tübingen, Germany, with histologically confirmed melanoma were identified in the Central Malignant Melanoma Registry (CMMR) database which prospectively records patients from more than 60 dermatological centers in Germany. 761 patients, with (a) archived serum samples taken between January 2008 and February 2012, (b) available follow-up data, and (c) history or presence of loco regional or distant metastasis at the time point of blood draw were selected. The aims and methods of data collection by the CMMR have previously been published in detail (Lasithiotakis, KG et al., Cancer / 107 / 1331-9. 2006). Data obtained for each patient included age, gender, the date of the last follow-up, and the date and cause of death, if applicable. All patients had given written informed consent to have clinical data recorded by the CMMR registry. The institutional ethics committee Tübingen has approved the study (ethic vote 125/2015BO2). Age, the pattern of distant metastasis (stage IV patients only), sub-stage (IIIA vs. IIIB vs. IIIC; stage III patients only) according to the AJCC classification (Balch, CM et al., J Clin Oncol / 27 / 6199-206. 2009), serum LDH and serum S100B (Elecsys S100 electrochemiluminescence immunoassay; Roche Diagnostics AG, Rotkreuz, Switzerland) were evaluated at the time of serum sampling. hGDF-15 serum concentrations were quantified in duplicates using a commercial ELISA kit according to the manufacturer's instructions (R&D systems, Wiesbaden, Germany):

Analysis of hGDF-15 Serum Levels by Enzyme-Linked Immunosorbent Assay (ELISA):

Human GDF-15 serum levels were measured by Enzyme-Linked Immunosorbent Assay (ELISA).

Buffers and reagents:

Blocking solution: 1% BSA (fraction V pH 7.0, PAA) in PBS

Wash solution: PBS-Tween (0.05%)

Standard: human GDF-15 (stock concentration 120 µg/ml, from R&D Systems)

Capture antibody: Human GDF-15 MAb (Clone 147627) from R&D Systems, Mouse IgG2B (catalog #MAB957, from R&D Systems, stock concentration 360 µg/ml)

Detection antibody: Human GDF-15 Biotinylated Affinity Purified PAb, Goat IgG (catalog #BAF940, from R&D Systems, stock concentration 9 µl/ml)

Streptavidin-HRP (Catalog #DY998, from R&D Systems)

Substrate solution: 10 ml 0.1 M NaOAc pH6.0 + 100 µl TMB + 2 µl H₂O₂

Stop solution: 1 M H₂SO₄

Analysis Procedure:**1. Plate Preparation:**

- a. The capture antibody was diluted to the working concentration of 2 µg/ml in PBS. A 96-well microplate (Nunc maxisorp®) was immediately coated with 50 µl per well of the diluted capture antibody excluding the outer rows (A and H). Rows A and H were filled with buffer to prevent evaporation of the samples during the experiment. The plate was gently tapped to

ensure that the bottom of each well was thoroughly covered. The plate was placed in a humid chamber and incubated overnight at room temperature (RT).

- b. Each well was aspirated and washed three times with PBS-Tween (0.05%).
- c. 150 μ l of blocking solution was added to each well, followed by incubation at RT for 1 hour.
- d. Each well was aspirated and washed three times with PBS-Tween (0.05%).

2. Assay Procedure:

- a. Standards were prepared. GDF-15 was diluted in buffered blocking solution to a final concentration of 1 ng/ml (4.17 μ l GDF + 496 μ l buffered blocking solution). 1:2 serial dilutions were made.
- b. Duplicate samples 1:20 (6 μ l + 114 μ l buffered blocking solution) were prepared.
- c. 50 μ l of diluted samples or standards were added per well, followed by incubation for 1 hour at RT.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	0	0	0	0	0	0	0	0	0
B	s1	s2	...									s12
C	s1	s2	...									s12
D	s13	s14	...									s24
E	s13	s14	...									s24
F	St	and	ard					dil	uti	on	s	
G					se	rial						
H	0	0	0	0	0	0	0	0	0	0	0	0

- a. Each well was aspirated and washed three times with PBS-Tween (0.05%).
- b. The detection antibody was diluted to a final concentration of 50 ng/ml (56 μ l + 10 ml buffered blocking solution). 50 μ l of the diluted detection antibody was added to each well, followed by incubation for 1 hour at RT.
- c. Each well was aspirated and washed three times with PBS-Tween (0.05%).
- d. Streptavidin-HRP was diluted 1:200 (50 μ l + 10 ml blocking buffer). 50 μ l of the working dilution of Streptavidin-HRP was added to each well, followed by incubation for 20 min at RT.
- e. Each well was aspirated and washed three times with PBS-Tween (0.05%).
- f. The substrate solution was prepared. 50 μ l of substrate solution was added to each well, followed by incubation for 20 min at RT.
- g. 50 μ l of stop solution was added to each well.
- h. The optical density of each well was determined immediately, using a microplate reader set to 450 nm.

3. Calculation of GDF-15 serum titer:

- a. Each sample/GDF-15 standard dilution was applied in duplicate. To determine GDF-15 titer, the average of the duplicates was calculated and the background (sample without GDF-15) subtracted.
- b. To create a standard curve, values from the linear range were plotted on an X-Y-diagram (X axis: GDF-15 concentration, Y axis: OD450), and a linear curve fit was applied. GDF-15 serum titer of the test samples was calculated by interpolating from the OD450 values of the standard dilutions with known concentration.

- c. To calculate the final GDF-15 concentration of the samples, the distinct dilution factor was considered. Samples yielding OD values below or above the standard range were re-analyzed at appropriate dilutions.

Statistical analysis

Follow-up time for survival analysis was defined from the date of blood sampling to the last follow-up or death. Cumulative survival probabilities according to Kaplan-Meier were calculated together with 95% confidence intervals (CIs) and compared using two-sided log-rank test statistics. For the analysis of OS, patients who were alive at the last follow-up were censored while patients who had died were considered an "event". To analyze the impact of sGDF-15 on OS, patients were randomly assigned to two cohorts using a 1:2 ratio (identification and validation cohort, respectively). In the identification cohort different cut-off points were applied to categorize patients according to sGDF-15 into two balanced groups comprising $\geq 25\%$ of patients each. Differences in OS between patients with high vs. low sGDF-15 were analyzed for each cut-off point and the one resulting in the lowest log rank p-value was selected, similarly to optimization algorithms published earlier (Camp, RL et al., Clin Cancer Res / 10 / 7252-9. 2004). The optimal cut-off point as defined in the identification cohort was thereafter analyzed in 507 patients of validation cohort.

Cox proportional hazard regression analysis was used to calculate the relative effect considering additional prognostic factors in the entire patient cohort. Age was dichotomized according to the median age of patients. Serum S100B levels and sLDH were categorized as elevated vs. normal according to cut-off values as used in clinical routine (upper limit of normal 0.10 $\mu\text{g/l}$ and 250 U/l, respectively). Patients with missing values were excluded from regression analysis. Results of the models were described by means of hazard ratios; p-values were based on the Wald test. All statistical analyses were carried out using the SPSS Version 22 (IBM SPSS, Chicago, Illinois, USA).

Results

Patients

Patients' characteristics are shown in Table 1. A total of 761 melanoma patients (52.0% male) was analyzed. The median age was 63 years. The median follow-up for patients who died was 10.3 months and 45.3 months for patients who were censored.

Stage IV patients (n=293) were assigned to the M-categories M1c (n=206; 70.3%), M1b (n=51; 17.4%), or M1a (n=36; 12.3%) based on the site of distant metastases and on serum LDH (sLDH) (Balch, CM et al., J Clin Oncol / 27 / 6199-206. 2009). The median survival estimate according to Kaplan Meier was 10.7 months. Survival probabilities were 46.4% at 1-year, 33.3% at 2-years, and 29.3% at 3-years.

A total of 468 stage III patients was included. Sub-stage was IIIA in 15.6%, IIIB in 37.2%, and IIIC in 47.2% of 422 patients with complete data for classification. Survival probabilities were 94.9% at 1-year, 85.0% at 2-years, and 72.8% at 5-years, respectively.

The median hGDF-15 serum concentration was 1.0 ng/mL considering all 761 patients (0.9 ng/mL for stage III vs. 1.5 ng/mL for stage IV patients). Mean sGDF-15 was 2.6 (1.1 ng/mL for stage III vs. 4.8 ng/mL for stage IV patients; $p < 0.001$).

Overall Survival according to hGDF-15 levels

Thirteen different cut-off points ranging from 0.7 ng/mL to 1.9 ng/mL at increments of 0.1 ng/mL were found to categorize patients of the identification cohort ($n=254$) according to sGDF-15 into two balanced groups (the smaller group had to comprise at least 25% of patients). The difference in prognosis was largest comparing 86 patients (33.9%) with hGDF-15 levels ≥ 1.5 ng/mL and poor OS to 168 (66.1%) patients with lower levels and favorable OS ($p < 0.001$; Figure 4A). The difference in OS applying this cut-off point for sGDF-15 was thereafter confirmed in the validation cohort ($n=508$; $p < 0.001$; Figure 4B).

This inverse correlation between sGDF-15 and OS was observed in tumor-free stage III patients and in unresectable stage IV patients (Figure 1A; 1B) but not in tumor-free stage IV patients (Figure 1C) considering all patients (both cohorts combined).

Considering stage III patients of both cohorts, the 1-, 2- and 5 year survival probability was 96.1%, 87.8% and 75.7% for those with sGDF-15 below 1.5 ng/mL ($n=369$, 78.8% of all stage III patients) but only 90.4%, 74.2% and 61.5% for patients with higher sGDF-15 ($n=99$, 21.2%) (Table 2).

For patients of both cohorts with unresectable distant metastases and high hGDF-15 levels the probability to survive one year after analysis was only 14.3%, but 45.0% for patients with low sGDF-15. Similarly, the 2-year and 5-year survival was 6.3% and 2.6% compared to 19.9% and 5.2%, respectively. Median survival was 5.7 months versus 11.0 months for unresectable stage IV patients with high and low sGDF-15, respectively (Table 3).

Table 1: Patient characteristics

Factor	Category	Stage III (n=468)		Stage IV tumor-free (n=87)		Stage IV unresectable (n=206)		Total (n=761)	
		N	(%)	N	(%)	N	(%)	N	(%)
Gender	Male	228	48.7	47	54.0	121	58.7	396	52.0
	Female	240	51.3	40	46.0	85	41.3	365	48.0
Age	≤ 50 years	120	25.6	16	18.4	57	27.7	193	25.4
	51 - 60 years	82	17.5	16	18.4	56	27.2	154	20.2
	61 - 70 years	117	25.0	28	32.2	38	18.4	183	24.0
	≥ 71 years	149	31.8	27	31.0	55	26.7	231	30.4
	Median age	64 years		66 years		59 years		63 years	
Stage (AJCC 2009)	IIIA	66	15.6					66	9.2
	IIIB	157	37.2					157	22.0
	IIIC	199	47.2					199	27.8
	Stage III – unknown substage	46						46	
	IV, M1a			23	26.4	13	6.3	36	5.0
	IV, M1b			21	24.1	30	14.6	51	7.1
	IV, M1c			43	49.4	163	79.1	206	28.8
S100B	Normal	409	87.8	74	89.2	65	33.7	548	73.9
	Elevated	57	12.2	9	10.8	128	66.3	194	26.1
	Unknown	2		4		13		19	
LDH	Normal	439	94.2	81	97.6	116	57.1	636	84.6
	Elevated	27	5.8	2	2.4	87	42.9	116	15.4
	Unknown	2		4		3		9	
Visceral involvement	Soft tissue only			23	26.4	22	10.7	45	5.9
	Lung			21	24.1	36	17.5	57	7.5
	Other organs			43	49.4	148	71.8	191	25.1
	None (stage III)	468						468	61.5

AJCC: American Joint Committee on Cancer; LDH: lactate dehydrogenase

Table 2: Overall survival in tumor-free stage III patients

Factor	Total	Categories	Univariable analysis						Multivariable analysis			
			n	%	1-year survival rate [95% CI] (%)	2-year survival rate [95% CI] (%)	5-year survival rate [95% CI] (%)	Log-rank p-value	Model 1 (n=415) Hazard ratio	Model 1 (n=415) Wald test p-value	Model 2 (n=415) Hazard ratio	Model 2 (n=415) Wald test p-value
sLDH	466	Normal	439	94.2	95.3 [93; 97]	85.2 [82; 89]	73.4 [68; 78]	0.391	1		1	
		Elevated	27	5.8	91.7 [81; 103]	82.7 [67; 98]	63.6 [41; 87]		0.9	0.892	1.2	0.634
sSI00B	466	Normal	409	87.8	96.9 [95; 99]	88.1 [85; 91]	76.1 [71; 81]	<0.001	1		1	
		Elevated	57	12.2	81.7 [71; 92]	63.5 [50; 77]	48.9 [32; 65]		3.2	<0.001	3.5	<0.001
Gender	468	Male	228	48.7	95.0 [92; 98]	85.8 [81; 91]	72.1 [65; 79]	0.894	Not considered		1	
		Female	240	51.3	94.8 [92; 98]	84.1 [79; 89]	73.6 [67; 80]				1.0	0.893
Stage	422	IIIA	66	15.6	96.9 [93; 101]	95.2 [90; 101]	79.7 [68; 91]	0.176	1		1	
		IIIB	157	37.2	96.0 [93; 99]	86.4 [81; 92]	71.9 [63; 81]					
		IIIC	199	47.2	92.2 [88; 96]	79.8 [74; 86]	69.0 [61; 77]		1.3	0.156	1.6	0.030
Age	468	<63 years	222	47.4	94.8 [92; 98]	85.3 [80; 90]	68.0 [60; 76]	0.196	Not considered		1.6	0.030
		≥63 years	246	52.6	94.9 [92; 98]	84.7 [80; 89]	77.3 [71; 83]				1	
sGDF-15	468	<1.5ng/mL	369	78.8	96.1 [94; 98]	87.8 [84; 91]	75.7 [70; 81]	0.001	1		1	
		≥1.5ng/mL	99	21.2	90.4 [84; 96]	74.2 [65; 83]	61.5 [51; 72]		2.3	<0.001	3.7	<0.001

CI: confidence interval

Table 3: Overall survival in unresectable stage IV patients

Factor	Total	Categories	Univariable analysis						Multivariable analysis				
			n	%	Median survival (months)	1-year survival rate [95% CI] (%)	2-year survival rate [95% CI] (%)	5-year survival rate [95% CI] (%)	Log-rank p-value	Model 1 (n=203)		Model 2 (n=193)	
										Hazard ratio	Wald test p-value	Hazard ratio	Wald test p-value
sLDH	203	Normal	116	57.1	9.2	36.2 [27; 45]	16.3 [10; 23]	0.0 [00; 00]	<0.001	1		1	
		Elevated	87	42.9	4.0	12.6 [06; 20]	5.7 [01; 11]	4.6 [00; 09]		1.6	0.002	1.1	0.442
sS100B	193	Normal	65	33.7	10.2	46.2 [34; 58]	26.1 [15; 37]	4.8 [00; 13]	<0.001	Not considered		1	
		Elevated	128	66.3	5.2	12.5 [07; 18]	3.1 [00; 06]	1.6 [00; 04]		Not considered		1.8	0.003
Gender	206	Male	121	58.7	7.4	27.3 [19; 35]	10.7 [05; 16]	3.7 [00; 09]	0.961	Not considered		1	
		Female	85	41.3	7.6	24.7 [16; 34]	12.9 [06; 20]	3.5 [00; 08]		Not considered		1.0	0.954
Pattern of distant metastasis	206	Soft-tissue/lung	58	28.2	10.0	43.1 [30; 56]	24.0 [13; 35]	10.6 [02; 19]	<0.001	1		1	
		Other visceral	148	71.8	6.1	19.6 [13; 26]	6.8 [03; 11]	1.8 [00; 05]		1.8	<0.001	1.7	0.005
Age	206	<63 years	118	57.3	7.7	32.2 [24; 41]	16.9 [10; 24]	4.3 [00; 11]	0.006	Not considered		0.7	0.044
		≥ 63 years	88	42.7	6.6	18.2 [10; 26]	4.5 [00; 09]	2.3 [00; 05]		Not considered		1	
sGDF-15	206	<1.5ng/mL	80	38.8	11	45.0 [34; 56]	19.9 [11; 29]	5.2 [00; 13]	<0.001	1		1	
		≥1.5ng/mL	126	61.2	5.7	14.3 [08; 20]	6.3 [02; 11]	2.6 [00; 06]		1.7	<0.001	1.7	0.002

CI: confidence interval; n.r.: not reached

Table 4: Overall survival of tumor-free stage IV patients (both cohorts combined)

Variable	Total	Categories	n	%	Median survival time (months)	1-year survival rate [95% CI] (%)	2-year survival rate [95% CI] (%)	5-year survival rate [95% CI] (%)	Log-rank p-value
LDH	83	Normal	81	97.6	n.d.	95.1 [90; 100]	86.4 [79; 94]	68.6 [57; 80]	0.471
		Elevated	2	2.4	n.d.	n.d.	n.d.	n.d.	
S100B	83	Normal	74	89.2	n.r.	95.9 [91; 100]	89.2 [82; 96]	73.2 [62; 85]	0.008
		Elevated	9	10.8	43.9	88.9 [68; 109]	66.7 [36; 97]	53.3 [19; 87]	
Gender	87	Male	47	54.0	n.r.	97.7 [94; 102]	85.1 [75; 95]	68.2 [53; 83]	0.749
		Female	40	46.0	n.r.	92.5 [84; 101]	87.5 [77; 98]	71.2 [56; 87]	
Pattern of visceral metastasis	87	Soft-tissue/Lung	44	50.6	n.r.	97.7 [93; 102]	86.4 [76; 97]	72.5 [58; 87]	0.822
		Other visceral	43	49.4	n.r.	93.0 [85; 101]	85.9 [75; 96]	67.2 [51; 83]	
Age	87	< 63 years	36	41.4	n.r.	94.4 [87; 102]	80.4 [67; 93]	61.7 [45; 78]	0.066
		≥ 63 years	51	58.6	n.r.	96.1 [91; 101]	90.2 [82; 98]	75.8 [62; 89]	
GDF-15	87	< 1.5ng/mL	64	73.6	n.r.	93.8 [88; 100]	85.9 [77; 94]	72.1 [60; 84]	0.651
		≥ 1.5ng/mL	23	26.4	n.r.	100 [100; 100]	87.0 [73; 101]	62.5 [39; 86]	

LDH: lactate dehydrogenase; CI: confidence interval; n.r.: not reached; n.d.: not determinable

The relative prognostic impact of sGDF-15 in stage III patients

Cox regression analyses of the entire cohort of stage III patients were performed to determine the relative impact of sGDF-15 compared to other prognostic factors (Table 3). In the first model the three biomarkers sGDF-15, sS100B, and sLDH were included to allow for direct comparison. Results were adjusted for sub-stage, as univariate analysis had revealed a trend towards better OS for sub-stages IIIA/B versus IIIC ($p=0.088$). In addition to elevated sGDF-15 (HR 2.3; $p<0.001$), elevated sS100B was strongly associated with poor OS (Figure 5A) and had independent negative impact on prognosis (HR 3.2; $p<0.001$) in multivariable analysis. One year survival rates were highest with 97.4% for patients with favorable results in both biomarkers in strong contrast to 56.2% for those with both markers elevated. The survival probabilities after one year of patients with either elevated sS100B or high sGDF-15 were 88.4% or 95.1%, respectively. In the second model age and gender were additionally considered. Here, stage IIIC and age >63 years had independent negative impact on prognosis in addition to sGDF-15 and sS100B. The number of unfavorable values considering those 4 factors was strongly associated with survival (Figure 5). As expected for stage III melanoma, sLDH showed no correlation with outcome in neither model.

The relative impact of hGDF-15 levels in stage IV patients

In stage IV patients without evidence of disease at the time point of blood sampling ($n=87$), no prognostic relevance was observed for sGDF-15. Neither the pattern of distant metastasis, nor sLDH were associated with OS (Table 4). Instead, sS100B was the only prognostic factor in this patient population (Figure 5C).

Looking at 203 thoroughly characterized stage IV melanoma patients with unresectable tumor burden, we applied Cox regression analysis to investigate the relative prognostic impact of sGDF-15 compared to other factors. In the first model, sGDF-15 was compared to the pattern of distant metastases and sLDH, which are both considered as prognostic factors in the AJCC classification (Table 3). Like in stage III melanoma, sGDF-15 had a strong independent impact on OS (HR 1.7; $p<0.001$) in conjunction with the pattern of distant metastases (HR 1.8; $p<0.001$) and sLDH (HR 1.6; $p=0.002$). The independent impact of sGDF-15 levels was evident both in M1a/b (Figure 3A) and in M1c patients (Figure 3B). The number of unfavorable values considering the three independent factors sLDH, sGDF-15 and the pattern of distant metastasis was strongly associated with OS (Figure 3C). Thereby, 47% of patients fell into a newly identified subgroup with an extremely poor (3.3%) probability to survive 1 year. The multivariate model 2 considered all analyzed variables (Table 3). Here, sS100B replaced sLDH as significant prognostic parameter and age had additional independent impact. Stratification according to the number of unfavorable factors considering sS100B, the M-category, hGDF-15, and age allowed identification of an 8% sub-group of patients with favorable prognosis and 1-year OS of 81.3%. In contrast, 16% of patients showing unfavorable values in all 4 independent factors had the poorest prognosis with a 1-year OS of 3.2% (Figure 7).

Example 2: Alternative Evaluation of the Patient Samples described in Example 1

As an alternative Example in accordance with the invention, the same patient samples, which were already described in Example 1, were evaluated in an alternative manner, as described in the following:

Patients, Materials and Methods:**Patients**

Patients from the Department of Dermatology, Tübingen, Germany, with histologically confirmed melanoma were identified in the Central Malignant Melanoma Registry (CMMR) database (Lasithiotakis et al., 2006). 761 patients, with (a) archived serum samples taken between January 2008 and February 2012, (b) available follow-up data, and either (c) history of loco-regional or (d) history or presence of distant metastasis at the time point of blood draw were selected. Serum used for analysis of sGDF-15 was sampled during routine blood draws for analysis of sS100B stage was defined according to the AJCC classification (Balch et al., 2009), serum LDH and serum S100B (Elecsys S100 electrochemiluminescence immunoassay; Roche Diagnostics, Rotkreuz, Switzerland) were categorized as elevated vs. normal according to cut-off values used in clinical routine (upper limits of normal 0.10 µg/l and 250 U/l, respectively). Distant soft tissue/lymph nodes, lung, brain, liver, bone, and other visceral organs were considered for the calculation of the number of involved distant sites. Thus the number could be between 1 and 6 for each stage IV patient. GDF-15 serum concentrations were quantified in duplicates using a commercial ELISA kit according to the manufacturer's instructions (R&D systems, Wiesbaden, Germany).

All patients had given written informed consent to have clinical data recorded by the CMMR registry. The institutional ethics committee Tübingen has approved the study (ethic vote 125/2015BO2).

Statistical analysis

Follow-up time was defined from the date of blood sampling to the last follow-up or death. Survival probabilities according to Kaplan-Meier were calculated together with 95% confidence intervals and compared using two-sided log-rank tests. Patients who were either alive at the last follow-up or died from reasons other than melanoma were censored. Patients were randomly assigned to two cohorts using a 1:2 ratio. In the identification cohort, differences in OS between patients with high vs. low sGDF-15 were analyzed for cut-off points which yield two balanced groups comprising ≥25% of patients each. Then, the cut-off point resulting in the lowest log rank p-value was selected, similar to optimization algorithms published earlier (Camp et al., 2004) and thereafter analyzed in the validation cohort.

Cox regression analysis was used excluding patients with missing values. Results of the multivariable models were described by means of HRs; p-values were based on the Wald test. Combination models were developed using the nomogram function in the survival package for R. Differences in sGDF-15 according to prior systemic treatments were analyzed by Mann-Whitney U Testing. All statistical analyses were carried out using SPSS Version 22 (IBM SPSS, Chicago, Illinois, USA) and R 3.2.1 (R Foundation for Statistical Computing, Vienna Austria).

Results:**Patients**

Patients' characteristics are shown in Table 5. A total of 761 melanoma patients was analyzed. The median follow-up for patients who died was 10.3 months and 45.3 months for patients who were alive at the time point of last follow-up.

Stage IV patients (n=293) were assigned to the M-categories M1c (n=206; 70.3%), M1b (n=51; 17.4%), or M1a (n=36; 12.3%). The median survival estimate according to Kaplan Meier was 10.7 months. Survival probabilities were 46.4% at 1 year, 33.3% at 2 years, and 29.3% at 3 years. Assessment for stage IV patients was within 12 weeks in 84 (28.7%), or within 12 months after first occurrence of distant metastasis in 96 (32.7%), or at later time points in 113 patients (38.6%). At the respective time-point 87 patients (29.7%) had no evidence of disease while 206 (70.3%) had unresectable tumor.

A total of 468 stage III patients was included. Sub-stage was IIIA in 15.6%, IIIB in 37.2%, and IIIC in 47.2% of 422 patients with complete data for classification. Survival probabilities were 94.9% at 1-year, 85.0% at 2-years, and 72.8% at 5-years, respectively. The time point of assessment was within 12 weeks for 55 patients (11.8%), within 12 months after first occurrence of loco regional metastasis for 100 (21.4%), or later for 313 patients (66.9%). None of the stage III patients had evidence of disease at the respective time point.

GDF-15 serum levels according to stage, tumor burden and prior treatments

Median sGDF-15 was 1.0 ng/mL considering all 761 patients (0.9 ng/mL for stage III vs. 1.5 ng/mL for stage IV patients). Stage IV patients with clinical or radiologic evidence of tumor had higher median sGDF-15 (2.1 ng/mL) than tumor-free stage IV or tumor-free stage III patients (both 0.9 ng/mL; Figure 10A). Among tumor-free stage IV patients, median sGDF-15 was not different between 13 patients who had ongoing complete responses after systemic treatments and 74 patients who were tumor-free after metastasectomy of distant metastases (both 0.9 ng/mL). sGDF-15 correlated with sLDH and the number of involved distant sites in unresectable stage IV patients (Figure 10B and 10C). In general, median sGDF-15 was not different in patients who had received systemic treatment within the last 4 weeks or any time before blood sampling (Table 8). A separate analysis about the impact of pre-treatment with chemotherapy, ipilimumab, other immunotherapy, BRAF/MEK inhibitors, or other systemic treatments showed lower sGDF-15 after BRAF/MEK inhibitors and a trend towards higher levels after ipilimumab in unresectable stage IV patients. No significant impact of prior systemic treatments was observed in tumor-free stage IV patients. A small but significant difference in sGDF-15 was observed comparing tumor-free stage III patients who had prior adjuvant treatment with Interferon- α to those without (0.8 ng/mL vs. 0.9 ng/mL; Table 8).

Overall survival according to GDF-15 levels

Thirteen different cut-off points of sGDF-15 ranging from 0.7 ng/mL to 1.9 ng/mL were tested in the identification cohort (n=254). The most significant difference in prognosis was observed when 86 patients (33.9%) with sGDF-15 ≥ 1.5 ng/mL and poor OS were compared to 168 (66.1%) patients with lower levels and favorable OS ($p < 0.001$; Figure 11A). The difference in OS using this cut-off point was thereafter confirmed in the validation cohort (n=507; $p < 0.001$; Figure 11B). A comparison of patient characteristics between the identification and the validation cohorts is provided in Table 9.

This inverse correlation between sGDF-15 and OS was observed in tumor-free stage III patients and in unresectable stage IV patients (Figure 1A; 1B) but not in tumor-free stage IV patients (Figure 1C) considering patients of both cohorts.

Among stage III patients, the 1-, 2- and 5-years OS probability was 96.1%, 87.8% and 75.7% for those with sGDF-15 < 1.5 ng/mL but only 90.4%, 74.2% and 61.5% for patients with higher sGDF-15 (Table 6 and Table 10). The association with OS was significant for patients who had been tumor-free for up to 6 months before serum sampling, or for 6 to 24 months. No difference in OS was observed for patients, who had been tumor-free for more than 24 months (Figure 12).

For patients with unresectable distant metastases and sGDF-15 ≥ 1.5 ng/mL the 1-year OS probability was only 14.3%, but 45.0% for those with low sGDF-15. Similarly, the 2-year and 5-year survival was 6.3% and 2.6% compared to 19.9% and 5.2%, respectively (Table 7 and Table 11). The association with OS was significant for patients whose assessment was within 6 months and between 6 and 24 months after first diagnosis of distant metastasis but not for those, who had been in stage IV for more than 24 months (Figure 13).

The relative prognostic impact of sGDF-15 in stage III patients

Cox regression analysis of all tumor-free stage III patients was performed to determine the relative impact of sGDF-15 compared to other prognostic factors. The hazard ratio (HR) was 2.2 ($p < 0.001$) for patients with sGDF15 ≥ 1.5 ng/mL when adjusted for the sub-stage according to American Joint Committee on Cancer (Table 6; model 1). In model 2, which considered a broad spectrum of factors, elevated sS100B was strongly associated with poor OS (Figure 14A) and had independent negative impact on OS (HR 4.0; $p < 0.001$) in addition to elevated sGDF-15 (HR 2.7; $p < 0.001$) and the pattern of loco-regional metastasis (HR=4.1; $p < 0.001$ for combined lymph-node and intransit/satellite involvement, HR=2.4; $p = 0.002$ for lymph-node involvement only; Table 6). To obtain an individual risk score, a nomogram accounting for the relative impact of these three factors was developed (Figure 8A). Two years OS was 96.1% for patients without lymph node involvement, normal sS100B, and sGDF-15 < 1.5 ng/mL (risk score 0), but only 40.2% for those with a risk score > 175 (Figure 8B). No significant associations with OS were observed for age, gender, sLDH, sub-stage, ulceration, or tumor thickness. OS was not different between patients who received prior adjuvant systemic treatments

compared to those without (Table 6). A similar impact of sGDF-15 on OS was observed, if the analysis was limited to stage III patients of the validation cohort (Table 12).

The relative impact of GDF-15 levels in stage IV patients

sGDF-15 had independent impact on OS among the entire cohort of stage IV patients (n=293). As expected, a prominent impact of the disease status at the time-point of serum sampling was observed (unresectable disease HR=8.6; $p<0.001$ vs. tumor-free; Table 13). Thus, unresectable stage IV patients and those which were tumor-free after metastasectomy or complete responses upon prior systemic treatments were analyzed separately.

In tumor-free stage IV patients (n=87), no impact on OS was observed for sGDF-15 (Table 14). Instead, increased sS100B (Figure 14B), ≥ 2 involved distant sites, and no prior systemic treatments were associated with poor OS in univariate and multivariate analysis. None of 13 patients with ongoing complete responses following systemic treatments died during follow-up. If the analysis was limited to the subgroup of patients who were tumor-free after complete metastasectomy the same factors remained independently associated with OS (Table 15).

Looking at 206 unresectable stage IV patients (Table 7), elevated sGDF-15 had a strong independent negative impact on OS (HR 1.9; $p<0.001$) in addition to the M category (HR 1.6; $p<0.001$ for M1c). The association of sGDF-15 with OS was evident both in M1a/b (Figure 9A) and in M1c patients (Figure 9B). In more detailed multivariable model 2, elevated sGDF-15, elevated sS100B (Figure 14C), CNS involvement, and ≥ 4 involved distant sites were independently associated with poorer OS (Table 7). Strong differences in OS were observed according to the nomogram-based risk score accounting for the relative impact of these four factors. 31.1% of patients with a risk score <100 had a 1-year OS of 48.3%. In contrast, none of 21.2% of patients who had a risk score ≥ 250 survived the first year after serum sampling (Figures 9C, 9D). Despite being associated with OS in univariate analysis, sLDH and the pattern of distant metastasis had no additional impact on OS when considered together with the other factors. OS of patients who received prior systemic treatment was not different compared to those without (Table 7) and a similar independent impact of sGDF-15 on OS was observed, if the analysis was limited to unresectable patients who were treatment-naïve (Table 16), or to those of the validation cohort only (Table 17). In patients with CNS-involvement GDF-15, sLDH and sS100B were associated with OS in univariate analysis but not independent factors when analyzed in combination (Table 18).

Table 5: Patient characteristics

Factor	Category	Stage III (n=468)		Stage IV tumor-free (n=87)		Stage IV unresectable (n=206)		Total (n=761)	
		N	(%)	N	(%)	N	(%)	N	(%)
Gender	Male	228	48.7	47	54.0	121	58.7	396	52.0
	Female	240	51.3	40	46.0	85	41.3	365	48.0
Age	≤50 years	120	25.6	16	18.4	57	27.7	193	25.4
	51 - 60 years	82	17.5	16	18.4	56	27.2	154	20.2
	61 - 70 years	117	25.0	28	32.2	38	18.4	183	24.0
	≥71 years	149	31.8	27	31.0	55	26.7	231	30.4
	Median age	64 years		66 years		59 years		63 years	
Stage (AJCC 2009)	IIIA	66	15.6					66	9.2
	IIIB	157	37.2					157	22.0
	IIIC	199	47.2					199	27.8
	Stage III – unknown sub-stage	46						46	
	IV, M1a			23	26.4	13	6.3	36	5.0
	IV, M1b			21	24.1	30	14.6	51	7.1
	IV, M1c			43	49.4	163	79.1	206	28.8
sS100B	Normal	409	87.8	74	89.2	65	33.7	548	73.9
	Elevated	57	12.2	9	10.8	128	66.3	194	26.1
	Unknown	2		4		13		19	
sLDH	Normal	439	94.2	81	97.6	116	57.1	636	84.6
	Elevated	27	5.8	2	2.4	87	42.9	116	15.4
	Unknown	2		4		3		9	
Visceral involvement	Soft tissue only			23	26.4	22	10.7	45	5.9
	Lung			21	24.1	36	17.5	57	7.5
	Other organs			43	49.4	148	71.8	191	25.1
	None (stage III)	468						468	61.5
Prior systemic treatments	Interferon-α (adjuvant)	228	48.7	35	32.4	67	32.5	330	37.6
	Chemotherapy	6	1.3	18	16.7	119	57.8	141	16.1
	Ipilimumab			5	4.6	11	5.3	16	1.8
	BRAF/MEK inhibitors					16	7.8	16	1.8
	Immunotherapy other than ipilimumab	5	1.1	17	15.7	34	16.5	56	6.4
	Other	1	0.2	2	1.9	6	2.9	9	1.0
	None	232	49.6	31	28.7	47	22.8	310	35.3
Ulceration	Yes	156	38.8	22	36.1	54	44.3	232	39.7
	No	246	61.2	39	63.9	68	55.7	353	60.3
	Unknown	66		26		84		176	
Pattern of locoregional metastasis	Only satellite/intransit	131	28.5	8	13.6	28	20.1	167	23.0
	Only lymph nodes	252	54.8	30	50.8	64	46.0	346	47.7
	Both	77	16.7	21	35.6	47	33.8	145	20.0
	Distant metastasis only			19		49		68	9.4
	Unknown	8		9		18		35	

Breslow's tumor thickness	≤1.00 mm	48	12.5	7	12.7	20	17.2	75	13.5
	1.01 - 2.00 mm	123	32.0	18	32.7	25	21.6	166	29.9
	2.01 - 4.00 mm	136	35.4	16	29.1	41	35.3	193	34.8
	>4.00 mm	77	20.1	14	25.5	30	25.9	121	21.8
	Unknown	84		32		90		206	
CNS involvement	Yes			14	16.1	77	37.4	91	12.0
	No			73	83.9	129	62.6	202	26.5
Number of involved distant sites	1			50	57.5	50	24.3	100	13.1
	2			24	27.6	53	25.7	77	10.1
	3			8	9.2	51	24.8	59	7.8
	≥4			5	5.7	52	25.2	57	7.5

Abbreviations: AJCC, American Joint Committee on Cancer; CNS, central nervous system; LDH, lactate dehydrogenase; sLDH, serum level of lactate dehydrogenase; sS100B, S100B in serum.

Table 6: Overall survival subsequent to serum sampling in tumor-free stage III patients

Factor	Total (n=468)	Categories	Univariable analysis					Multivariable analysis						
			n	%	1-year survival rate (%) [*]	2-year survival rate (%) [*]	5-year survival rate (%) [*]	Log-rank p-value	Model 1 (n=417)		Model 2 (n=374)			
sLDH	466	Normal	439	94.2	95.3	85.2	73.4	0.391	Not considered	1	1	Wald test p- value		
		Elevated	27	5.8	91.7	82.7	63.6						1.2	0.698
sS100B	466	Normal	409	87.8	96.9	88.1	76.1	<0.001	Not considered	1	1			
		Elevated	57	12.2	81.7	63.5	48.9						4.0	<0.001
Gender	468	Male	228	48.7	95.0	85.8	72.1	0.894	Not considered	1	1			
		Female	240	51.3	94.8	84.1	73.6						1.2	0.468
Stage	422	IIIA	66	15.6	96.9	95.2	79.7	0.176	1	1.3	0.460	Not considered		
		IIIB	157	37.2	96.0	86.4	71.9						1.6	0.152
		IIIC	199	47.2	92.2	79.8	69.0							
Age	468	≤50 years	120	25.6	96.4	87.8	71.2	0.093	Not considered	1	1.3	0.487		
		51 - 60 years	82	17.5	95.1	84.5	66.1						1.2	0.557
		61 - 70 years	117	25.0	96.5	88.0	81.8						1.3	0.490
		≥71 years	149	31.8	92.4	80.6	70.7						1	1
sGDF-15	468	<1.5ng/mL	369	78.8	96.1	87.8	75.7	0.001	1	2.2	<0.001	3.3	<0.001	
		≥1.5ng/mL	99	21.2	90.4	74.2	61.5							
Ulceration	402	No	246	61.2	94.0	87.6	75.1	0.221	Not considered	1	1.2	0.471		
		Yes	156	38.8	94.1	81.3	68.6							
Pattern of locoregional metastasis	460	Only satellite/intransit	131	28.5	96.8	90.8	80.1	<0.001	Not considered	1	1.7	0.143		
		Only lymph nodes	252	54.8	95.1	87.8	76.9						1.7	0.155
		Both	77	16.7	91.8	72.3	53.1						4.0	<0.001
Breslow's tumor thickness	384	≤1.00 mm	48	12.5	93.7	86.7	71.3	0.396	Not considered	1	1.7	0.143		
		1.01 - 2.00 mm	123	32.0	94.1	89.4	77.5						1.7	0.155
		2.01 - 4.00 mm	136	35.4	93.1	83.8	73.4						1.5	0.336
		>4.00 mm	77	20.1	95.9	78.1	66.7						1	
Prior adjuvant systemic treatment	468	Yes	236	50.4	95.6	85.4	73.6	0.821	Not considered	1	1.0	0.849		
		No	232	49.6	93.1	84.6	72.1							

Abbreviations: sGDF-15, serum levels of growth and differentiation factor 15; sLDH, serum level of lactate dehydrogenase; sS100B, S100B in serum. *The 95% confidence intervals are presented in Table 10.

Table 7: Overall survival subsequent to serum sampling in unresectable stage IV patients

Factor	Total (n=206)	Categories	Univariable analysis					Multivariable analysis				
			n	%	1-year survival rate (%)*	2-year survival rate (%)*	5-year survival rate (%)*	Log- rank p-value	Model 1 (n=203)		Model 2 (n=193)	
									Hazar d ratio	Wald test p-value	Hazard ratio	Wald test p-value
sLDH	203	Normal	116	57.1	36.2	16.3	0.0	<0.001	Not considered		1	
		Elevated	87	42.9	12.6	5.7	4.6			1.3	0.202	
sS100B	193	Normal	65	33.7	46.2	26.1	4.8	<0.001	Not considered		1	
		Elevated	128	66.3	12.5	3.1	1.6			1.9	0.003	
Gender	206	Male	121	58.7	27.3	10.7	3.7	0.961	Not considered		1	
		Female	85	41.3	24.7	12.9	3.5			1.0	0.846	
Pattern of distant metastasis	206	Soft-tissue/ Lung	58	28.2	43.1	24.0	10.6	<0.001	Not considered		1	
		Other visceral	148	71.8	19.6	6.8	1.8			1.0	0.915	
Age	206	≤50 years	57	27.7	31.6	21.1	0.0	0.010	Not considered		1	
		51 - 60 years	56	27.2	33.9	4.5	10.2			1.2	0.392	
		61 - 70 years	38	18.4	10.5	0.0	0.0			1.5	0.103	
		≥71 years	55	26.7	23.6	7.3	3.6			1.3	0.232	
sGDF-15	206	<1.5ng/mL	80	38.8	45.0	19.9	5.2	<0.001	1		1	
		≥1.5ng/mL	126	61.2	14.3	6.3	2.6		1.9	<0.001	1.5	0.036
M-category	206	M1a/b	43	20.9	46.5	25.4	9.1	0.001	1		Not considered	
		M1c	163	79.1	20.9	8.0	2.9		1.6	<0.001		
CNS involvement	206	No	129	62.6	31.0	15.5	4.9	<0.001	Not considered		1	
		Yes	77	37.4	18.2	5.2	2.6			1.6	0.013	
Number of involved distant sites	206	1	50	24.3	44.0	20.0	4.6	<0.001	Not considered		1	
		2	53	25.7	34.0	17.0	10.6				1.1	0.592
		3	51	24.8	21.6	7.8	2.0				1.5	0.154
		≥4	52	25.2	5.8	1.9	1.9				1.9	0.035
Prior systemic treatment	206	Yes	134	65.0	25.4	12.6	6.3	0.703	Not considered		1	
		No	72	35.0	27.8	9.7	0.0			1.1	0.693	

Abbreviations: CNS, central nervous system; sGDF-15, serum levels of growth and differentiation factor 15; sLDH, serum level of lactate dehydrogenase; sS100B, S100B in serum.*The 95% confidence intervals are presented in Table 11.

Table 8: GDF-15 serum levels according to systemic treatments applied

	Within 4 weeks before blood draw					Any time before blood draw					
		n	% of patients	Median sGDF-15	p-value		n	% of patients	Median sGDF-15	p-value	
Stage IV unresectable (n=206)	Any systemic treatment	Yes	98	47.6	1.6	0.579	Yes	134	65.0	1.8	0.336
		No	108	52.4	1.7		No	72	35.0	1.4	
	Chemotherapy	Yes ¹	76	36.9	1.8	0.329	Yes	119	57.8	1.9	0.104
		No	129	62.6	1.5		No	87	42.2	1.3	
	Ipilimumab	Yes	5	2.4	7.4	0.058	Yes	11	5.3	4.9	0.122
		No	201	97.6	1.6		No	195	94.7	1.6	
	BRAF/MEK inhibition	Yes	15	7.3	0.7	0.010	Yes	16	7.8	1.0	0.035
		No	191	92.7	1.8		No	190	92.2	1.8	
	Immunotherapy other than ipilimumab	Yes ²	13	6.3	1.5	0.845	Yes	35	17.0	1.7	0.677
		No	191	92.7	1.7		No	171	83.0	1.6	
	Other	Yes	2	1.0	1.6	0.863	Yes	6	2.9	3.1	0.300
		No	204	99.0	1.7		No	200	97.1	1.6	
Stage IV tumor-free (n=87)	Any systemic treatment	Yes	3	3.4	2.1	0.418	Yes	33	37.9	1.3	0.075
		No	84	96.6	0.9		No	54	62.1	0.8	
	Chemotherapy	Yes	2	2.3	1.8	0.820	Yes	18	20.7	1.1	0.297
		No	85	97.7	0.9		No	69	79.3	0.9	
	Ipilimumab	Yes	0	0			Yes	5	5.7	1.3	0.693
		No	87	100	0.9		No	82	94.3	0.9	
	BRAF/MEK inhibition	Yes	0	0			Yes	0	0		
		No	87	100	0.9		No	87	100	0.9	
	Immunotherapy other than ipilimumab	Yes	2	2.3	2.6	0.069	Yes	17	19.5	1.3	0.151
		No	85	97.7	0.9		No	70	80.5	0.8	
	Other	Yes	0	0			Yes	2	2.3	1.0	0.947
		No	87	100	0.9		No	85	97.7	0.9	
Stage III (n=468)	Any systemic adjuvant treatment	Yes	65	13.9	0.9	0.998	Yes	236	50.4	0.8	0.029
		No	403	86.1	0.9		No	232	49.6	0.9	
	Interferon-α (adjuvant)	Yes ³	61	13.2	0.9	0.387	Yes	228	48.7	0.8	0.023
		No	402	86.8	0.9		No	240	51.3	0.9	

1: data not available in one patient; 2: not available in two patients; 3: not available in five patients

Table 9: Patient characteristics

Factor	Category	Identification Cohort (n=254)		Validation Cohort (n=507)		Total (n=761)	
		N	(%)	N	(%)	N	(%)
Gender	Male	133	52.4	263	51.9	396	52.0
	Female	121	47.6	244	48.1	365	48.0
Age	≤50 years	67	26.4	126	24.9	193	25.4
	51 - 60 years	57	22.4	97	19.1	154	20.2
	61 - 70 years	52	20.5	131	25.8	183	24.0
	≥71 years	78	30.7	153	30.2	231	30.4
	Median age	61 years		64 years		63 years	
Stage (AJCC 2009)	IIIA	23	9.8	43	8.9	66	9.2
	IIIB	47	20.1	110	22.9	157	22.0
	IIIC	58	24.8	141	29.3	199	27.8
	Stage III – unknown sub-stage	20		26		46	
	IV, M1a	15	6.4	21	4.4	36	5.0
	IV, M1b	24	10.3	27	5.6	51	7.1
	IV, M1c	67	28.6	139	28.9	206	28.8
sS100B	Normal	196	79.7	352	71.0	548	73.9
	Elevated	50	20.3	144	29.0	194	26.1
	Unknown	8		11		19	
sLDH	Normal	209	83.3	427	85.2	636	84.6
	Elevated	42	16.7	74	14.8	116	15.4
	Unknown	3		6		9	
Visceral involvement	Soft tissue only	16	15.1	29	15.5	45	5.9
	Lung	26	24.5	31	16.6	57	7.5
	Other organs	64	60.4	127	67.9	191	25.1
	None (stage III)	148		320		468	61.5
Prior systemic treatments	Interferon-α (adjuvant)	104	34.9	226	38.8	330	37.5
	Chemotherapy	47	15.8	96	16.5	143	16.2
	Ipilimumab	6	2.0	10	1.7	16	1.8
	BRAF/MEK inhibitors	7	2.3	9	1.5	16	1.8
	Immunotherapy other than ipilimumab	25	8.4	32	5.5	57	6.5
	Other	3	1.0	6	1.0	9	1.0
	None	106	35.6	204	35.0	310	35.2
Clinical situation	Stage III tumor-free	148	58.3	320	63.1	468	61.5
	Stage IV tumor-free	34	13.4	53	10.5	87	11.4
	Stage IV unresectable	72	28.3	134	26.4	206	27.1
Ulceration	Yes	87	45.3	145	36.9	232	39.7
	No	105	54.7	248	63.1	353	60.3
	Unknown	62		114		176	

Pattern of locoregional metastasis	Only satellite/intransit	56	22.0	111	21.9	167	21.9
	Only lymph nodes	114	44.9	232	45.8	346	45.5
	Both	48	18.9	97	19.1	145	19.1
	Distant metastasis only	24	9.4	44	8.7	68	8.9
	Unknown	12	4.7	23	4.5	35	4.6
Breslow's tumor thickness	≤1.00 mm	21	11.5	54	14.5	75	13.5
	1.01 - 2.00 mm	52	28.6	114	30.6	166	29.9
	2.01 - 4.00 mm	65	35.7	128	34.3	193	34.8
	>4.00 mm	44	24.2	77	20.6	121	21.8
	Unknown	72		134		206	
CNS involvement (Stage IV only)	Yes	34	32.1	57	30.5	91	31.1
	No	72	67.9	130	69.5	202	68.9
Number of involved distant sites (Stage IV only)	1	41	38.7	59	31.6	100	34.1
	2	24	22.6	53	28.3	77	26.3
	3	19	17.9	40	21.4	59	20.1
	≥4	22	20.8	35	18.7	57	19.5

Table 10: Overall survival subsequent to serum sampling in tumor-free stage III patients

Factor	Total (n=468)	Categories	Univariable analysis						Log-rank p-value
			n	%	Median survival (months)	1-year survival rate [95% CI] (%)	2-year survival rate [95% CI] (%)	5-year survival rate [95% CI] (%)	
sLDH	466	Normal	439	94.2	n.r.	95.3 [93; 97]	85.2 [82; 89]	73.4 [68; 78]	0.391
		Elevated	27	5.8	n.r.	91.7 [81; 100]	82.7 [67; 98]	63.6 [41; 87]	
sS100B	466	Normal	409	87.8	n.r.	96.9 [95; 99]	88.1 [85; 91]	76.1 [71; 81]	<0.001
		Elevated	57	12.2	n.r.	81.7 [71; 92]	63.5 [50; 77]	48.9 [32; 65]	
Gender	468	Male	228	48.7	n.r.	95.0 [92; 98]	85.8 [81; 91]	72.1 [65; 79]	0.894
		Female	240	51.3	n.r.	94.8 [92; 98]	84.1 [79; 89]	73.6 [67; 80]	
Stage	422	IIIA	66	15.6	n.r.	96.9 [93; 100]	95.2 [90; 100]	79.7 [68; 91]	0.176
		IIIB	157	37.2	n.r.	96.0 [93; 99]	86.4 [81; 92]	71.9 [63; 81]	
		IIIC	199	47.2	n.r.	92.2 [88; 96]	79.8 [74; 86]	69.0 [61; 77]	
Age	468	≤50 years	120	25.6	n.r.	96.4 [93; 100]	87.8 [82; 94]	71.2 [61; 81]	0.093
		51 - 60 years	82	17.5	n.r.	95.1 [90; 100]	84.5 [76; 93]	66.1 [53; 79]	
		61 - 70 years	117	25.0	n.r.	96.5 [93; 100]	88.0 [82; 94]	81.8 [74; 90]	
		≥71 years	149	31.8	n.r.	92.4 [88; 97]	80.6 [74; 87]	70.7 [62; 79]	
sGDF-15	468	<1.5ng/mL	369	78.8	n.r.	96.1 [94; 98]	87.8 [84; 91]	75.7 [70; 81]	0.001
		≥1.5ng/mL	99	21.2	n.r.	90.4 [84; 96]	74.2 [65; 83]	61.5 [51; 72]	
Ulceration	402	No	246	61.2	n.r.	94.0 [91; 97]	87.6 [83; 92]	75.1 [60; 78]	0.221
		Yes	156	38.8	n.r.	94.1 [90; 98]	81.3 [75; 88]	68.6 [60; 812]	
Pattern of locoregional metastasis	460	Only satellite/intransit	131	28.5	n.r.	96.8 [94; 100]	90.8 [86; 96]	80.1 [72; 88]	<0.001
		Only lymph nodes	252	54.8	n.r.	95.1 [92; 98]	87.8 [84; 92]	76.9 [71; 83]	
Breslow's tumor thickness	384	Both	77	16.7	65.2	91.8 [86; 98]	72.3 [62; 83]	53.1 [38; 68]	0.396
		≤1.00 mm	48	12.5	n.r.	93.7 [87; 100]	86.7 [77; 97]	71.3 [56; 86]	
		1.01 - 2.00 mm	123	32.0	n.r.	94.1 [90; 98]	89.4 [84; 95]	77.5 [69; 87]	
		2.01 - 4.00 mm	136	35.4	n.r.	93.1 [89; 98]	83.8 [77; 90]	73.4 [65; 82]	
Prior adjuvant systemic treatment	468	>4.00 mm	77	20.1	n.r.	95.9 [91; 100]	78.1 [68; 88]	66.7 [55; 79]	0.821
		Yes	236	50.4	n.r.	95.6 [94; 99]	85.4 [81; 90]	73.6 [67; 80]	
		No	232	49.6	n.r.	93.1 [90; 96]	84.6 [80; 90]	72.1 [65; 79]	

CI: confidence interval; n.r.: not reached.

Table 11: Overall survival subsequent to serum sampling in unresectable stage IV patients

Factor	Total (n=206)	Categories	Univariable analysis						Log-rank p-value
			n	%	Median survival (months)	1-year survival rate [95% CI] (%)	2-year survival rate [95% CI] (%)	5-year survival rate [95% CI] (%)	
sLDH	203	Normal	116	57.1	9.2	36.2 [27; 45]	16.3 [10; 23]	0.0 [0; 0]	<0.001
		Elevated	87	42.9	4.0	12.6 [6; 20]	5.7 [1; 11]	4.6 [0; 9]	
sS100B	193	Normal	65	33.7	10.2	46.2 [34; 58]	26.1 [15; 37]	4.8 [0; 13]	<0.001
		Elevated	128	66.3	5.2	12.5 [7; 18]	3.1 [0; 6]	1.6 [0; 4]	
Gender	206	Male	121	58.7	7.4	27.3 [19; 35]	10.7 [5; 16]	3.7 [0; 9]	0.961
		Female	85	41.3	7.6	24.7 [16; 34]	12.9 [6; 20]	3.5 [0; 8]	
Pattern of distant metastasis	206	Soft-tissue/lung	58	28.2	10.0	43.1 [30; 56]	24.0 [13; 35]	10.6 [2; 19]	<0.001
		Other visceral	148	71.8	6.1	19.6 [13; 26]	6.8 [3; 11]	1.8 [0; 5]	
Age	206	≤50 years	57	27.7	7.6	31.6 [20; 44]	21.1 [11; 32]	0.0 [0; 0]	0.010
		51 - 60 years	56	27.2	8.5	33.9 [22; 46]	4.5 [2; 24]	10.2 [2; 18]	
		61 - 70 years	38	18.4	6.6	10.5 [1; 20]	0.0 [0; 0]	0.0 [0; 0]	
		≥71 years	55	26.7	7.0	23.6 [12; 35]	7.3 [0; 14]	3.6 [0; 9]	
		<1.5ng/mL	80	38.8	11	45.0 [34; 56]	19.9 [11; 29]	5.2 [0; 13]	
sGDF-15	206	≥1.5ng/mL	126	61.2	5.7	14.3 [8; 20]	6.3 [2; 11]	2.6 [0; 6]	<0.001
		M1a/b	43	20.9	11.6	46.5 [32; 61]	25.4 [12; 38]	9.1 [0; 18]	
M-category	206	M1c	163	79.1	6.6	20.9 [15; 27]	8.0 [4; 12]	2.9 [0; 6]	0.001
		No	129	62.6	8.5	31.0 [23; 39]	15.5 [9; 22]	4.9 [0; 10]	
CNS involvement	206	Yes	77	37.4	4.7	18.2 [10; 27]	5.2 [0; 10]	2.6 [0; 6]	<0.001
		1	50	24.3	9.2	44.0 [30; 58]	20.0 [9; 31]	4.6 [0; 12]	
Number of involved distant sites	206	2	53	25.7	7.8	34.0 [21; 47]	17.0 [7; 27]	10.6 [2; 19]	<0.001
		3	51	24.8	6.4	21.6 [10; 33]	7.8 [1; 15]	2.0 [0; 6]	
		≥4	52	25.2	4.0	5.8 [0; 12]	1.9 [0; 6]	1.9 [0; 6]	
Prior systemic treatment	206	Yes	134	65.0	6.8	25.4 [18; 33]	12.6 [7; 18]	6.3 [2; 11]	0.703
		No	72	35.0	8.6	27.6 [17; 38]	9.7 [3; 17]	0.0 [0; 0]	

CI: confidence interval.

Table 12: Overall survival of tumor-free stage III patients in the validation cohort

Factor	Total (n=320)	Categories	Univariable analysis					Multivariable analysis					
			n	%	1-year survival rate [95% CI] (%)	5-year survival rate [95% CI] (%)	Log-rank p-value	Model 1 (n=294)		Model 2 (n=263)			
								Hazard ratio	Wald test p-value	Hazard ratio	Wald test p-value		
sLDH	318	Normal	300	94.3	95.5	[93, 98]	73.6	[68, 80]	Not considered	1	Not considered	1	0.980
		Elevated	18	5.7	94.1	[83, 100]	56.8	[25, 89]					
sS100B	318	Normal	274	86.2	97.7	[96, 100]	76.7	[71, 83]	Not considered	1	Not considered	1	<0.001
		Elevated	44	13.8	80.7	[69, 93]	47.2	[28, 66]					
Gender	320	Male	160	50.0	94.8	[92, 98]	71.4	[63, 80]	Not considered	1	Not considered	1	0.218
		Female	160	50.0	95.4	[92, 99]	74.2	[66, 82]					
Stage	294	IIIA	43	14.6	97.7	[93, 100]	88.8	[76, 100]	1	Not considered	Not considered	Not considered	Not considered
		IIIB	110	37.4	96.2	[93, 100]	67.2	[56, 79]					
		IIIC	141	48.0	92.6	[88, 97]	70.0	[61, 79]					
Age	320	≤50 years	77	24.1	97.2	[93, 100]	77.0	[66, 88]	Not considered	1	Not considered	1	0.544
		51 - 60 years	52	16.3	96.2	[91, 100]	63.6	[46, 81]					
		61 - 70 years	85	26.6	95.1	[90, 100]	78.2	[69, 88]					
		≥71 years	106	33.1	93.2	[88, 98]	69.0	[58, 80]					
sGDF-15	320	<1.5ng/mL	252	78.8	95.9	[93, 98]	76.2	[70, 83]	1	Not considered	Not considered	1	0.005
		≥1.5ng/mL	68	21.3	92.1	[86, 99]	59.9	[46, 73]					
		0.014	0.014										
Ulceration	282	No	182	64.5	94.3	[91, 98]	74.1	[66, 82]	Not considered	1	Not considered	1	0.295
		Yes	100	35.5	94.8	[91, 99]	69.8	[58, 81]					
Pattern of locoregional metastasis	318	Only satellite/intransit	90	28.3	97.7	[95, 100]	80.0	[70, 90]	Not considered	1	Not considered	1	0.091
		Only lymph nodes	172	54.1	95.2	[92, 98]	75.1	[67, 83]					
		Both	56	17.6	90.5	[83, 98]	53.2	[38, 69]					
Breslow's tumor thickness	269	≤1.00 mm	38	14.1	94.7	[88, 100]	63.6	[44, 83]	Not considered	1	Not considered	1	0.078
		1.01 - 2.00 mm	87	32.3	94.0	[89, 99]	79.1	[68, 90]					
		2.01 - 4.00 mm	93	34.6	92.2	[87, 98]	72.1	[61, 93]					
		>4.00 mm	51	19.0	97.9	[94, 100]	71.7	[58, 68]					
Prior adjuvant systemic treatment	320	Yes	161	50.3	96.9	[94, 100]	74.7	[67, 82]	Not considered	1	Not considered	1	0.640
		No	159	49.7	93.3	[90, 97]	71.2	[63, 80]					

LDH: lactate dehydrogenase, CI: confidence interval.

Table 13: Overall survival subsequent to serum sampling in all stage IV patients

Factor	Total (n=293)	Categories	Univariable analysis					Multivariable analysis			
			n	%	1-year survival rate [95% CI] (%)	2-year survival rate [95% CI] (%)	Log-rank p-value	Model 1 (n=293) Hazard ratio	Model 1 (n=293) Wald test p-value	Model 2 (n=276) Hazard ratio	Model 2 (n=276) Wald test p-value
sLDH	286	Normal	197	68.9	60.4	45.1	<0.001	Not considered	Not considered	1	0.195
		Elevated	89	31.1	14.6	7.5					
sS100B	276	Normal	139	50.4	72.7	60.4	<0.001	Not considered	Not considered	1	0.001
		Elevated	137	49.6	17.5	7.0					
Gender	293	Male	168	57.3	47.0	31.3	0.525	Not considered	Not considered	1	0.993
		Female	125	42.7	46.4	36.7					
Disease status	293	tumor-free	87	29.7	95.4	86.2	<0.001	Not considered	Not considered	1	<0.001
		unresectable	206	70.3	26.2	11.6					
Pattern of distant metastasis	293	Soft-tissue/lung	102	34.8	66.7	51.9	<0.001	Not considered	Not considered	1	0.692
		Other visceral	191	65.2	36.1	24.4					
Age	293	≤50 years	73	24.9	46.6	36.9	0.684	Not considered	Not considered	1	0.481
		51-60 years	72	24.6	45.8	26.2					
		61-70 years	66	22.5	48.5	37.8					
		≥71 years	82	28.0	46.3	34.0					
		<1.5ng/mL	144	49.1	69.4	49.2					
sGDF-15	293	≥1.5ng/mL	149	50.9	27.5	18.6	<0.001	1	<0.001	1.5	0.017
		M1a/b	87	29.7	72.4	56.2					
M-category	293	M1c	206	70.3	35.9	24.1	<0.001	2.3	<0.001	Not considered	Not considered
		No	202	68.9	54.5	41.4					
CNS involvement	293	Yes	91	31.1	29.7	16.5	<0.001	2.1	<0.001	1	0.003
		1	100	34.1	70.0	54.8					
Number of involved distant sites	293	2	77	26.3	53.2	36.4	<0.001	Not considered	Not considered	1	0.053
		3	59	20.1	30.5	16.6					
		≥4	57	19.5	14.0	10.5					
		No	126	43.0	55.6	39.4					
Prior systemic treatment	293	Yes	167	57.0	40.1	29.9	0.042	Not considered	Not considered	1	0.059
		1.3									

LDH: lactate dehydrogenase, CI: confidence interval.

Table 14: Overall survival subsequent to serum sampling of tumor-free stage IV patients

Factor	Total (n=87)	Categories	Univariable analysis					Multivariable analysis			
			n	%	1-year survival rate [95% CI] (%)	2-year survival rate [95% CI] (%)	Log-rank p-value	Model 1 (n=87) Hazard ratio	Model 1 (n=87) Wald test p-value	Model 2 (n=83) Hazard ratio	Model 2 (n=83) Wald test p-value
sLDH	83	Normal	81	97.6	95.1	[90; 100]	86.4	[79; 94]	Not considered	Not considered	Not considered
		Elevated	2	2.4	n.d.	n.d.	n.d.	n.d.			
sS100B	83	Normal	74	89.2	95.9	[91; 100]	89.2	[82; 96]	Not considered	1	0.015
		Elevated	9	10.8	88.9	[88; 100]	66.7	[36; 97]			
Gender	87	Male	47	54.0	97.7	[94; 100]	85.1	[75; 95]	Not considered	1	0.663
		Female	40	46.0	92.5	[94; 100]	87.5	[77; 98]			
Pattern of distant metastasis	87	Soft-tissue/lung	44	50.6	97.7	[93; 100]	86.4	[76; 97]	Not considered	1	0.938
		Other visceral	43	49.4	93.0	[85; 100]	85.9	[75; 96]			
Age	87	≤50 years	16	18.4	100.0		93.8	[82; 100]	Not considered	1	0.827
		51 - 60 years	16	18.4	87.5	[71; 100]	68.8	[46; 92]			
		61 - 70 years	28	32.2	100.0		89.3	[78; 100]			
		≥71 years	27	31.0	92.6	[83; 100]	88.9	[77; 100]			
sGDF-15	87	<1.5ng/mL	64	73.6	93.8	[88; 100]	85.9	[77; 94]	1	1.2	0.969
		≥1.5ng/mL	23	26.4	100	[100; 100]	87.0	[73; 100]			
M-category	87	M1a/b	44	50.6	97.7	[93; 100]	86.4	[76; 97]	1	1.1	Not considered
		M1c	43	49.4	93.0	[85; 100]	85.9	[76; 96]			
CNS involvement	87	No	73	83.9	95.9	[91; 100]	87.6	[80; 95]	Not considered	1	0.790
		Yes	14	16.1	92.9	[79; 100]	78.6	[57; 100]			
Number of involved distant sites	87	1	50	57.5	96.0	[91; 100]	90.0	[82; 98]	Not considered	1	0.004
		≥2	37	42.5	94.6	[87; 100]	81.0	[68; 94]			
Prior systemic treatment	87	Yes	33	37.9	97.0	[91; 100]	97.0	[91; 100]	Not considered	1	0.001
		No	54	62.1	92.6	[86; 100]	79.5	[69; 90]			

LDH: lactate dehydrogenase; CI: confidence interval; n.d.: not done.

Table 15: Overall survival of stage IV patients who were tumor-free after complete metastasectomy

Factor	Total (n=74)	Categories	Univariable analysis					Multivariable analysis			
			n	%	1-year survival rate [95% CI] (%)	2-year survival rate [95% CI] (%)	Log-rank p-value	Model 1 (n=74) Hazard ratio	Model 1 (n=74) Wald test p-value	Model 2 (n=71) Hazard ratio	Model 2 (n=71) Wald test p-value
sLDH	71	Normal	70	98.6	94.3 [89, 100]	84.2 [76, 93]	0.679	Not considered	Not considered	Not considered	Not considered
		Elevated	1	1.4	n.d.	n.d.					
sS100B	71	Normal	63	88.7	95.2 [90, 100]	87.3 [79, 96]	0.008	Not considered	Not considered	1	0.023
		Elevated	8	11.3	87.5 [65, 100]	62.5 [29, 96]					
Gender	74	Male	39	52.7	97.4 [93, 100]	82.0 [70, 94]	0.614	Not considered	Not considered	1	0.893
		Female	35	47.3	91.4 [82, 100]	85.7 [74, 97]					
Pattern of distant metastasis	74	Soft-tissue/lung	39	52.7	97.4 [93, 100]	84.6 [73, 96]	0.563	Not considered	Not considered	1	0.809
		Other visceral	35	47.3	91.4 [82, 100]	82.7 [70, 95]					
Age	74	≤50 years	15	20.3	100	93.3 [81, 100]	0.624	Not considered	Not considered	1	0.405
		51-60 years	15	20.3	86.7 [70, 100]	66.7 [43, 91]					
		61-70 years	23	31.1	100	87.0 [73, 100]					
		≥71 years	21	28.4	90.5 [78, 100]	85.7 [71, 100]					
sGDF-15	74	<1.5ng/mL	56	75.7	92.9 [86, 100]	83.9 [74, 94]	0.442	1	0.443	1	1.0
		≥1.5ng/mL	18	24.3	100	83.3 [68, 100]					
M-category	74	M1a/b	39	52.7	97.4 [93, 100]	84.6 [73, 96]	0.563	1	0.562	Not considered	Not considered
		M1c	35	47.3	91.4 [82, 100]	82.7 [70, 95]					
GNS involvement	74	No	60	81.1	95.0 [90, 100]	84.9 [76, 94]	0.738	Not considered	Not considered	1	0.849
		Yes	14	18.9	92.9 [79, 100]	78.6 [57, 100]					
Number of involved distant sites	74	1	45	60.8	95.6 [90, 100]	88.9 [80, 98]	0.003	Not considered	Not considered	1	0.16
		≥2	29	39.2	93.1 [94, 100]	75.7 [60, 91]					

LDH: lactate dehydrogenase, CI: confidence interval; n.d.: not done.

Table 16: Overall survival of treatment naïve, unresectable stage IV patients

Factor	Total (n=72)	Categories	Univariable analysis					Multivariable analysis			
			n	%	1-year survival rate [95% CI] (%)	2-year survival rate [95% CI] (%)	Log-rank p-value	Model 1 (n=72) Hazard ratio	Model 1 (n=72) Wald test p-value	Model 2 (n=66) Hazard ratio	Model 2 (n=66) Wald test p-value
sLDH	69	Normal	44	63.8	31.8 [18; 46]	11.4 [2; 21]	0.135	Not considered	Not considered	1	
		Elevated	25	36.2	20.0 [4; 36]	8.0 [0; 19]				1.7	0.160
sS100B	66	Normal	26	39.4	38.5 [20; 57]	19.2 [4; 36.5]	0.055	Not considered	Not considered	1	
		Elevated	40	60.6	17.5 [6; 29]	5.0 [0; 12]				1.2	0.658
Gender	72	Male	43	59.7	27.9 [15; 47]	11.6 [2; 21]	0.407	Not considered	Not considered	1	
		Female	29	40.3	27.6 [11; 44]	6.9 [0; 16]				2.2	0.010
Pattern of distant metastasis	72	Soft-tissue/lung	26	36.1	26.9 [10; 44]	11.5 [0; 24]	0.715	Not considered	Not considered	1	
		Other visceral	46	63.9	28.3 [15; 41]	8.7 [1; 17]				1.7	0.179
Age	72	≤50 years	20	27.8	40.0 [19; 62]	25.0 [6; 44]	0.059	Not considered	Not considered	1	
		51-60 years	16	22.2	37.5 [14; 61]	6.3 [0; 18]				1.3	0.512
		61-70 years	11	15.3	9.1 [0; 26]	0.0 [0; 0]				1.7	0.265
		≥71 years	25	34.7	20.0 [4; 36]	4.0 [0; 12]				1.5	0.315
sGDF-15	72	<1.5ng/mL	33	45.8	45.5 [29; 63]	15.2 [3; 27]	0.006	1		1	
		≥1.5ng/mL	39	54.2	12.8 [2; 23]	5.1 [0; 12]		4.2	0.012	2.2	0.015
M-category	72	M1a/b	18	25.0	33.3 [12; 55]	11.1 [0; 26]	0.348	1		Not considered	
		M1c	54	75.0	25.9 [14; 38]	9.3 [2; 17]		1.1	0.759		
CNS involvement	72	No	51	70.8	27.5 [15; 40]	11.8 [3; 21]	0.047	Not considered	Not considered	1	
		Yes	21	29.2	28.6 [9; 48]	4.8 [0; 14]				2.2	0.022
Number of involved distant sites	72	1	31	43.1	41.9 [25; 60]	19.4 [5; 33]	0.008	Not considered	Not considered	1	
		2	16	22.2	31.3 [9; 54]	0.0 [0; 0]				2.3	0.049
		3	12	16.7	16.7 [0; 38]	8.3 [0; 24]				2.7	0.033
		≥4	13	18.1	0.0 [0; 0]	0.0 [0; 0]				3.5	0.018

LDH: lactate dehydrogenase, CI: confidence interval.

Table 17: Overall survival of unresectable stage IV patients in the validation cohort

Factor	Total (n=134)	Categories	Univariable analysis					Multivariable analysis			
			n	%	1-year survival rate [95% CI] (%)	2-year survival rate [95% CI] (%)	Log-rank p-value	Model 1 (n=134) Hazard ratio	Model 1 (n=134) Wald test p-value	Model 2 (n=128) Hazard ratio	Model 2 (n=128) Wald test p-value
sLDH	133	Normal	79	59.4	32.9 [23; 43]	12.7 [5; 20]	0.001	Not considered	Not considered	1	
		Elevated	54	40.6	11.1 [3; 20]	5.6 [0; 12]				1.3	0.180
sS100B	128	Normal	34	26.6	47.1 [30; 64]	23.5 [9; 38]	<0.001	Not considered	Not considered	1	
		Elevated	94	73.4	12.8 [6; 20]	3.2 [0; 7]				2.0	0.003
Gender	134	Male	72	53.7	22.2 [13; 32]	8.3 [2; 15]	0.756	Not considered	Not considered	1	
		Female	62	46.3	25.8 [15; 37]	11.3 [3; 19]				1.0	0.912
Pattern of distant metastasis	134	Soft-tissue/lung	33	24.6	36.4 [20; 53]	18.2 [5; 31]	0.014	Not considered	Not considered	1	
		Other visceral	101	75.4	19.8 [12; 28]	6.9 [2; 12]				1.3	0.377
Age	134	≤50 years	37	27.6	27.0 [13; 41]	21.6 [8; 35]	0.022	Not considered	Not considered	1	
		51-60 years	34	25.4	35.3 [19; 51]	8.8 [0; 18]				1.5	0.156
		61-70 years	30	22.4	10.0 [0; 21]	0.0 [0; 0]				1.3	0.295
		≥71 years	33	24.6	21.2 [7; 35]	6.1 [0; 14]				1.4	0.261
sGDF-15	134	<1.5ng/mL	51	38.1	41.2 [28; 55]	13.7 [4; 23]	0.001	1		1	
		≥1.5ng/mL	83	61.9	13.3 [6; 21]	7.2 [2; 13]		1.8	0.002	1.8	0.018
M-category	134	M1a/b	21	15.7	42.9 [22; 64]	19.0 [2; 36]	0.034	1		Not considered	
		M1c	113	84.3	20.4 [13; 28]	8.0 [3; 13]		1.6	0.066		
CNS involvement	134	No	88	65.7	26.1 [17; 35]	11.4 [5; 18]	0.023	Not considered	Not considered	1	
		Yes	46	34.3	19.6 [8; 31]	6.5 [0; 14]				1.6	0.038
Number of involved distant sites	134	1	28	20.9	42.9 [25; 61]	17.9 [4; 32]	0.024	Not considered	Not considered	1	
		2	38	28.4	26.3 [12; 40]	10.5 [1; 20]				1.3	0.451
		3	34	25.4	20.6 [7; 34]	8.8 [0; 18]				1.3	0.543
		≥4	34	25.4	8.8 [0; 18]	2.9 [0; 9]				1.9	0.110
Prior systemic treatment	134	Yes	88	65.7	27.3 [18; 37]	12.5 [6; 19]	0.820	Not considered	Not considered	1	
		No	46	34.3	21.7 [10; 34]	4.3 [0; 10]				1.1	0.516

LDH: lactate dehydrogenase, CI: confidence interval.

Table 18: Overall survival of unresectable stage IV patients with CNS involvement

Factor	Total (n=77)	Categories	Univariable analysis				Multivariable analysis								
			n	%	1-year survival rate [95% CI] (%)	2-year survival rate [95% CI] (%)	Log- rank p-value	Model 1 (n=73)		Model 2 (n=73)		Model 3 (n=76)		Model 4 (n=73)	
								Hazard ratio	Wald test p-value	Hazard ratio	Wald test p-value	Hazard ratio	Wald test p-value	Hazard ratio	Wald test p-value
sLDH	76	Normal	37	48.7	32.4 [17.48]	10.8 [1.21]	<0.001	1			1				Not considered
		Elevated	39	51.3	2.6 [0.8]	0.0 [0.0]		1.8	0.062			3.1	<0.001		
sS100B	73	Normal	18	24.7	38.9 [16.61]	16.7 [0.34]	0.001	1			Not considered	Not considered	Not considered	1	0.001
		Elevated	55	75.3	5.5 [0.12]	0.0 [0.0]		1.9	0.187						
Gender	77	Male	47	61.0	21.3 [10.33]	6.4 [0.124]	0.893	1			1			1	
		Female	30	39.0	13.3 [1.26]	3.3 [0.10]		1.2	0.517	1.1	0.728	1.0	0.986	1.3	0.310
Age	77	≤50 years	23	29.9	26.1 [8.44]	13.0 [0.27]	0.711	1			1			1	
		51-60 years	24	31.2	12.5 [0.26]	4.2 [0.12]		1.0	0.953	1.1	0.778	1.2	0.654	1.1	0.869
		61-70 years	16	20.8	12.5 [0.29]	0.0 [0.0]		1.2	0.581	1.2	0.525	1.1	0.692	1.3	0.422
		≥71 years	14	18.2	21.4 [0.43]	0.0 [0.0]		1.2	0.599	1.3	0.519	1.1	0.812	1.5	0.317
sGDF-15	77	<1.5ng/mL	27	35.1	37.0 [19.55]	11.1 [0.23]	0.001	1			1			Not considered	Not considered
		≥1.5ng/mL	50	64.9	8.0 [1.16]	2.0 [0.6]		1.5	0.365	2.3	0.006				
Number of involved distant sites	77	1	5	6.5	40.0 [0.83]	0.0 [0.0]	0.265	1			1			1	
		2	15	19.5	33.3 [9.57]	13.3 [0.31]		2.1	0.326	1.2	0.799	2.2	0.208	2.9	0.116
		3	25	32.5	16.0 [2.30]	4.0 [0.12]		1.3	0.683	1.6	0.385	1.6	0.634	1.5	0.528
		≥4	32	41.6	9.4 [0.20]	3.1 [0.9]		1.5	0.663	1.4	0.525	1.3	0.700	1.7	0.438
Prior systemic treatment	77	Yes	56	72.7	14.3 [5.24]	5.4 [0.11]	0.929	1			1			1	
		No	21	27.3	28.6 [9.48]	4.8 [0.14]		1.3	0.480	1.4	0.306	1.3	0.331	1.0	0.940

LDH: lactate dehydrogenase, CI: confidence interval.

Summary:

In the current study, the inventors surprisingly found that the serum concentration of hGDF-15 is a powerful prognostic biomarker for patients with metastatic melanoma.

For instance, the inventors found that hGDF-15 serum concentrations above 1.5 ng/mL most strongly predicted poor overall survival in a cohort of 761 patients with metastatic melanoma.

In tumor-free stage III patients, no world-wide accepted prognostic biomarkers are used in daily clinical routine. Estimation of the individual prognosis is mainly based on clinical and histopathological characteristics considered for the definition of the sub-stages IIIA, IIIB, or IIIC, respectively (Balch, CM et al., J Clin Oncol / 27 / 6199-206. 2009). Serum LDH does not harbor prognostic information in tumor-free patients after surgery of loco-regional metastases (Wevers, KP et al., Ann Surg Oncol / 20 / 2772-9. 2013). Serum levels of S100B are only analyzed for early detection of recurrences mainly in Europe (Pflugfelder, A et al., J Dtsch Dermatol Ges / 11 / 563-602. 2013), despite a large body of evidence of its prognostic impact in melanoma patients (Mocellin, S et al., Int J Cancer / 123 / 2370-6. 2008). In the current study, the inventors surprisingly found that sGDF15 and sS100B are both independent prognostic markers for these patients and are greatly superior to the clinical sub-stage for the identification of patients who will likely die from the disease.

The analysis of sGDF-15 alone allowed to identify 21% of all tumor-free stage III patients with high serum concentrations, who had a 2-fold increased risk to die within three years after blood draw compared to patients with low levels (33% vs 16%, respectively). The combined consideration of sGDF-15 and sS100B increased the proportion of patients at risk from 21% (sGDF-15 elevated irrespective of sS100B) to 31% (either one or both biomarkers elevated) and further enlarged the difference in OS between biomarker categories. In detail, the risk to die within 3 years with normal sS100B and low sGDF-15 was only 14% compared to 33% for patients with at least one biomarker elevated. The blood draw was taken at times without clinical or radiological evidence of disease in these patients thereby especially the combined analysis of both biomarkers may allow to identify patients which might profit from more intense surveillance or adjuvant therapies.

Thus, according to the invention, the use of hGDF-15 as a biomarker for the prediction of survival, e.g. in combination with S100B as a further biomarker, is highly advantageous even for sub-groups of melanoma patients, for which no reliable prognosis of survival has yet been available.

In unresectable stage IV melanoma patients, the pattern of visceral metastasis and sLDH are regularly used to classify patients into prognostically different M-categories M1a, M1b, or M1c (Balch, CM et al., J Clin Oncol / 27 / 6199-206. 2009). In the present study, the consideration of sGDF-15 in combination with these two established prognostic factors significantly improved the estimation of prognosis for the individual patient (HR

1.7; $p < 0.001$; pattern of visceral metastasis: HR 1.8; $p < 0.001$; sLDH: HR 1.6; $p = 0.002$) and allowed the identification of a relevant subgroup (comprising 30% of all patients with unresectable distant metastasis) with an extremely poor probability to survive 1 year (3.3%). In contrast, the worst biomarker category without consideration of sGDF-15 (visceral metastases other than lung and elevated sLDH; 35% of all unresectable stage IV patients) indicated a 1-year survival estimate of 8.3%. The additional consideration of sGDF-15 added prognostic information for M1a/b as well as for M1c patients. The gain in prognostic information based on the consideration of sGDF-15 is valuable for patient counselling and stratification within clinical trials, and might impact the individual risk/benefit assessment for therapeutic decisions. Considering the availability (and emergence) of various therapeutic options for advanced melanoma and the inevitable trade-off between efficacy and side effects, enhanced prognosis prediction most likely becomes instrumental for the further guidance of individualized therapy.

In conclusion, according to the invention, sGDF-15 is a powerful prognostic biomarker in patients with melanoma such as metastatic melanoma.

In tumor-free stage III patients the consideration of sGDF-15 alone or in combination with sS100B allows to identify individuals with increased risk to die from disease who might profit from more intense patient surveillance or adjuvant treatments. In patients with unresectable stage IV melanoma sGDF-15, sLDH and the pattern of visceral metastasis are independent prognostic factors. The combined consideration of these three factors improves the individual estimate of prognosis compared to the M-category alone and may influence individualized treatment decisions.

Industrial Applicability

The apparatuses and the kits according to the present invention may be industrially manufactured and sold as products for the itemed prediction methods, in accordance with known standards for the manufacture of diagnostic products. Accordingly, the present invention is industrially applicable.

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Claims

1. A method for predicting the probability of survival of a human melanoma patient, wherein the method comprises the steps of:
 - a) determining the level of hGDF-15 in a human blood sample obtained from said patient; and
 - b) predicting said probability of survival based on the determined level of hGDF-15 in said human blood sample; wherein a decreased level of hGDF-15 in said human blood sample indicates an increased probability of survival,wherein step b) comprises comparing said level of hGDF-15 determined in step a) with a hGDF-15 threshold level, wherein said probability is predicted based on the comparison of said level of hGDF-15 determined in step a) with said hGDF-15 threshold level; and wherein a level of hGDF-15 in said human blood sample which is decreased compared to said hGDF-15 threshold level indicates that the probability of survival is increased compared to a probability of survival at or above said hGDF-15 threshold level, wherein step a) comprises determining the level of hGDF-15 by using one or more antibodies capable of binding to hGDF-15 or an antigen-binding portion thereof, and wherein the human melanoma patient is a tumor-free stage III melanoma patient or an unresectable stage IV melanoma patient.
2. The method according to claim 1, wherein the human blood sample is a human serum sample.
3. The method according to claim 2, wherein the hGDF-15 threshold level is a level selected from the range of between 1.1 ng/ml and 2.2 ng/ml.
4. The method according to claim 2, wherein the hGDF-15 threshold level is a hGDF-15 level selected from the range of between 1.2 ng/ml and 2.0 ng/ml.
5. The method according to claim 2, wherein the hGDF-15 threshold level is a hGDF-15 level selected from the range of between 1.3 ng/ml and 1.8 ng/ml.
6. The method according to claim 2, wherein the hGDF-15 threshold level is a hGDF-15 level selected from the range of between 1.4 ng/ml and 1.6 ng/ml.
7. The method according to claim 6, wherein the hGDF-15 threshold level is 1.5 ng/ml.
8. The method according to claim 2, wherein the hGDF-15 threshold level is a level selected from the range of between 3.3 ng/ml and 4.3 ng/ml.
9. The method according to claim 2, wherein the hGDF-15 threshold level is a level selected from the range of between 3.6 ng/ml and 4.0 ng/ml.
10. The method according to claim 9, and wherein the hGDF-15 threshold level is 3.8 ng/ml.

11. The method according to any one of claims 1 to 10,
 wherein step a) further comprises determining the level of lactate dehydrogenase in said human blood sample, and
 wherein in step b), said probability of survival is also predicted based on the determined level of lactate dehydrogenase in said human blood sample; and wherein a decreased level of lactate dehydrogenase in said human blood sample indicates an increased probability of survival.
12. The method according to claim 11, wherein step b) comprises comparing said level of lactate dehydrogenase determined in step a) with a lactate dehydrogenase threshold level, wherein said probability is also predicted based on the comparison of said level of lactate dehydrogenase determined in step a) with said lactate dehydrogenase threshold level; and wherein a level of lactate dehydrogenase in said human blood sample which is decreased compared to said lactate dehydrogenase threshold level or is at said lactate dehydrogenase threshold level indicates that the probability of survival is increased compared to a probability of survival above said lactate dehydrogenase threshold level.
13. The method according to any one of claims 1 to 12,
 wherein step a) further comprises determining the level of S100B in said human blood sample, and
 wherein in step b), said probability of survival is also predicted based on the determined level of S100B in said human blood sample; and wherein a decreased level of S100B in said human blood sample indicates an increased probability of survival.
14. The method according to claim 13, wherein step b) comprises comparing said level of S100B determined in step a) with a S100B threshold level, wherein said probability is predicted based on the comparison of said level of S100B determined in step a) with said S100B threshold level; and wherein a level of S100B in said human blood sample which is decreased compared to said S100B threshold level or is at said S100B threshold level indicates that the probability of survival is increased compared to a probability of survival above said S100B threshold level.
15. The method according to any of claims 1 to 14, wherein in step b), said probability of survival is also predicted based on the age of said patient; and wherein an increased age indicates a decreased probability of survival.
16. The method according to claim 15, wherein step b) comprises comparing said age of said patient to a threshold age, wherein said probability is predicted based on the comparison of said age of said patient with said threshold age; and wherein an age of said patient which is equal to or increased compared to said threshold age indicates that the probability of survival is decreased compared to a probability of survival below said threshold age.
17. The method according to claim 16, wherein said threshold age is selected from the range of 60 to 65 years.

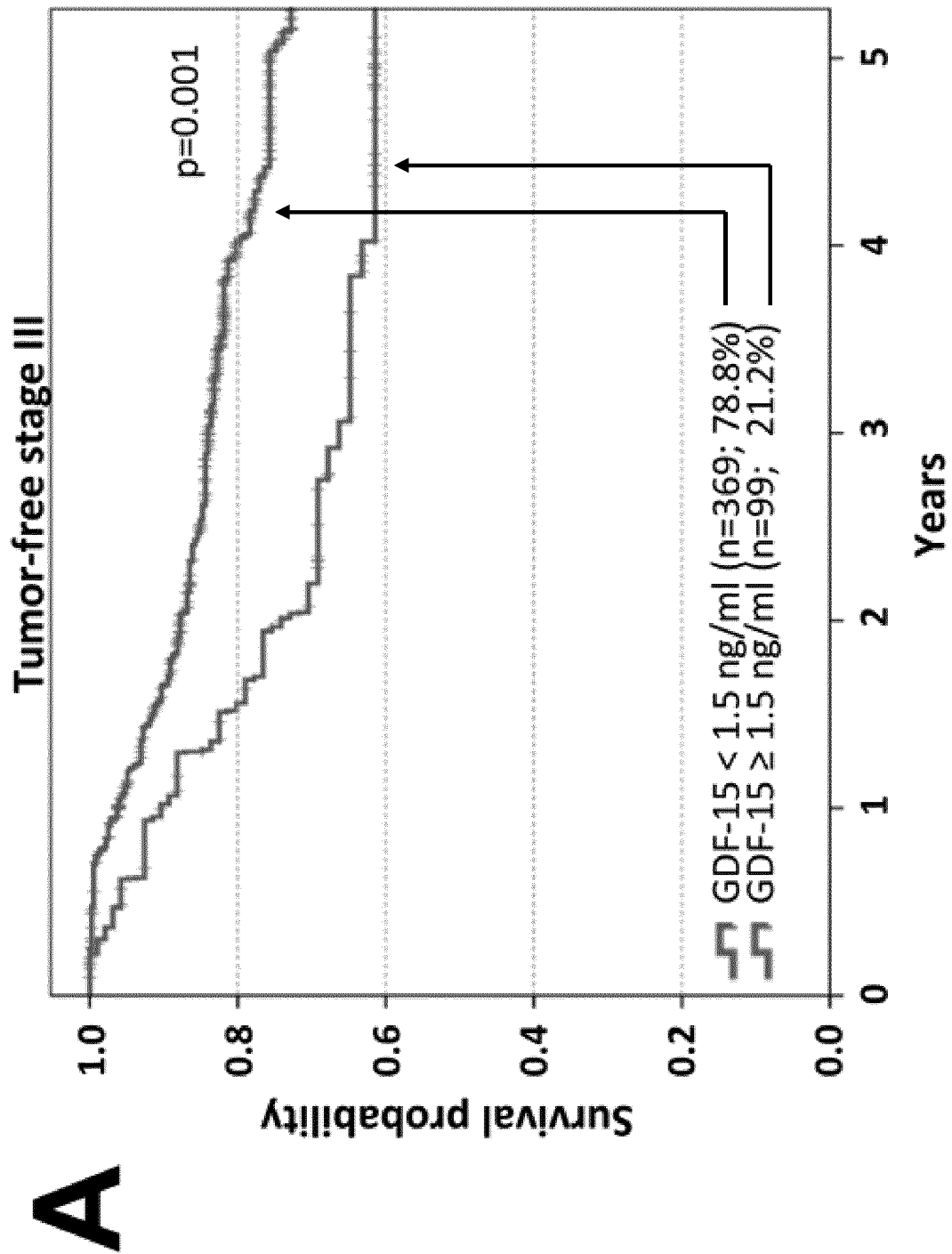
18. The method according to claim 17, wherein said threshold age is 63 years.
19. The method according to any one of claims 1 to 18, wherein in step b), said probability of survival is also predicted based on metastasis; and wherein the presence of metastases in visceral organs other than lung indicates that the probability of survival is decreased as compared to the probability of survival when metastases are absent from visceral organs other than lung.
20. The method according to any one of claims 1 to 19, wherein the human melanoma patient is a tumor-free stage III melanoma patient.
21. The method according to any one of claims 1 to 19, wherein the human melanoma patient is an unresectable stage IV melanoma patient.
22. The method according to any one of claims 1 to 21, wherein the one or more antibodies comprise at least one polyclonal antibody.
23. The method according to any one of claims 1 to 22, wherein the one or more antibodies or the antigen-binding portion comprise at least one monoclonal antibody or an antigen-binding portion thereof.
24. The method according to claim 23, wherein the binding is binding to a conformational or discontinuous epitope on hGDF-15, and wherein the conformational or discontinuous epitope is comprised by the amino acid sequences of SEQ ID No: 25 and SEQ ID No: 26.
25. The method according to claim 23 or 24, wherein the antibody or antigen-binding portion thereof comprises a heavy chain variable domain which comprises a CDR1 region comprising the amino acid sequence of SEQ ID NO: 3, a CDR2 region comprising the amino acid sequence of SEQ ID NO: 4 and a CDR3 region comprising the amino acid sequence of SEQ ID NO: 5, and wherein the antibody or antigen-binding portion thereof comprises a light chain variable domain which comprises a CDR1 region comprising the amino acid sequence of SEQ ID NO: 6, a CDR2 region comprising the amino acid sequence ser-ala-ser and a CDR3 region comprising the amino acid sequence of SEQ ID NO: 7.
26. The method according to any one of claims 1 to 25, wherein in step a), the level of hGDF-15 is determined by capturing hGDF-15 with an antibody or antigen-binding fragment thereof according to any one of claims 23 to 25 and by detecting hGDF-15 with a polyclonal antibody, or by detecting hGDF-15 with a monoclonal antibody or antigen-binding fragment thereof which binds to a different epitope than the antibody which captures hGDF-15.
27. The method according to any one of claims 1 to 26, wherein the method is an *in vitro* method.
28. The method according to any one of claims 1 to 27, wherein in step a), the level of hGDF-15 in the human blood sample is determined by an enzyme linked immunosorbent assay.

29. The method according to any one of claims 1-28, wherein in step a), the level of hGDF-15 in the human blood sample is determined by an electrochemiluminescence assay.
30. The method according to claim 29, wherein the electrochemiluminescence assay is a sandwich detection method comprising a step of forming a detection complex between
 - (A) streptavidin-coated beads;
 - (B) a biotinylated first antibody or antigen-binding portion thereof capable of binding to hGDF-15;
 - (C) hGDF-15 from the sample; and
 - (D) a ruthenium complex-labelled second antibody or antigen-binding portion thereof capable of binding to hGDF-15;
 wherein said detection complex has the structure (A)-(B)-(C)-(D), and wherein the biotinylated first antibody or antigen-binding portion thereof binds to a first hGDF-15 epitope and the ruthenium complex-labelled second antibody or antigen-binding portion thereof binds to a second hGDF-15 epitope which is different from said first hGDF-15 epitope, wherein the method further comprises a step of detecting the detection complex by measuring electrochemiluminescence, and wherein the level of hGDF-15 in the human blood sample is determined based on the electrochemiluminescence measurement.
31. Use of an apparatus to perform the method according to any one of claims 1-30.
32. The use according to claim 31, wherein the apparatus is an electrochemiluminescence analyzer configured to perform the method according to claim 29 or claim 30.
33. Use of a detection kit in a method according to any one of claims 1-30, the detection kit comprising:
 - (i) streptavidin-coated beads;
 - (ii) a biotinylated first antibody or antigen-binding portion thereof capable of binding to hGDF-15;
 - (iii) recombinant hGDF-15;
 - (iv) a ruthenium complex-labelled second antibody or antigen-binding portion thereof capable of binding to hGDF-15; and
 - (v) instructions for use in a method according to claims 1-30,
 wherein the biotinylated first antibody or antigen-binding portion thereof is capable of binding to a first hGDF-15 epitope and the ruthenium complex-labelled second antibody or antigen-binding portion thereof is capable of binding to a second hGDF-15 epitope which is different from said first hGDF-15 epitope.
34. The use according to claim 33, wherein the (iii) recombinant hGDF-15 is in form of a buffered solution comprising recombinant hGDF-15, the buffered solution having a pH in the range of 4 to 7.

35. The use according to claim 33 or 34, further comprising:
 - (vi) a container containing said recombinant hGDF-15, wherein the surface of the container which is in contact with recombinant hGDF-15 is coated with a non-adhesive material.
36. The use according to any one of claims 33 to 35, wherein one of the first antibody or antigen-binding portion thereof capable of binding to hGDF-15 and second antibody or antigen-binding portion thereof capable of binding to hGDF-15 is an antibody or antigen-binding portion thereof according to any one of claims 24 to 26.

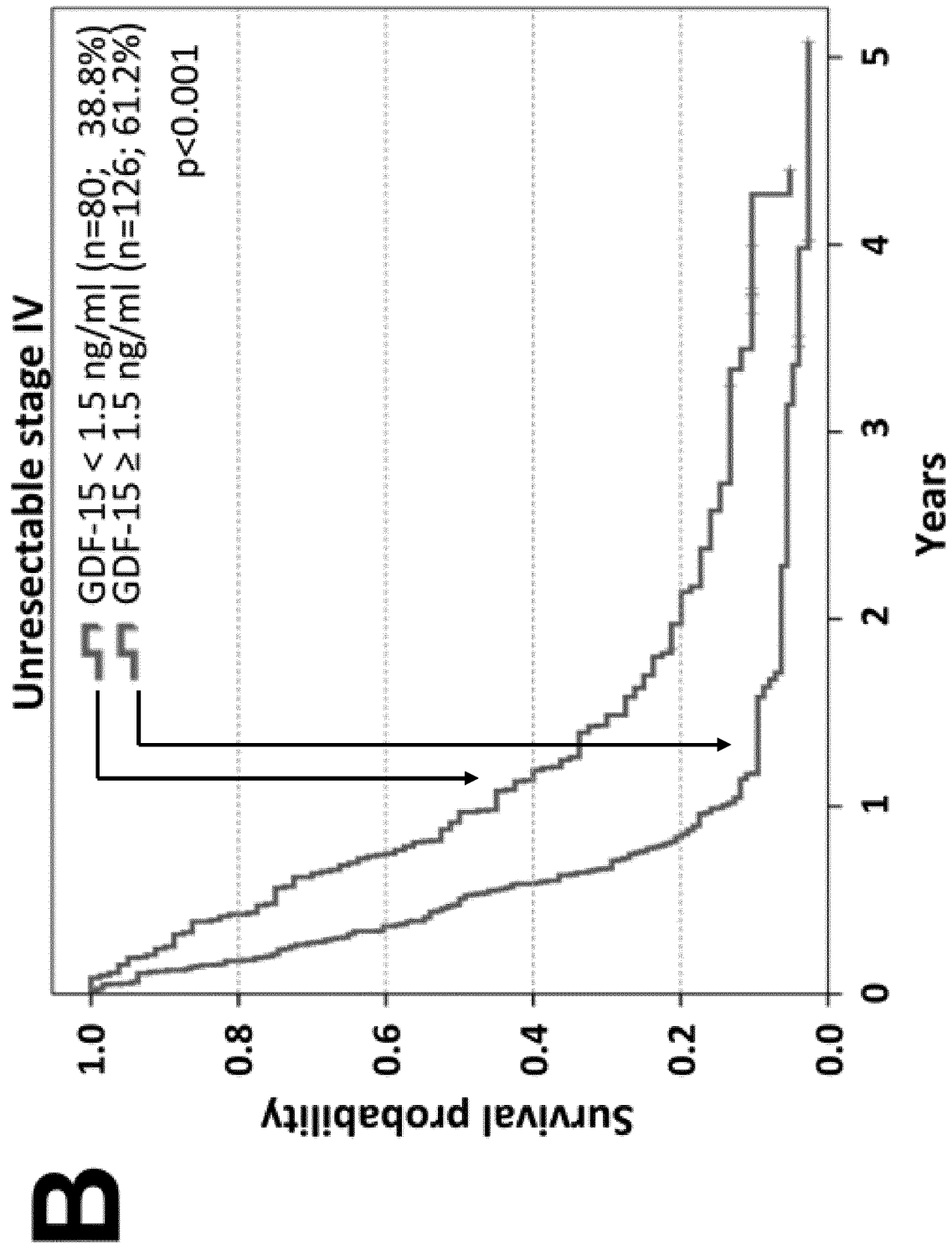
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Figure 1



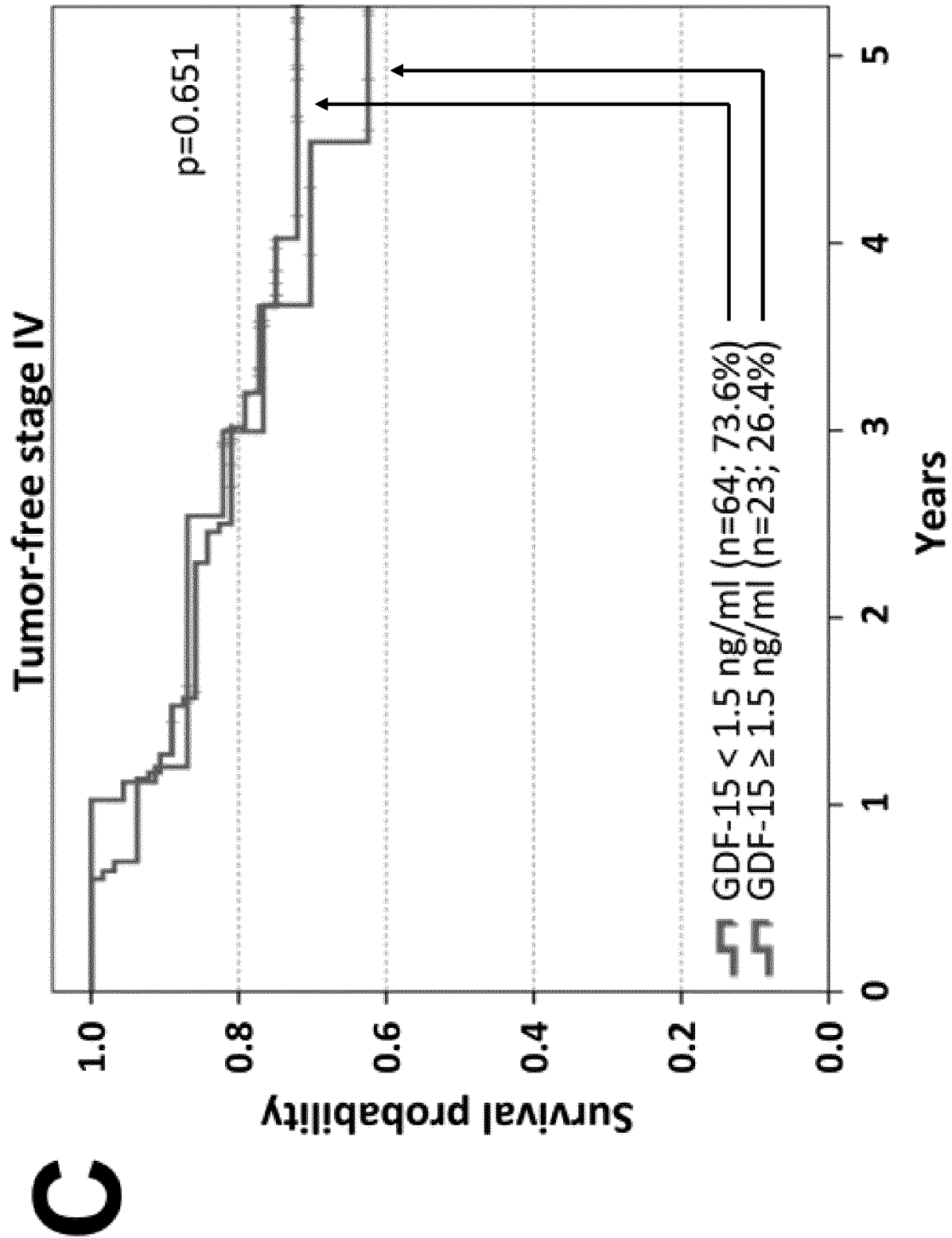
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Figure 1, continued



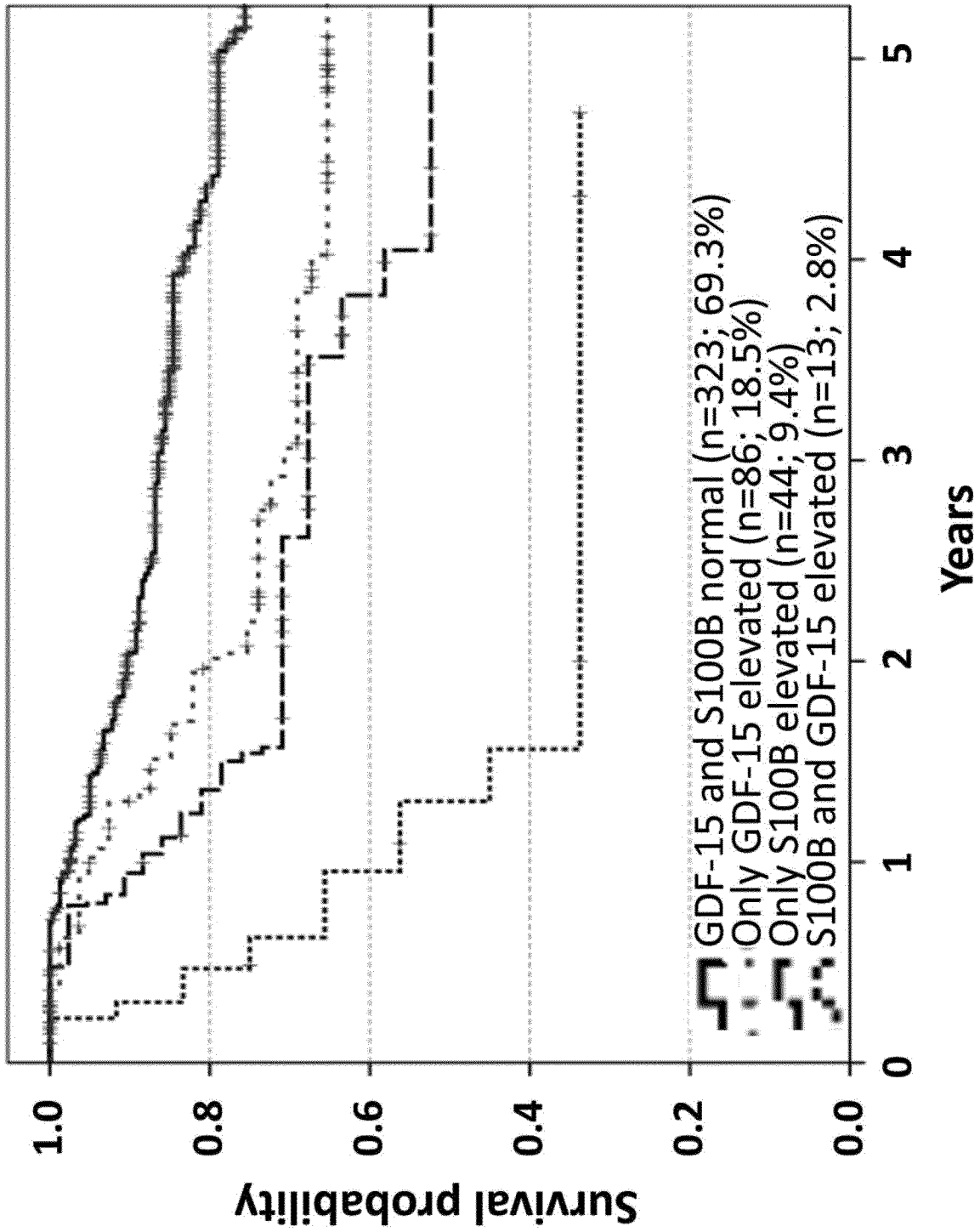
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Figure 1, continued



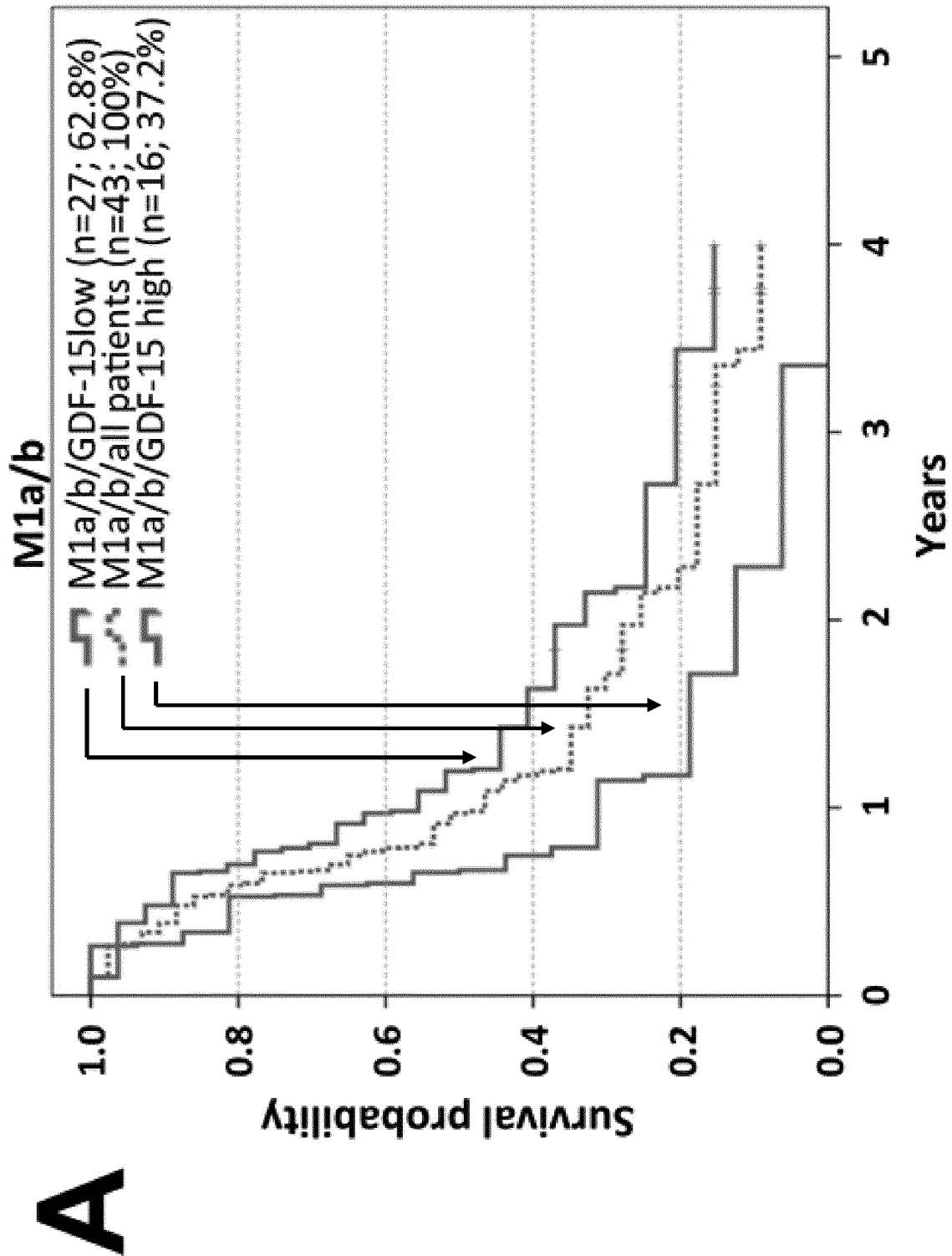
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Figure 2



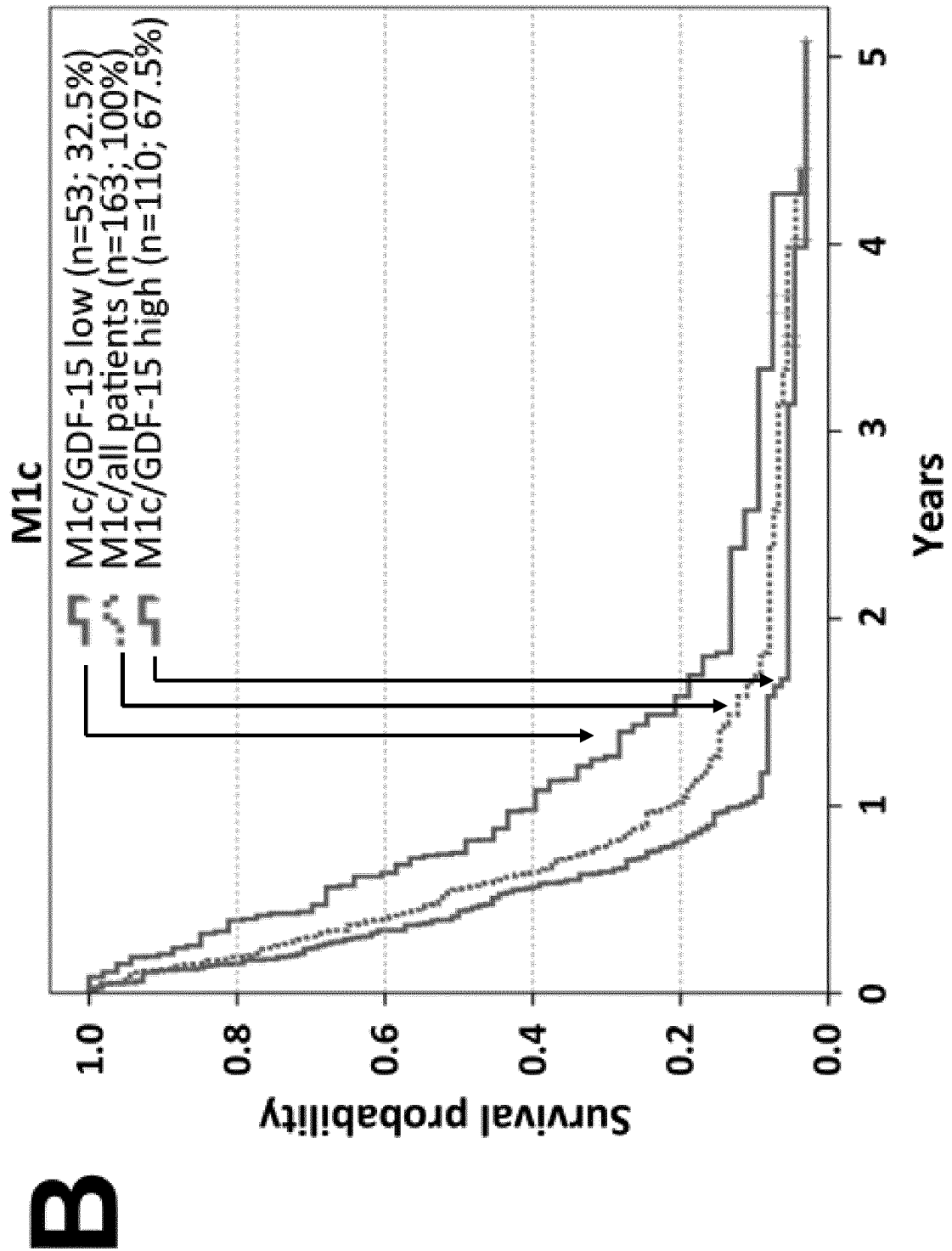
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Figure 3



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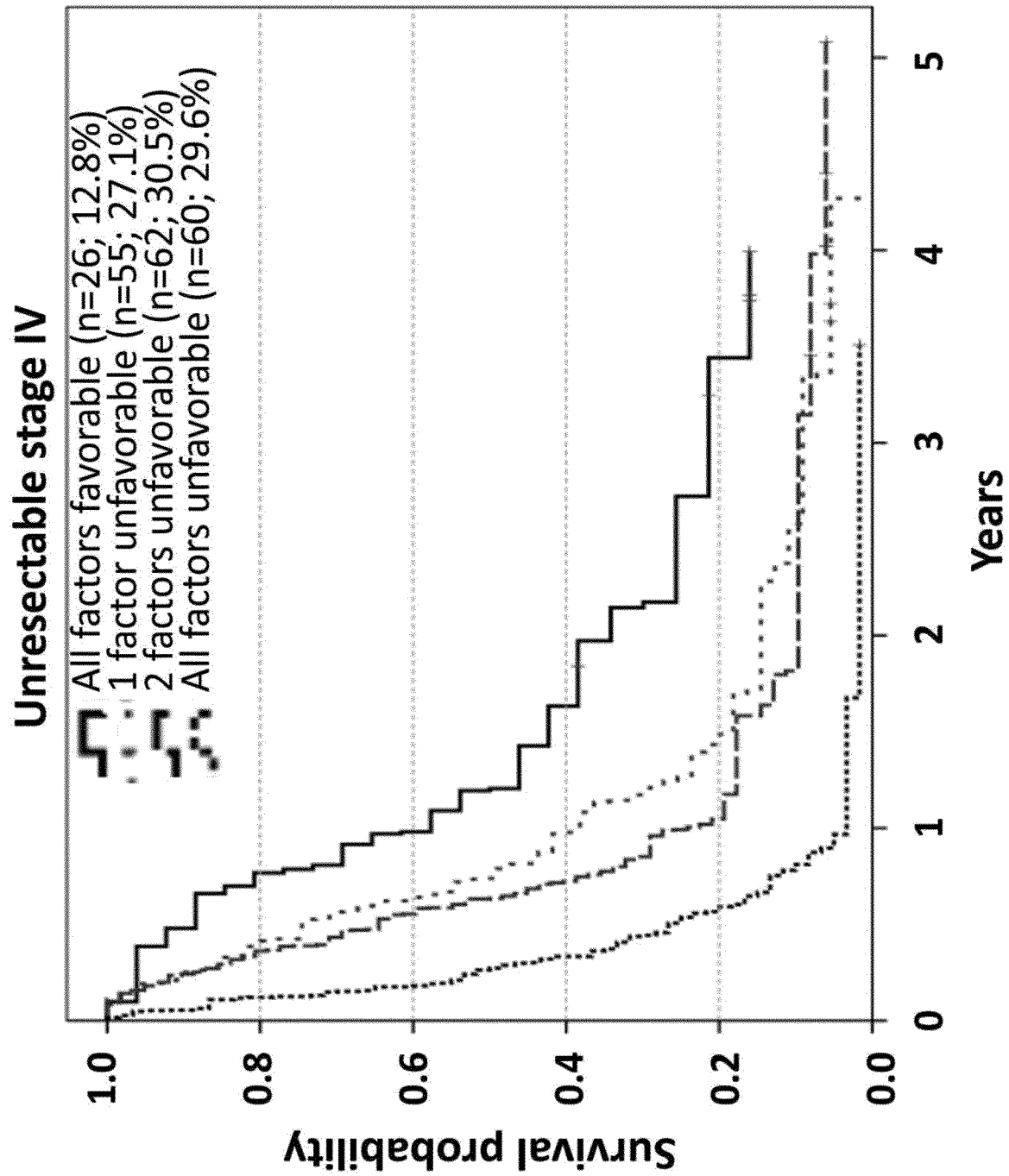
Figure 3, continued



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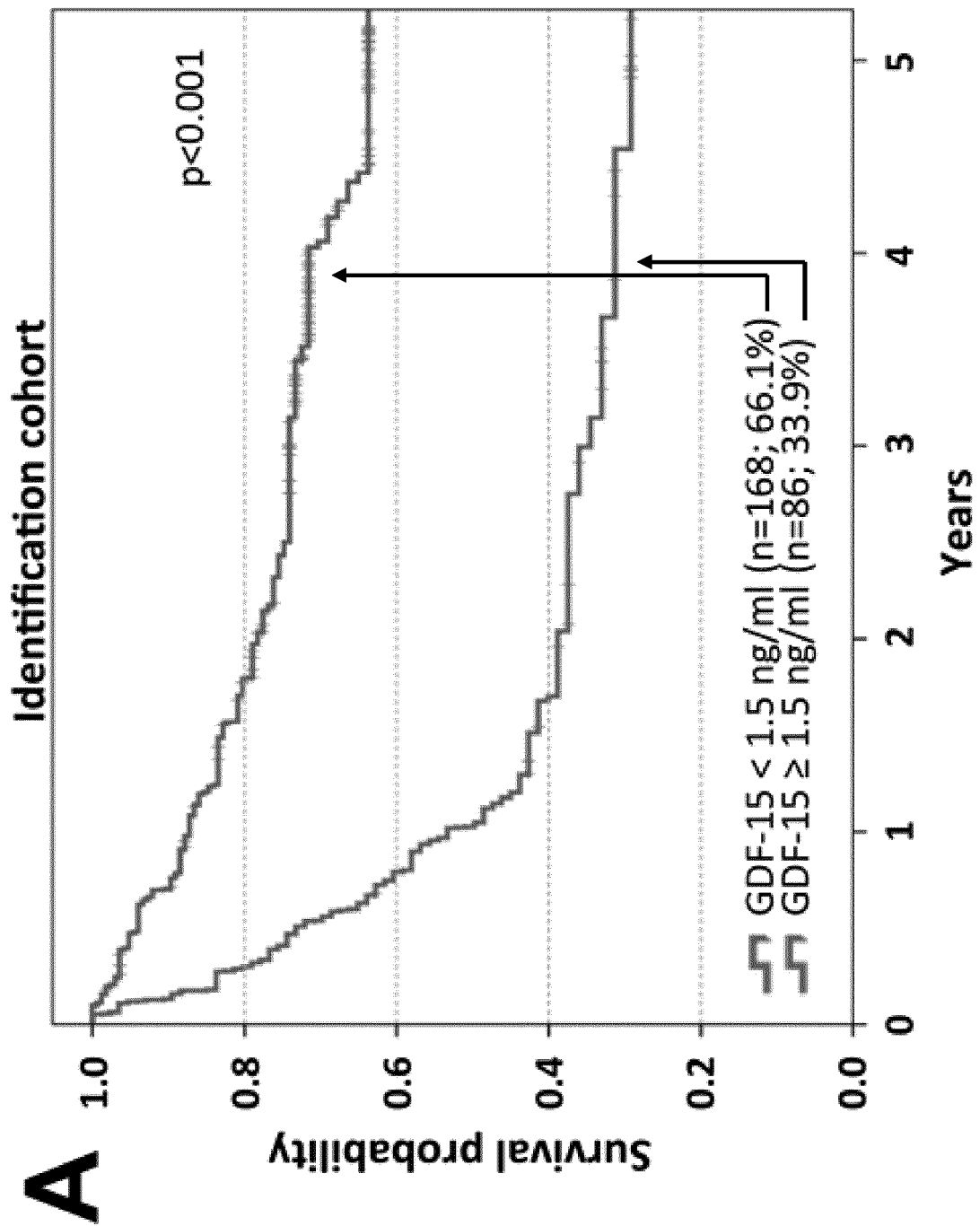
Figure 3, continued

C)



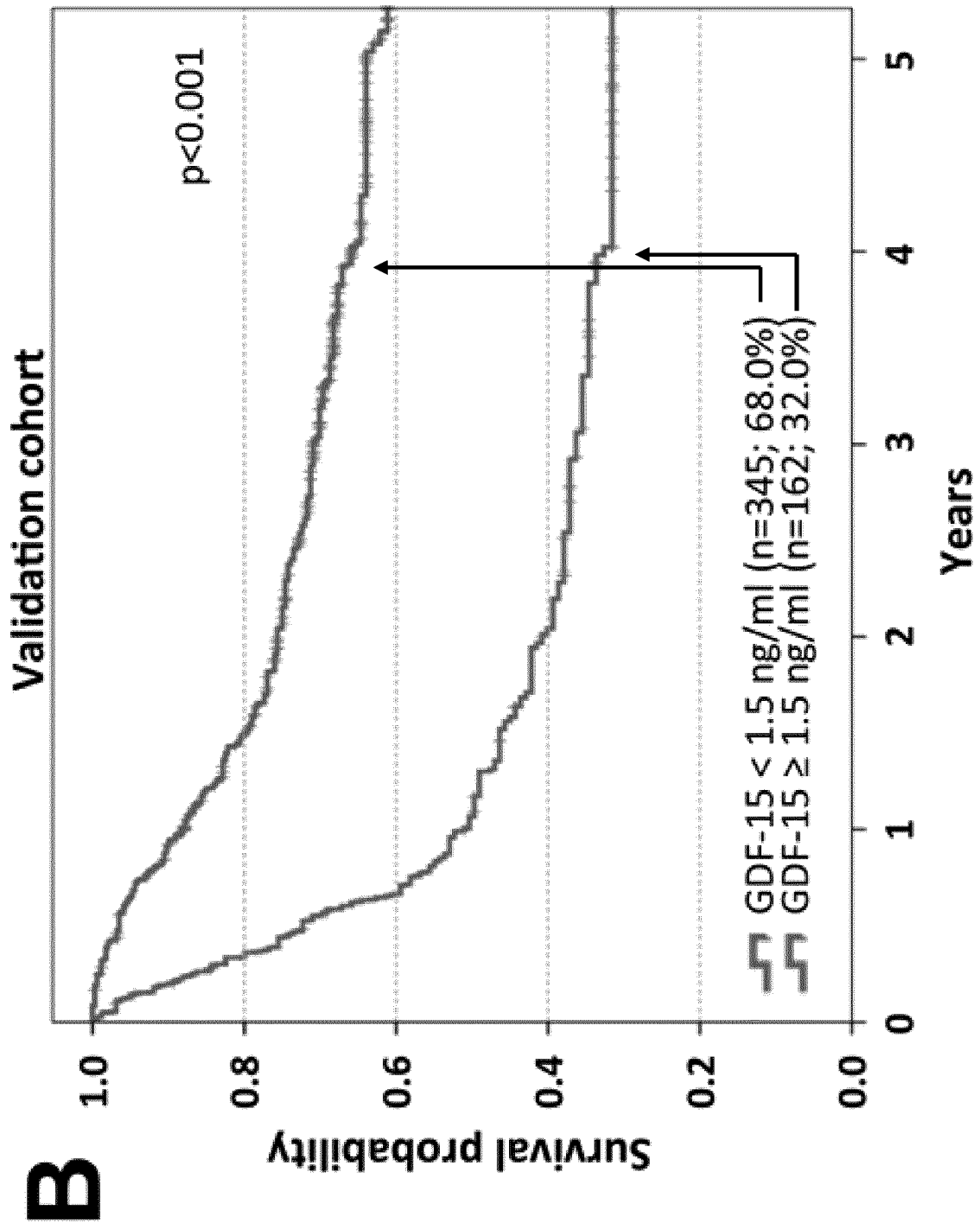
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Figure 4



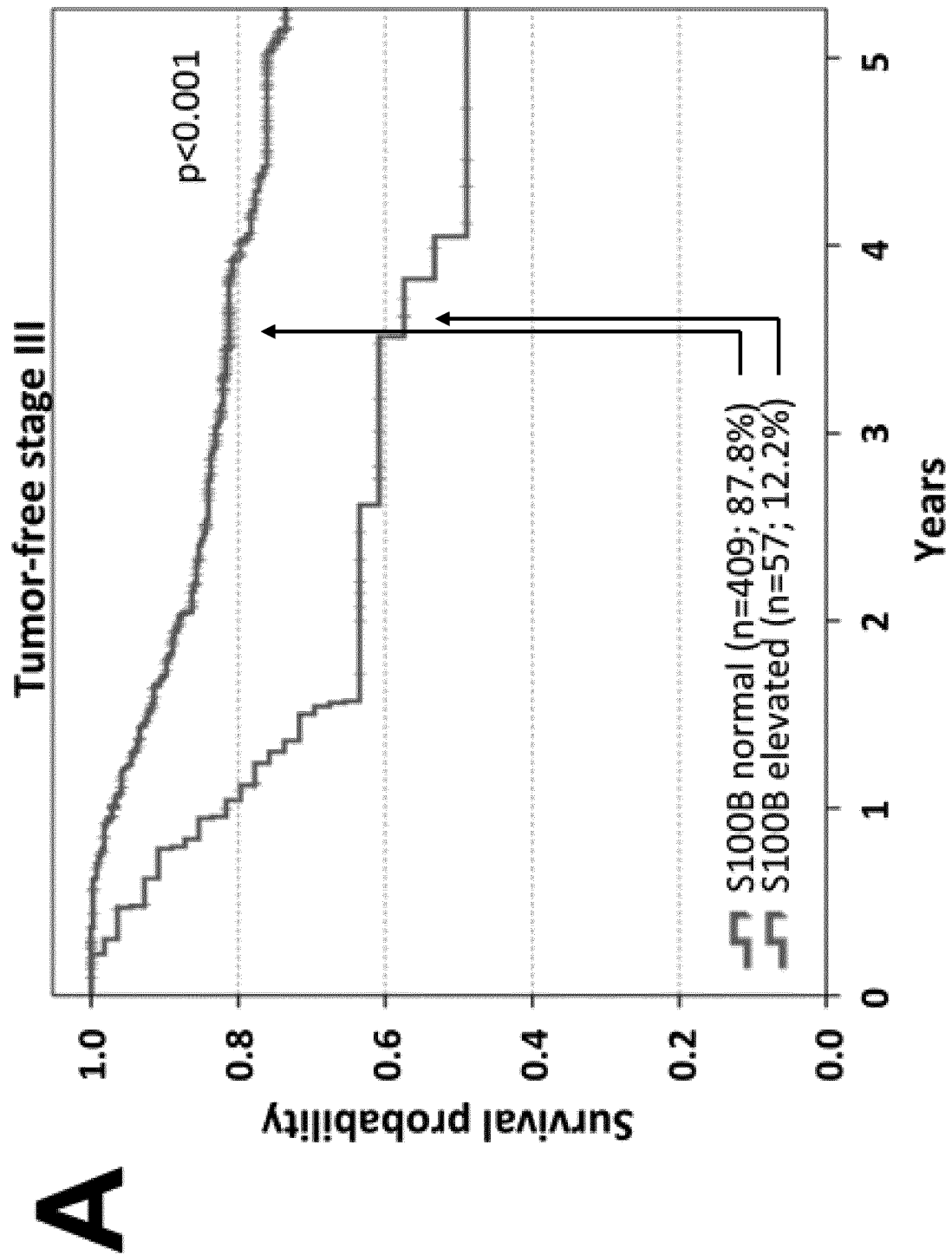
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Figure 4, continued



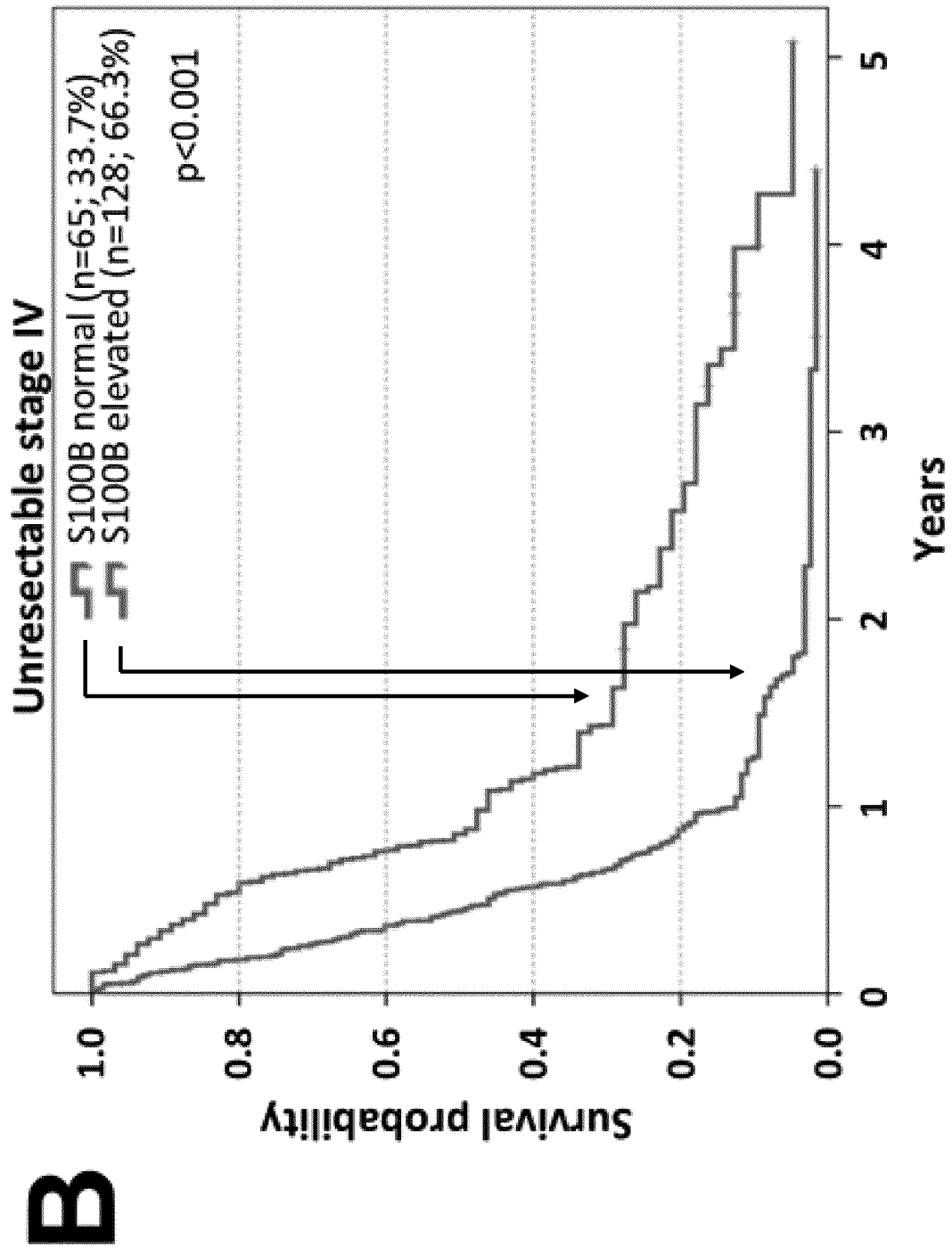
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Figure 5



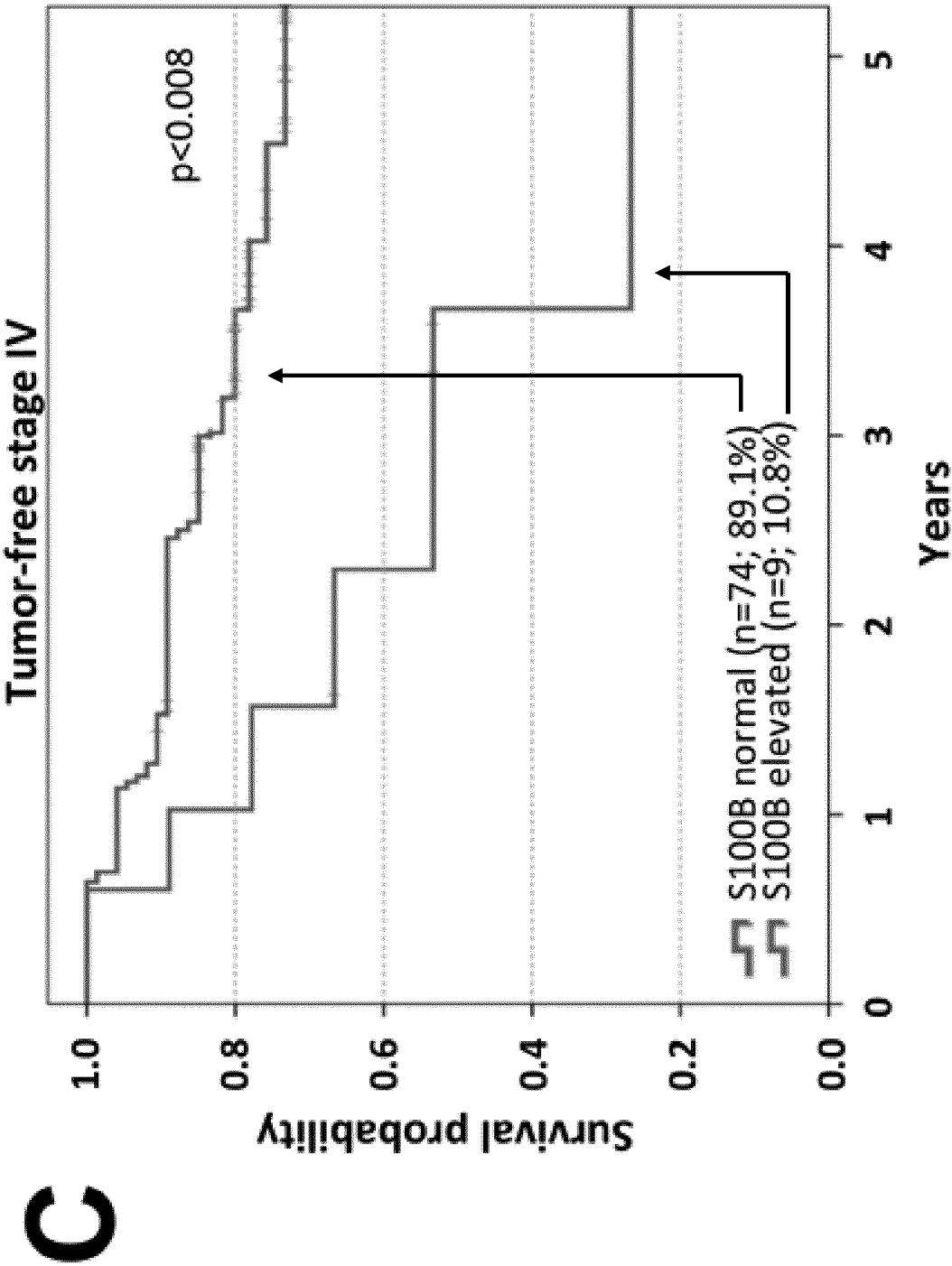
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Figure 5, continued



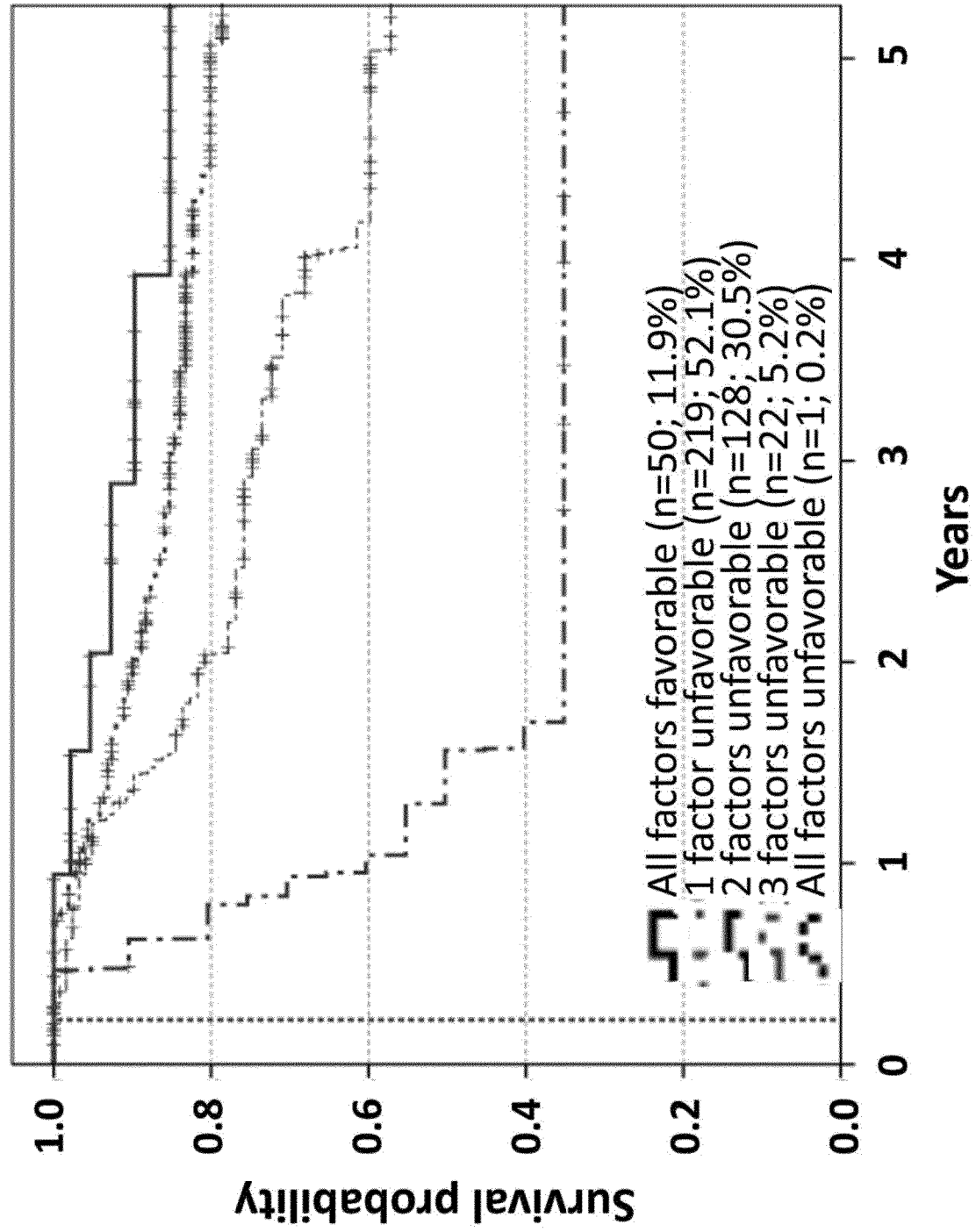
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Figure 5, continued



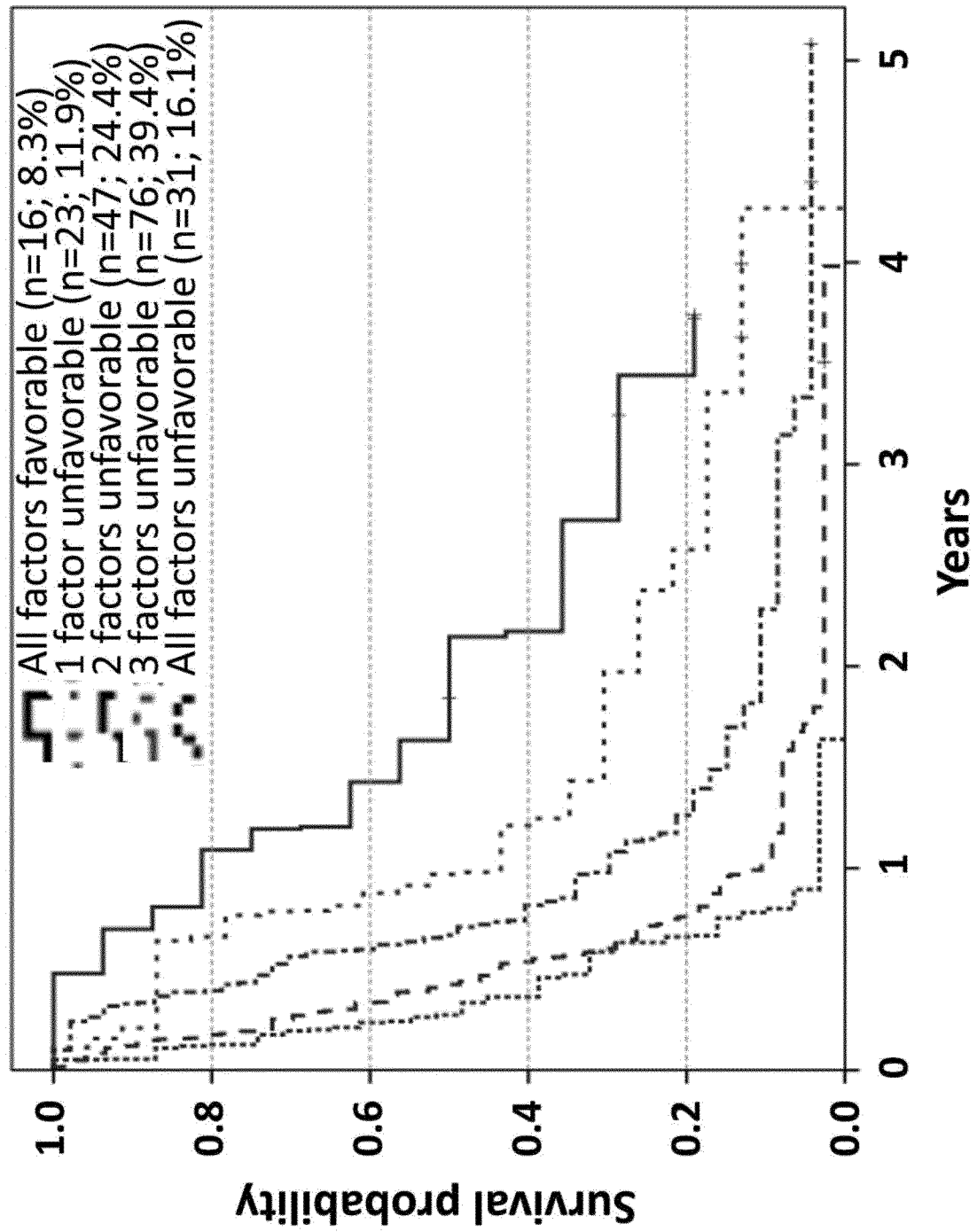
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Figure 6



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Figure 7



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Figure 8

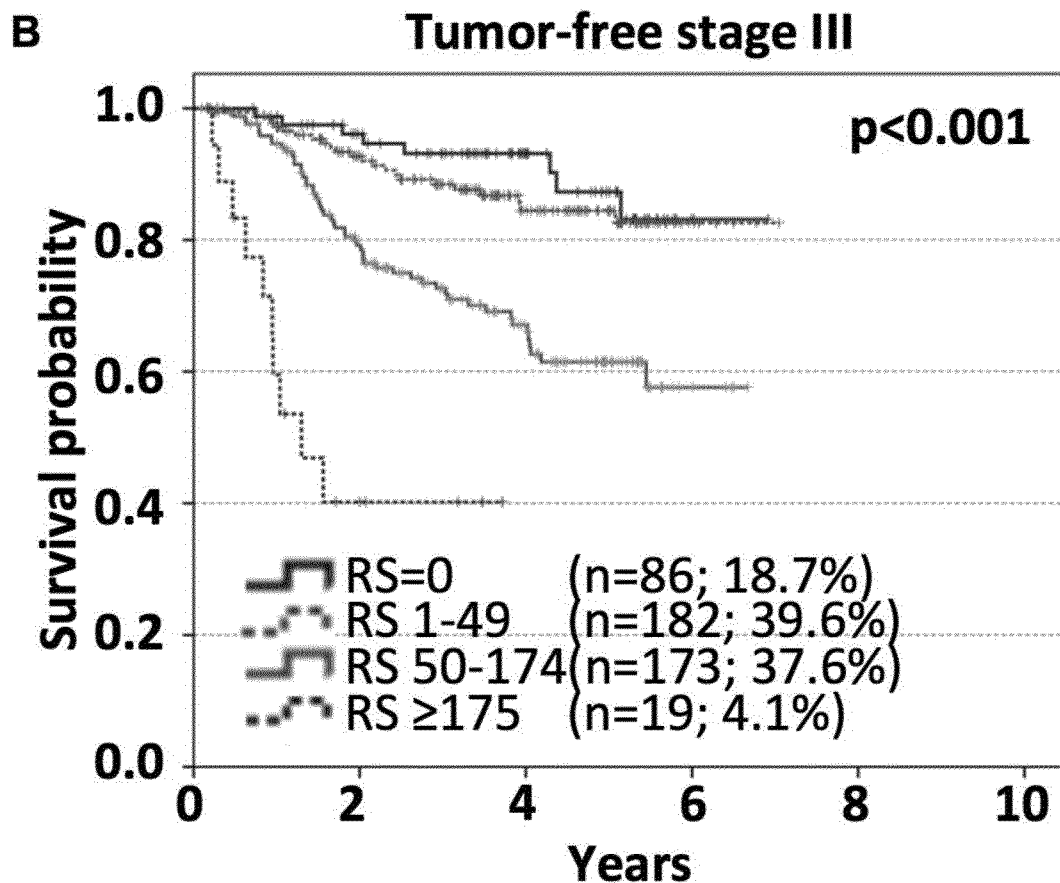
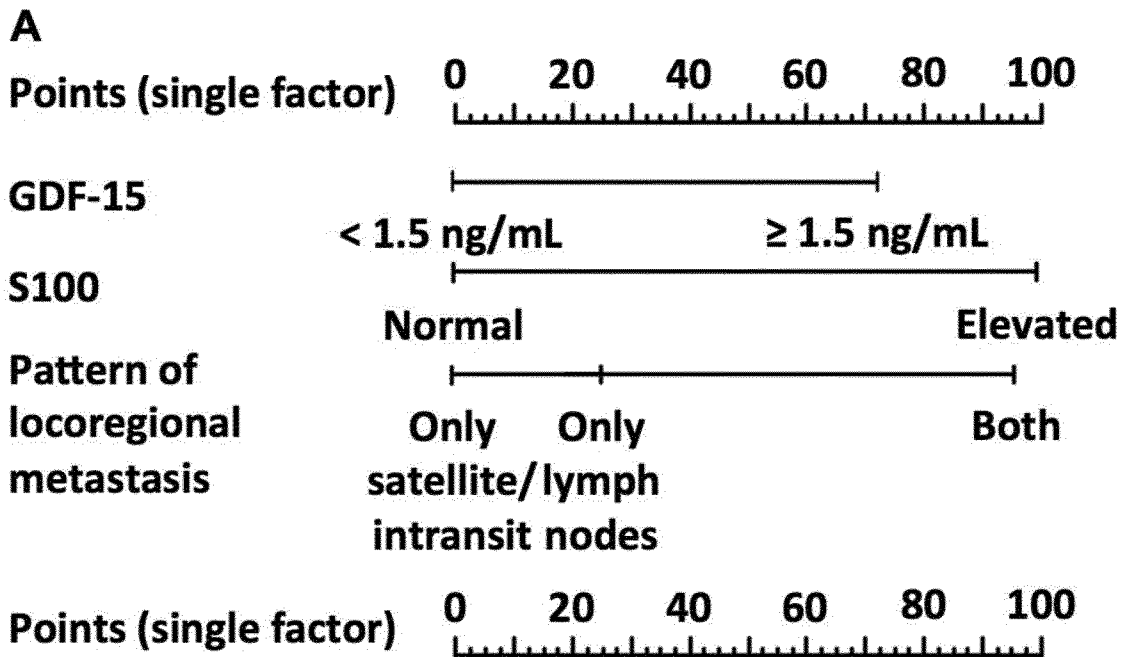


Figure 9

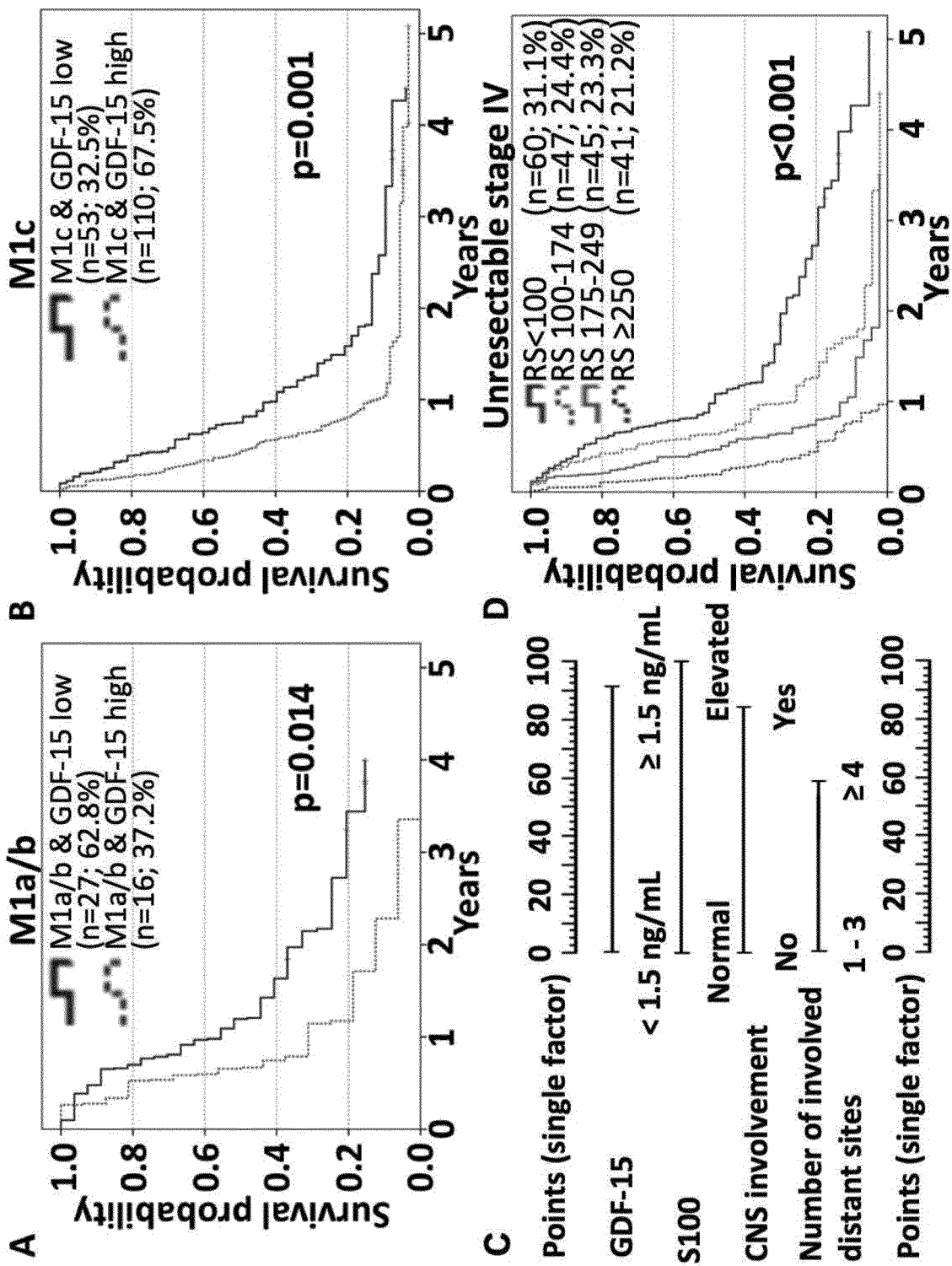
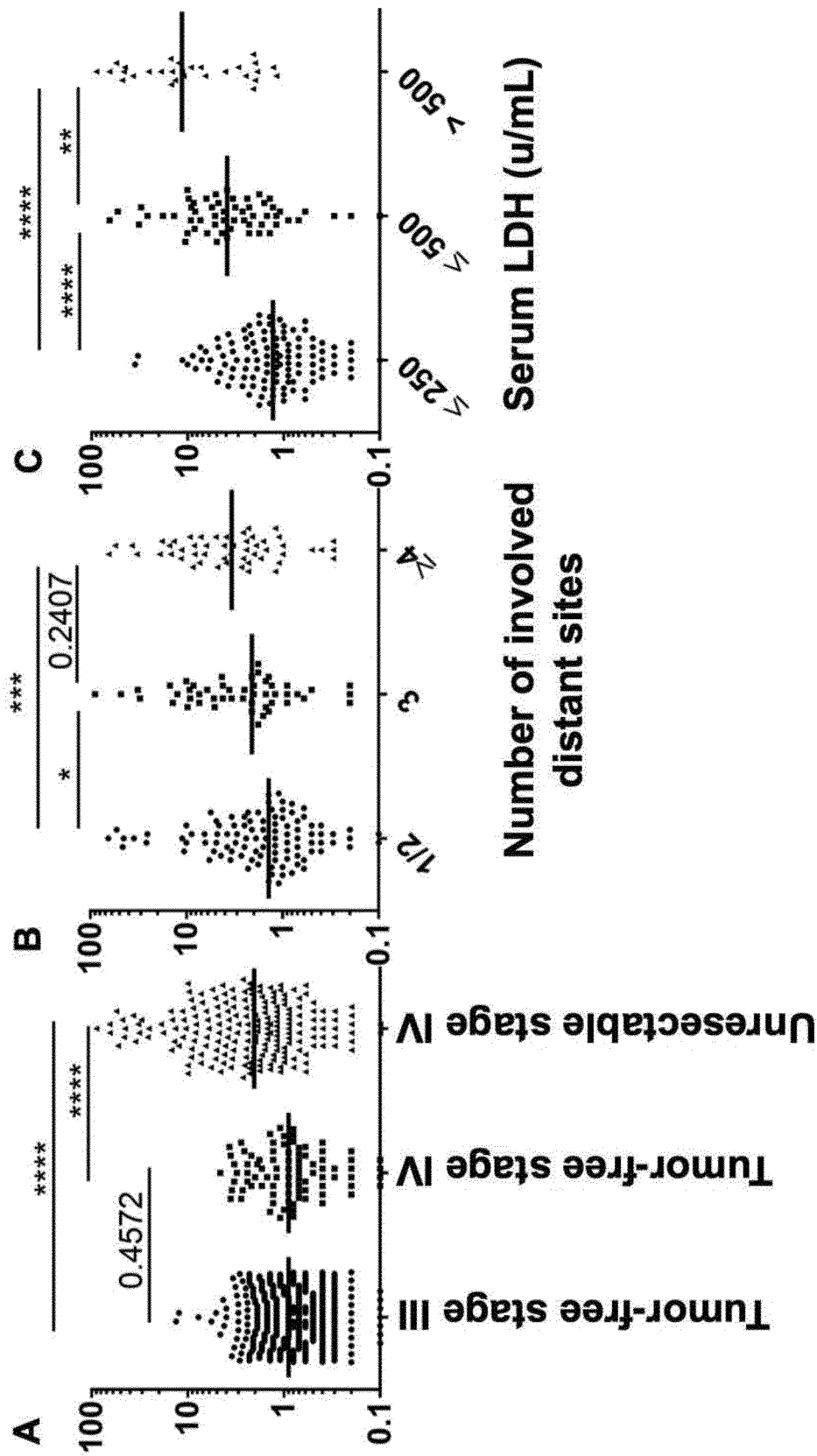
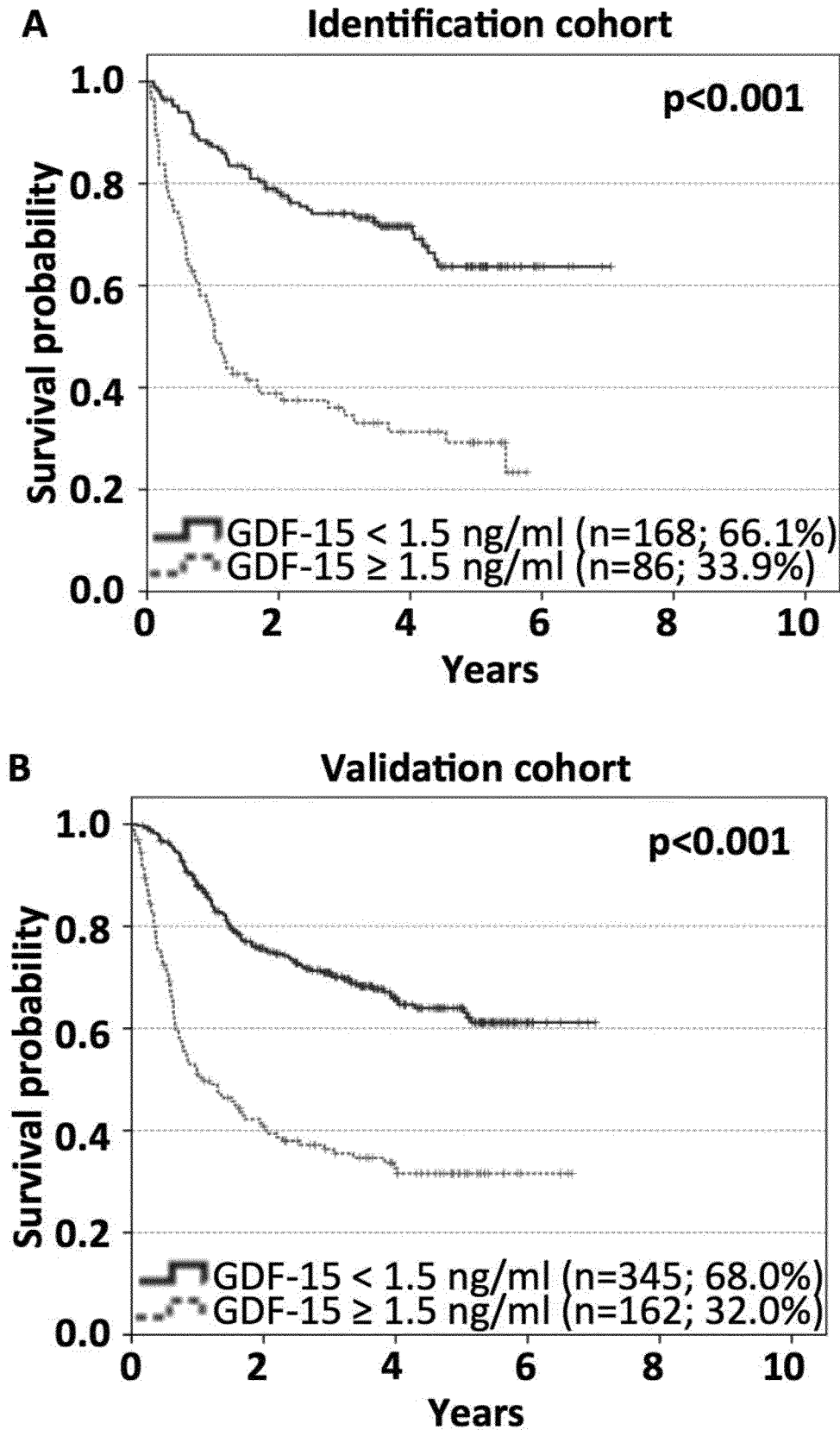


Figure 10



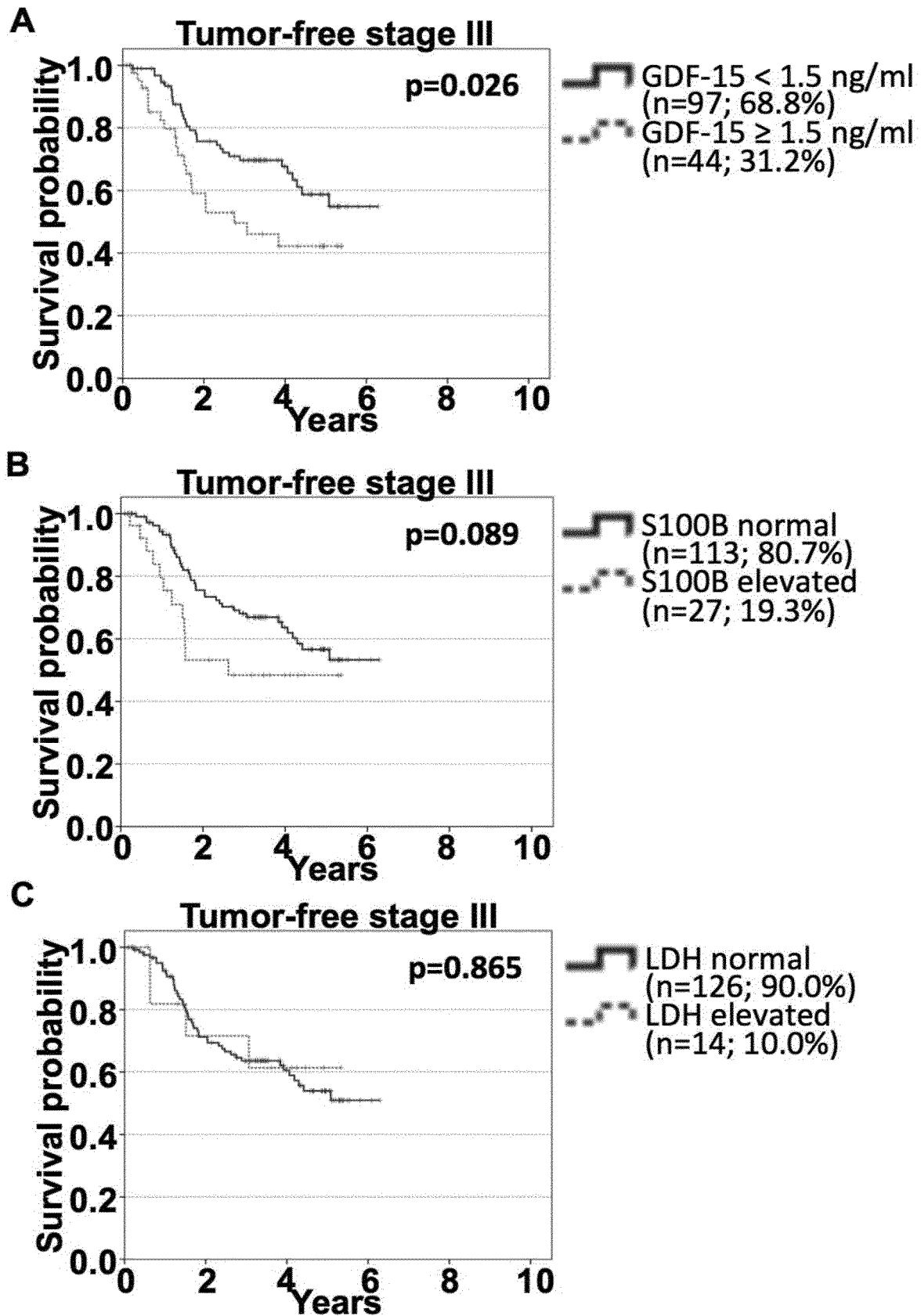
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Figure 11



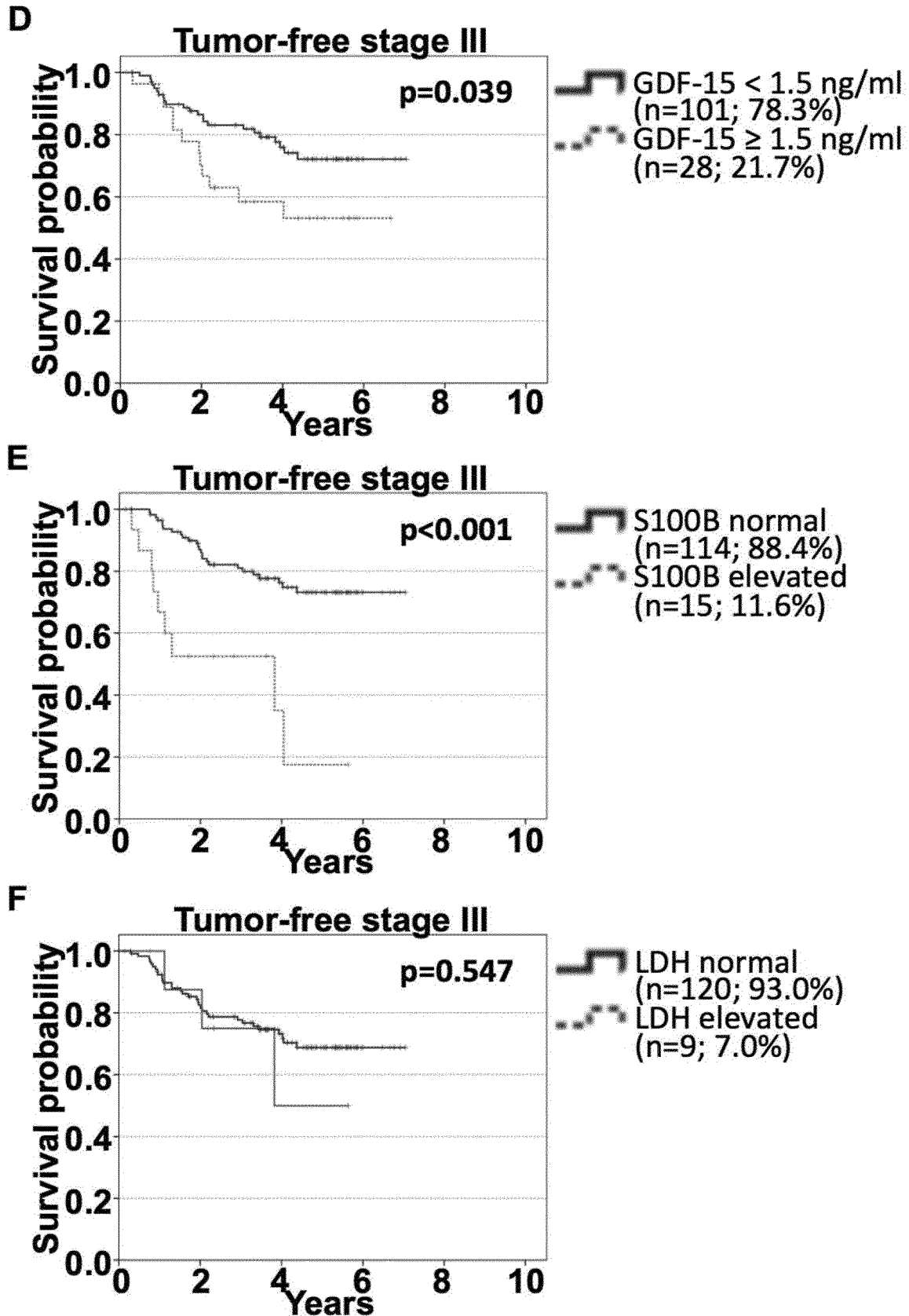
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Figure 12



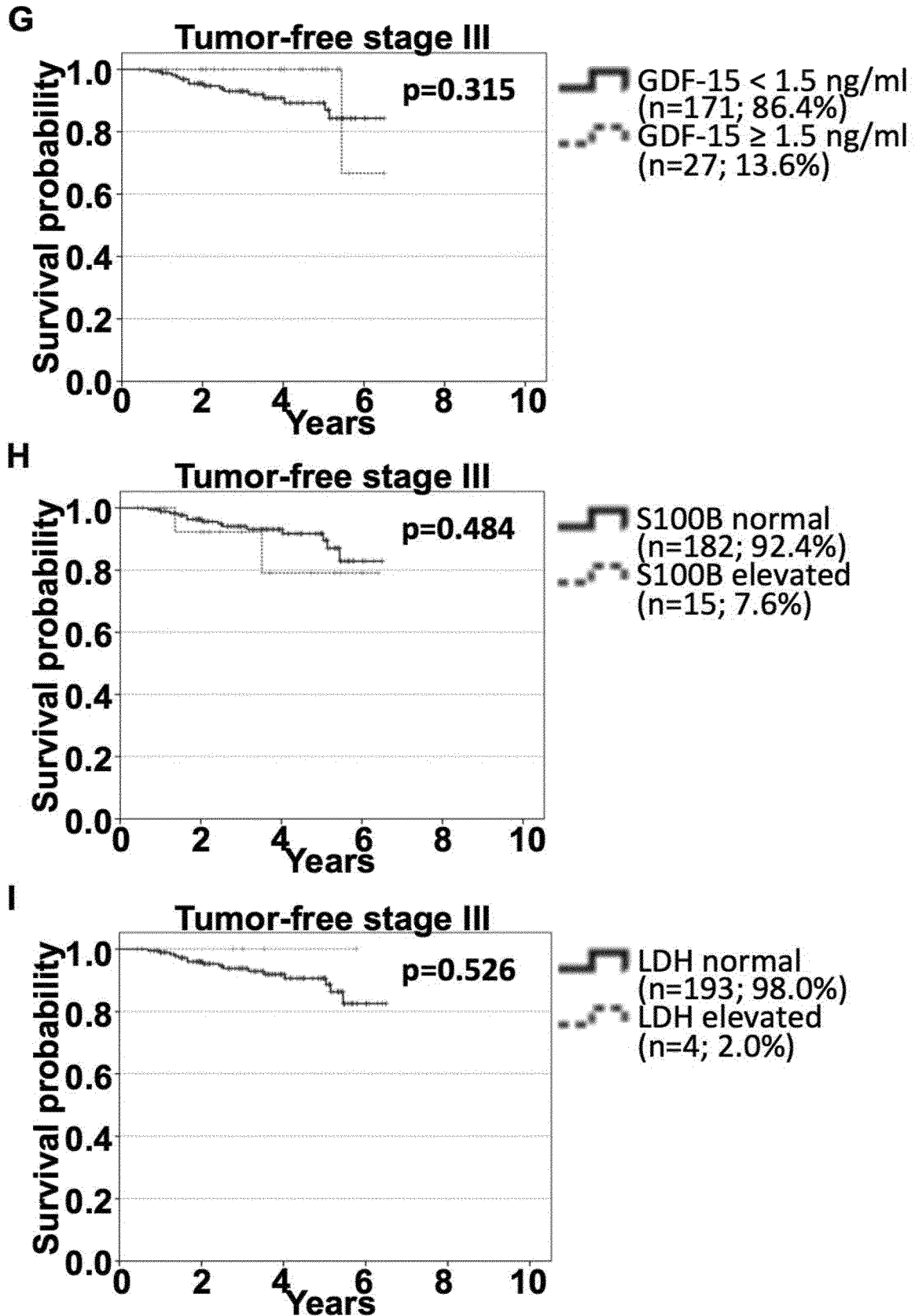
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Figure 12, continued



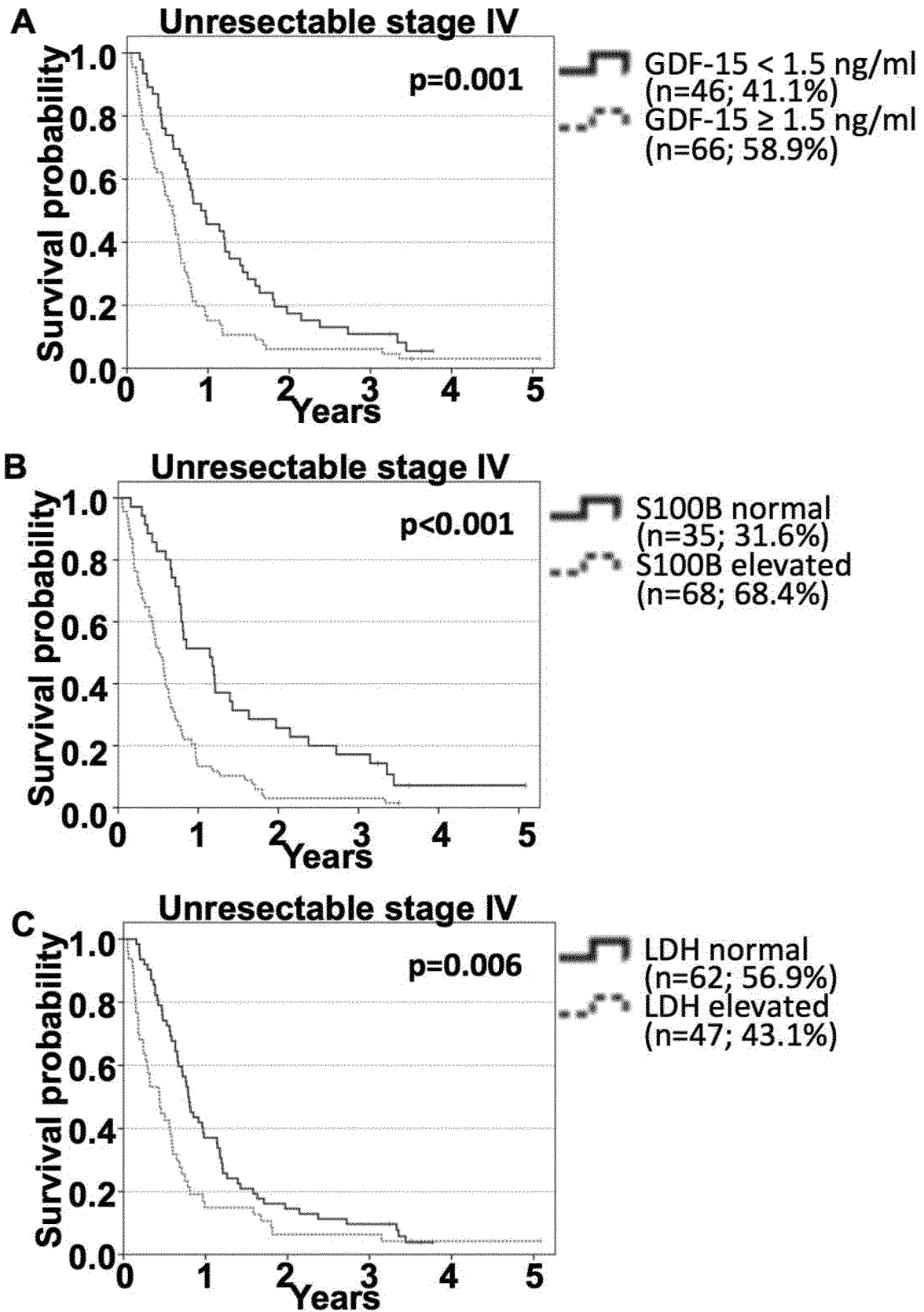
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Figure 12, continued



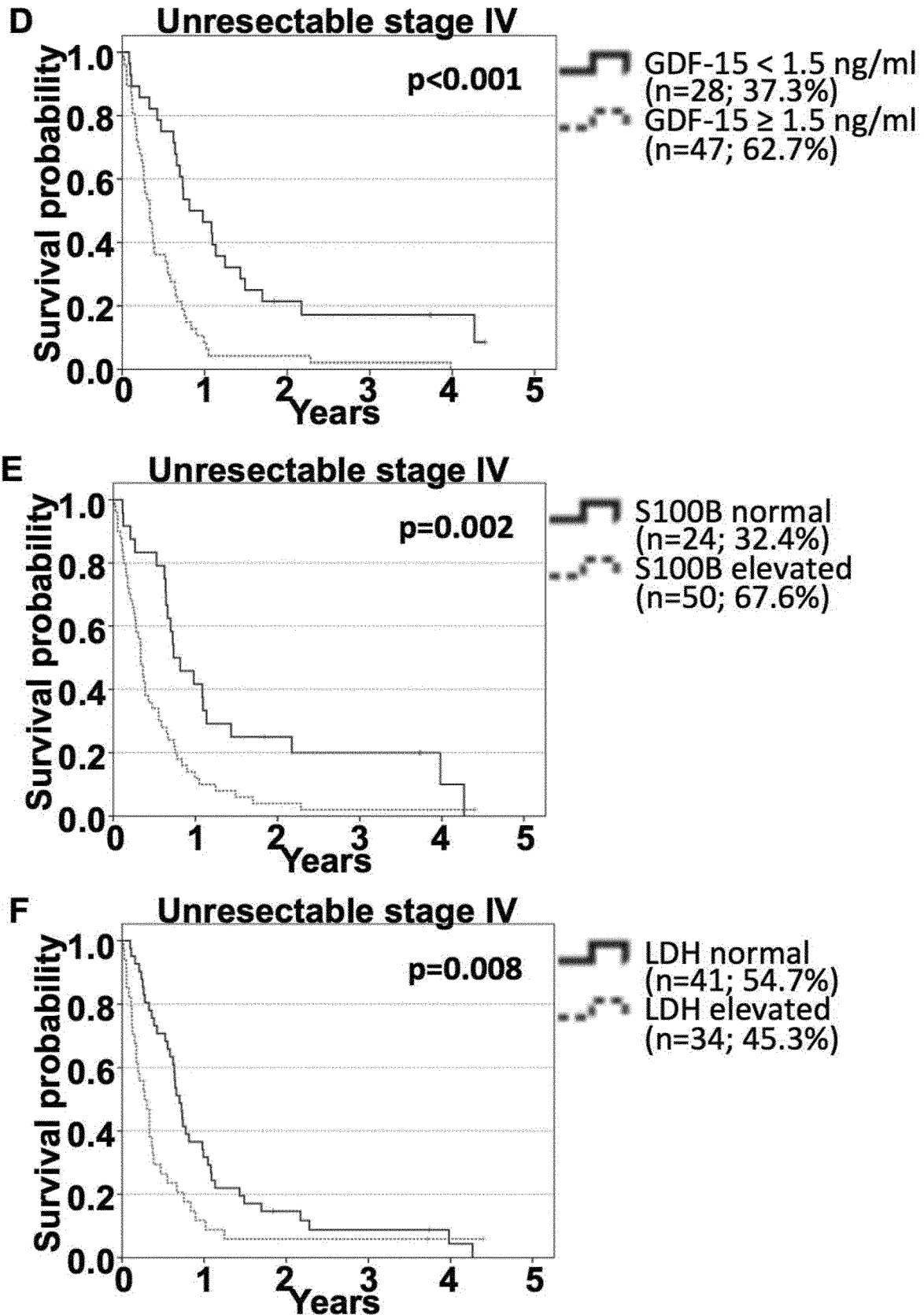
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Figure 13



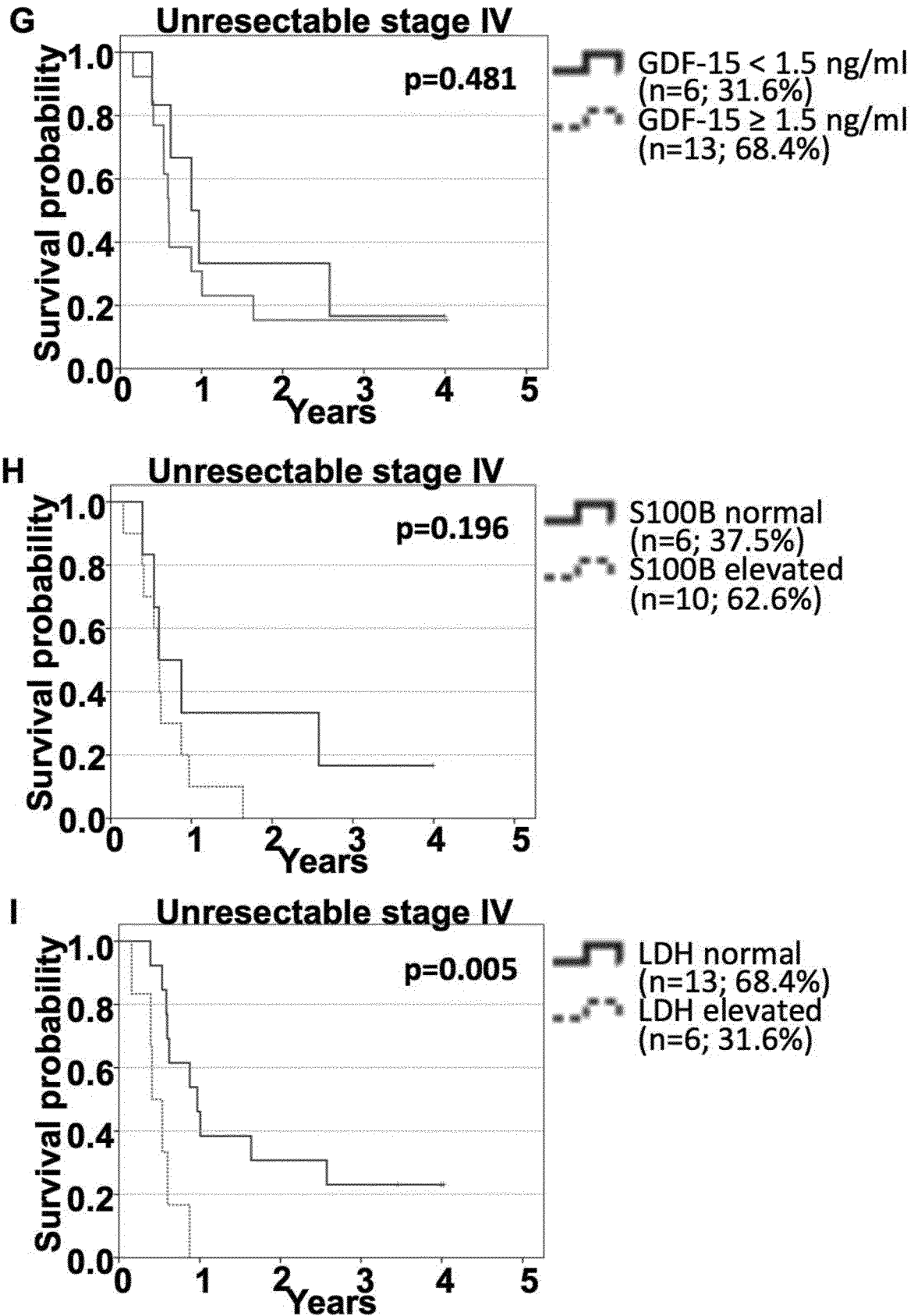
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Figure 13, continued



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Figure 13, continued



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Figure 14

