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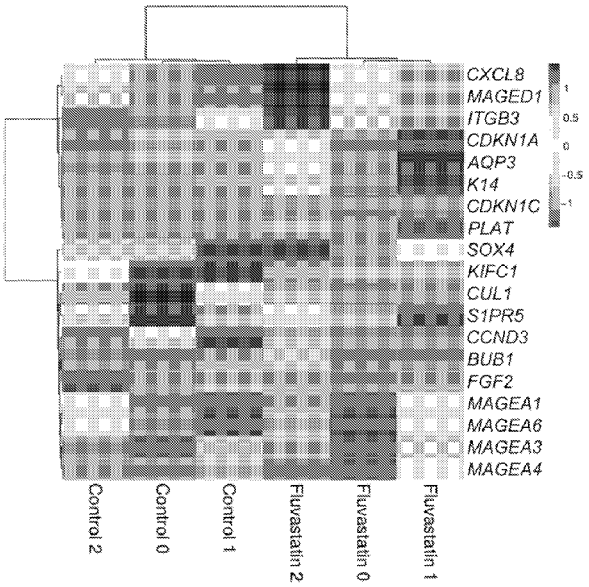
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(54) **Title:** METHODS FOR PREDICTING LYMPH NODE STATUS, LIKELIHOOD OF METASTASIS, AND/OR OVERALL PROGNOSIS IN PATIENTS WITH MELANOMA

FIG. 2



(57) **Abstract:** Described are methods taking advantage of the discovery that a melanoma patient's history of taking an inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (that is, a statin drug) influences their risk of future melanoma metastasis, lymph node status, prognosis, and/or disease progression. The methods involve considering whether (or not) the subject has taken a statin, and based on that information, assigning the subject to relatively higher risk (if no statin has been taken or is being taken) or to relatively lower risk (if a statin has been taken or is being taken) for future melanoma metastasis and/or progression. Optionally, analysis of the subject's risk further involves measurement of a gene expression signature in the primary melanoma, and/or additional clinicopathological measurements.

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METHODS FOR PREDICTING LYMPH NODE STATUS, LIKELIHOOD OF METASTASIS, AND/OR OVERALL PROGNOSIS IN PATIENTS WITH MELANOMA

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to and the benefit of the earlier filing of U.S. Provisional Application No. 63/227,930, filed on July 30, 2021, which is incorporated by reference herein in its entirety.

FIELD OF THE DISCLOSURE

[0002] The present disclosure relates to use of medication history (such as use of statin(s)) as a predictor of lymph node status, likelihood of future metastasis, and overall prognosis in patients with melanoma.

BACKGROUND OF THE DISCLOSURE

[0003] Cutaneous melanoma is an aggressive form of skin cancer with over 100,000 US cases diagnosed in 2021 (Siegel *et al.*, *CA Cancer J Clin*. 2021;71(1):7-33. doi:10.3322/caac.21654); annually, melanoma causes over 7000 deaths. Tumor stage is determined by histopathologic and clinical factors, which include the Breslow depth of the tumor, ulceration status, spread of disease from the primary tumor to lymph nodes, and the presence of metastasis (*Id.*). These factors are summarized into an overall stage denoted by a Roman numeral from 0 to IV, with Stage 0 disease being the earliest and Stage IV being the most advanced (*Id.*). Patients within a stage should theoretically have similar outcomes, but there is still significant heterogeneity within each stage. For example, most patients with Stage I disease will be cured by surgery, but a significant minority (5-10%) may go on to develop metastasis at a later time.

[0004] Up to one-third of melanoma deaths are caused by early, localized melanomas (American Joint Committee on Cancer (AJCC) Stage 1-2) that progress to metastasis despite adequate surgical treatment (Whiteman *et al.*, *J Invest Dermatol*. 2015;135(4):1190-1193. doi:10.1038/jid.2014.452). In addition, metastatic disease accounts for over 40% of the annual economic burden of melanoma.

[0005] Gene expression signatures have been identified that predict which melanomas will recur or metastasize (Gerami *et al.*, *Clin Cancer Res*. 2015. doi:10.1158/1078-0432.CCR-13-3316; Zager *et al.*, *BMC Cancer*. 2018. doi:10.1186/s12885-018-4016-3; Greenhaw *et al.*, *Dermatologic Surg*. 2018. doi:10.1097/DSS.0000000000001588). Gene expression profiling has been used to better identify patients at risk of future melanoma metastasis. Examples include the DecisionDx-Melanoma test (see U.S. Patent Application Publication No.

US20200362419A1) and the Merlin Assay (see WO2020022895A2). These tests generally stratify patients into either high- or low-risk groups, and are used for prognosis.

[0006] The clinical utility of gene expression profiling (GEP) has been limited by the lack of proven treatments for patients who are determined to be at high-risk for future progression or metastasis. A recent melanoma expert consensus statement determined that there is no clinical action associated with the result (Grossman *et al.*, *JAMA Dermatology*. 2020. doi:10.1001/jamadermatol.2020.1729; Marchetti *et al.*, *J Am Acad Dermatol*. 2019;80(6):e161-e162. doi:10.1016/j.jaad.2018.11.063; Marchetti *et al.*, *JAMA Dermatology*. 2020;156(9):953. doi:10.1001/jamadermatol.2020.1731). Gene expression tests have never been used to select melanoma patients for treatments to reduce future metastasis. One recent expert review states that a "major challenge of GEP testing [is] determining [...] how results should affect patient management. It is not currently known whether a high-risk GEP classification is associated with improved response to [...] systemic therapies." (Grossman *et al.*, *Melanoma Manag*. 2019;6(4):MMT32. doi:10.2217/mmt-2019-0016).

[0007] The National Comprehensive Cancer Network® (NCCN) Clinical Practice Guideline (CPG) in Oncology® recommendations for the clinical staging and workup of cutaneous melanoma (V2. 2019) states: "While there is interest in newer prognostic molecular techniques such as gene expression profiling to differentiate melanomas at low- versus high-risk for metastasis, routine (baseline) genetic testing of primary cutaneous melanomas [...] is not recommended outside of a clinical study (trial)." The updated 2021 NCCN Clinical Practice Guidelines reiterate this sentiment, stating: "the impact of these tests on treatment outcomes or follow-up schedules has not been established." The 2020 Medical Policy of multiple insurers such as Anthem BlueCross and Wellmark state that GEP testing "may improve our ability to determine prognosis in individuals with primary cutaneous melanoma, but how this would alter disease management and health outcomes remains unclear." (Anthem BlueCross Medical Policy Regarding Gene Expression Profiling of Melanomas. Available online at anthem.com/dam/medpolicies/abc/active/policies/mp_pw_c148391.html. Accessed June 17, 2021). The 2019 guideline by the American Academy of Dermatology (AAD) regarding care for the management of primary cutaneous melanoma states: "Evidence is lacking that molecular classification should be used to alter patient management." (Swetter *et al.*, *J Am Acad Dermatol*. 2019;80(1):208-250. doi:10.1016/j.jaad.2018.08.055).

[0008] Previous studies on statins in melanoma have focused on melanoma initiation and primary prevention rather than metastasis prevention and have had mixed results. Two large cardiovascular trials demonstrated a small reduction in melanoma incidence with statin use, but this effect was not observed in the Women's Health Initiative or two Dutch epidemiologic studies (Splichal *et al.*, *Semin Thromb Hemost*. 2003. doi:10.1055/s-2003-40964; Rubins *et*

al., *N Engl J Med*. 1999. doi:10.1056/NEJM199908053410604; Jagtap *et al.*, *Cancer*. 2012. doi:10.1002/cncr.27497). Two meta-analyses also demonstrated no reduction in melanoma incidence with statin use, and a randomized controlled trial of lovastatin for melanoma prevention did not identify any significant decreases in melanocytic atypia or other melanoma initiation markers (Bonovas *et al.*, *Eur J Epidemiol.* 2010. doi:10.1007/s10654-009-9396-x; Freeman *et al.*, *J Natl Cancer Inst*. 2006. doi:10.1093/jnci/djj412; Linden *et al.*, *Cancer Prev Res (Phila).* 2014;7(5):496-504. doi:10.1158/1940-6207.CAPR-13-0189). Recently, a Mendelian randomization analysis using the UK Biobank demonstrated that individuals with variants in the HMGCR region, which represent proxies for statin use, had decreased overall cancer risk but did not reach statistical significance for any site-specific cancers (Carter *et al.*, *medRxiv*. 2020. doi:https://doi.org/10.1101/2020.02.28.20028902). In summary, the current evidence available in the literature and the public domain suggest that statins have little to no effect on melanoma initiation and progression.

[0009] Several studies of *in vitro* and animal models have suggested that statins might decrease tumor cell migration, decrease cell adhesion, and increase immunogenicity and prevent progression of melanoma metastasis (Collisson *et al.*, *Mol Cancer Ther*. 2003; Pich *et al.*, *Front Immunol. 2013.* doi:10.3389/fimmu.2013.0006; Kidera *et al.*, *J Exp Clin Cancer Res*. 2010. doi:10.1186/1756-9966-29-127; Zanfardino *et al.*, *Int J Oncol.* 2013. doi:10.3892/ijo.2013.2126). However, when these studies were followed up in large human clinical cohorts, the effect was not observed and there was no statistically significance decrease in metastasis (Koomen *et al.*, *Eur J Cancer.* 2007. doi:10.1016/j.ejca.2007.09.004). Thus, statins are currently not used in metastasis prevention because there is no evidence that they have any treatment effect in unselected populations of melanoma patients; in fact, past attempts to find any beneficial effect of statins in treatment of melanoma have failed. The finding that statins could have a beneficial effect on selected melanoma patients would thus be novel and surprising.

[0010] There is a need in the art for methods to predict lymph node status and future prognosis in patients with melanoma.

SUMMARY OF THE DISCLOSURE

[0011] Provided herein in a first embodiment is a method including: determining whether a subject with a primary melanoma has taken an inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCoA reductase, or HMGCR) (commonly referred to as a statin), and classifying the subject as: having a relatively lower risk of sentinel lymph node metastasis and/or a relatively positive prognosis if the subject has taken a statin; and having a relatively higher risk of sentinel lymph node metastasis and/or a relatively negative prognosis if the subject has not taken a statin. Optionally, one or more additional factors or

clinicopathological measurements are also considered, such as one or more of Breslow depth of the primary melanoma tumor, and/or ulceration status of the primary melanoma tumor of the subject, and/or age of the subject, in classifying the subject. Further, the method may include considering at least one additional measure or marker of melanoma disease state, disease severity, or disease progression in classifying the subject.

[0012] In examples of these embodiments, classifying the subject further includes analyzing a genetic expression profile (GEP) of the primary melanoma. For instance, the GEP in some cases includes measurement of the expression level of one or more genes selected from: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6. Alternatively, the GEP includes measurement of the expression level of: at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least 10, at least 15, at least 20, at least 25, or at least 30 genes selected from GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6. Further, the GEP in some instances includes measurement of the expression level of all of the following genes: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0013] In additional embodiments, classifying the subject further includes analyzing a GEP of the primary melanoma that includes measurement of the expression level of any one or more of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI; or that includes measurement of the expression level of each of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI. Also contemplated are methods, wherein the GEP includes measurement of the expression level of any or more of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6; or wherein GEP includes measurement of the expression level of each of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0014] In any of these method embodiments, classifying the subject may be used to determining a treatment and/or diagnostic work-up schedule for the subject.

[0015] In any of these method embodiments, the statin can include at least one of fluvastatin, pitavastatin, atorvastatin, simvastatin, lovastatin, rosuvastatin, or pravastatin.

[0016] In any of these method embodiments, the gene expression levels are measured using one or more of polymerase chain reaction (PCR), real-rime polymerase chain reaction (RT-PCR), direct DNA expression in microarray, Sanger sequencing analysis, Northern blot, direct RNA expression detection, serial analysis of gene expression (SAGE), or next-generation RNA-sequencing. In certain preferred embodiments, the gene expression levels are measured using RT-PCR.

[0017] Another provided embodiment is a method for assessing pre-test (that is, prior to carrying out the biopsy) probability of a positive sentinel lymph node biopsy (SLNB) for a subject with cutaneous melanoma, including: determining whether a subject has taken a statin, and classifying the subject as: having a relatively lower risk of a positive SLNB if the subject has taken a statin; and having a relatively higher risk of positive SLNB if the subject has not taken a statin. Optionally, one or more additional factors or clinicopathological measurements are also considered, such as one or more of Breslow depth of the primary melanoma tumor, and/or ulceration status of the primary melanoma tumor of the subject, and/or age of the subject, in classifying the subject. Further, the method may include considering at least one additional measure or marker of melanoma disease state, disease severity, or disease progression in classifying the subject.

[0018] In examples of these embodiments, the method further includes analyzing a genetic expression profile (GEP) of the primary melanoma. For instance, the GEP in some cases includes measurement of the expression level of one or more genes selected from: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSl, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6. Alternatively, the GEP includes measurement of the expression level of: at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least 10, at least 15, at least 20, at least 25, or at least 30 genes selected from GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSl, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6. Further, the GEP in some instances includes measurement of the expression level of all of the following genes: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSl, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1,

CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0019] In additional embodiments, the method further includes analyzing a GEP of the primary melanoma that includes measurement of the expression level of any one or more of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI; or that includes measurement of the expression level of each of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI. Also contemplated are methods, wherein the GEP includes measurement of the expression level of any or more of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6; or wherein GEP includes measurement of the expression level of each of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0020] In any of these method embodiments, classifying the subject may be used to determining a treatment and/or diagnostic work-up schedule for the subject.

[0021] In any of these method embodiments, the statin can include at least one of fluvastatin, pitavastatin, atorvastatin, simvastatin, lovastatin, rosuvastatin, or pravastatin.

[0022] In any of these method embodiments, the gene expression levels are measured using one or more of polymerase chain reaction (PCR), real-rime polymerase chain reaction (RT-PCR), direct DNA expression in microarray, Sanger sequencing analysis, Northern blot, direct RNA expression detection, serial analysis of gene expression (SAGE), or next-generation RNA-sequencing. In certain preferred embodiments, the gene expression levels are measured using RT-PCR.

[0023] Also provided are improved methods of classifying a subject with melanoma, the improvement including taking into account in the classifying whether the subject has a history of taking a statin, wherein: a history of taking a statin classifies the subject as having one or more of: a relatively lower risk of a positive sentinel lymph node biopsy, a relatively low risk of sentinel lymph node metastasis, and a relatively good prognosis; and no history of taking a statin classifies the subject as having one or more of: a relatively higher risk of positive sentinel lymph node biopsy, a relatively high risk of sentinel lymph node metastasis, and a relatively poor prognosis.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] Some of the drawings submitted herein may be better understood in color. Applicant considers the color versions of the drawings as part of the original submission and reserves the right to present color images of the drawings in later proceedings.

[0025] FIG. 1. Heatmap of the top 100 differentially expressed genes after fluvastatin treatment. K, keratin; MMP, matrix metalloproteinase. In order, the 100 illustrated genes are: ADAMTS16, CTB-47B8.1, RP11-98F14.11, AVIL, OLFML2A, RASA4, RN7SKP55, DACT1, GJB2, CEACAM1, DUSP8, CYSLTR1, CALB1, FAM83A, RN7SL865P, CAPS, BMF, PTPRH, JUP, KRT14, C10orf10, MIR210HG, AQP3, LGR6, KRT15, AKR1C1, CRISPLD2, WFDC3, ELF3, TNS4, SLCO4A1-AS1, LYPD3, PCDH1, CTD-2587H24.5, MYO15B, KLF6, AKR1C2, CLDN4, IL1B, RHOB, WNT9A, TSPAN1, NDRG1, AHNAK2, OSBP2, TMC7, TSC22D3, RP11-443P15.2, CDKN1C, SERPINB2, ADRA1B, BMP6, GJB3, HAS2, CYP27C1, ACE, GALNT9, DSC2, TRAF1, RP11-96B2.1, SNAI2, BTNL8, MMP1, ATP2B4, MAST4, RP5-1063M23.2, C11orf96, RP11-757F18.5, NGEF, SH3RF2, ABHD11-AS1, IL32, KLF2, KDR, CNN1, TCF7, HCN1, GHRL, ZRANB2-AS2, MIR600HG, MAGI2, CACNG8, AC004941.5, AC004941.5, PDE3B, ZNF385D, KCNJ15, WT1-AS, LRRC17, SCN5A, CYP24A1, EFEMP1, KCNK2, HIGD1AP1, IL7R, ST18, APLN, ANKRD1, VDR, RNF152, and THBS1

[0026] FIG. 2: Heatmap of differentially expressed genes, illustrating that fluvastatin significantly affected the expression of genes previously shown to be involved in metastasis.

DETAILED DESCRIPTION

[0027] It has surprisingly been discovered that a melanoma patient's medication history, and particularly their history of taking an inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCoA reductase, or HMGCR), can serve as a predictor of lymph node status, likelihood of future metastasis, and overall prognosis.

[0028] Provided herein in a first embodiment is a method including: determining whether a subject with a primary melanoma has taken a statin, and classifying the subject as: having a relatively lower risk of sentinel lymph node metastasis and/or a relatively positive prognosis if the subject has taken a statin; and having a relatively higher risk of sentinel lymph node metastasis and/or a relatively negative prognosis if the subject has not taken a statin. Optionally, one or more additional factors or clinicopathological measurements are also considered, such as one or more of Breslow depth of the primary melanoma tumor, and/or ulceration status of the primary melanoma tumor of the subject, and/or age of the subject, in classifying the subject. Further, the method may include considering at least one additional measure or marker of melanoma disease state, disease severity, or disease progression in classifying the subject.

[0029] In examples of these embodiments, classifying the subject further includes analyzing a genetic expression profile (GEP) of the primary melanoma. For instance, the GEP in some cases includes measurement of the expression level of one or more genes selected from: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6. Alternatively, the GEP includes measurement of the expression level of: at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least 10, at least 15, at least 20, at least 25, or at least 30 genes selected from GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6. Further, the GEP in some instances includes measurement of the expression level of all of the following genes: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0030] In additional embodiments, classifying the subject further includes analyzing a GEP of the primary melanoma that includes measurement of the expression level of any one or more of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI; or that includes measurement of the expression level of each of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI. Also contemplated are methods, wherein the GEP includes measurement of the expression level of any or more of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6; or wherein GEP includes measurement of the expression level of each of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0031] In any of these method embodiments, classifying the subject may be used to determining a treatment and/or diagnostic work-up schedule for the subject.

[0032] In any of these method embodiments, the statin can include at least one of fluvastatin, pitavastatin, atorvastatin, simvastatin, lovastatin, rosuvastatin, or pravastatin.

[0033] In any of these method embodiments, the gene expression levels are measured using one or more of polymerase chain reaction (PCR), real-rime polymerase chain reaction (RT-PCR), direct DNA expression in microarray, Sanger sequencing analysis, Northern blot, direct RNA expression detection, serial analysis of gene expression (SAGE), or next-generation RNA-sequencing. In certain preferred embodiments, the gene expression levels are measured using RT-PCR.

[0034] Another provided embodiment is a method for assessing pre-test (that is, prior to carrying out the biopsy) probability of a positive sentinel lymph node biopsy (SLNB) for a subject with cutaneous melanoma, including: determining whether a subject has taken a statin, and classifying the subject as: having a relatively lower risk of a positive SLNB if the subject has taken a statin; and having a relatively higher risk of positive SLNB if the subject has not taken a statin. Optionally, one or more additional factors or clinicopathological measurements are also considered, such as one or more of Breslow depth of the primary melanoma tumor, and/or ulceration status of the primary melanoma tumor of the subject, and/or age of the subject, in classifying the subject. Further, the method may include considering at least one additional measure or marker of melanoma disease state, disease severity, or disease progression in classifying the subject.

[0035] In examples of these embodiments, the method further includes analyzing a genetic expression profile (GEP) of the primary melanoma. For instance, the GEP in some cases includes measurement of the expression level of one or more genes selected from: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6. Alternatively, the GEP includes measurement of the expression level of: at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least 10, at least 15, at least 20, at least 25, or at least 30 genes selected from GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6. Further, the GEP in some instances includes measurement of the expression level of all of the following genes: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0036] In additional embodiments, the method further includes analyzing a GEP of the primary melanoma that includes measurement of the expression level of any one or more of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI; or that includes measurement of the expression level of each of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI. Also contemplated are methods, wherein the GEP includes measurement of the expression level of any or more of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6; or wherein GEP includes measurement of the expression level of each of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0037] In any of these method embodiments, classifying the subject may be used to determining a treatment and/or diagnostic work-up schedule for the subject.

[0038] In any of these method embodiments, the statin can include at least one of fluvastatin, pitavastatin, atorvastatin, simvastatin, lovastatin, rosuvastatin, or pravastatin.

[0039] In any of these method embodiments, the gene expression levels are measured using one or more of polymerase chain reaction (PCR), real-rime polymerase chain reaction (RT-PCR), direct DNA expression in microarray, Sanger sequencing analysis, Northern blot, direct RNA expression detection, serial analysis of gene expression (SAGE), or next-generation RNA-sequencing. In certain preferred embodiments, the gene expression levels are measured using RT-PCR.

[0040] Also provided are improved methods of classifying a subject with melanoma, the improvement including taking into account in the classifying whether the subject has a history of taking a statin, wherein: a history of taking a statin classifies the subject as having one or more of: a relatively lower risk of a positive sentinel lymph node biopsy, a relatively low risk of sentinel lymph node metastasis, and a relatively good prognosis; and no history of taking a statin classifies the subject as having one or more of: a relatively higher risk of positive sentinel lymph node biopsy, a relatively high risk of sentinel lymph node metastasis, and a relatively poor prognosis.

[0041] One embodiment is a method for assessing the pre-test probability of a positive sentinel lymph node biopsy (SLNB) and/or prognosis for a patient with cutaneous melanoma by considering whether the individual has a history of statin use. Optionally, the method involves considering at least one additional measure or marker of melanoma disease state, disease severity, or disease progression. For instance, in an embodiment, the method

includes considering whether the individual has a history of statin use, the Breslow depth and ulceration status of the primary melanoma tumor, and the age of the patient.

[0042] In another embodiment, the method for assessing the pre-test probability of a positive SLNB and prognosis for a patient with cutaneous melanoma involves considering the patient's history of statin use and a genetic expression profile (GEP) including one or more markers in the primary melanoma tumor. For instance, the GEP of the primary tumor may include measurement of the expression level of any one or more of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI. In another embodiment, the GEP of the primary tumor includes measurement of the expression level of any or more of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6. Optionally, the Breslow depth and ulceration status of the primary tumor and the age of the patient are also considered. Additional clinicopathological features or measurements, such as those known to one of ordinary skill in the art for the diagnosis or staging of melanoma, may also be considered.

[0043] Analysis of clinicopathologic factors and genetic expression levels for estimating the probability of a positive SLNB may be accomplished by any computational method known or developed in the art, including radial basis machine and/or partition tree analysis, LRA, K-nearest neighbor, or other algorithmic approaches.

[0044] The data displayed in Table 1, and discussed in the Example 1 as well as in Yu et al. (*J Invest Dermatol* 2021. doi:10.0116/j.jid.2020.12.015), support embodiments provided herein. As shown, patients with a history of taking statins had a 34% relative risk reduction (12.9% absolute risk reduction) for regional or distant metastasis at the time of melanoma diagnosis (24.7% taking statins vs 37.6% not taking statins, p=0.038). This result remained significant in multivariate analysis, after controlling for age, Breslow depth, ulceration, and mitotic rate (p=0.016, Table 1). Importantly, patients taking statins had thicker primary melanomas with higher mitotic count, implying that history of statin use is a stronger predictor than traditional risk factors such as age, Breslow depth, and ulceration status.

[0045] Table 1. Multivariate analysis for factors that predispose to metastasis at initial workup.

Factors	**Estimate**	**Standard error**	**p-value**
Age (days)	-7.631×10^{-6}	1.163×10^{-5}	0.5118
Depth of melanoma (mm)	0.1054	0.05044	0.037
Ulceration present	0.2682	0.2742	0.328
Dermal mitotic rate	0.02826	0.03572	0.429
Taking statin	-0.7580	0.3156	0.016

[0046] The gene expression signatures useful in the provided methods may include two or more of the genes listed in Table 10. For instance, a gene expression signature may include those genes listed in Table 2, Table 3, Table 4, Table 4, or any combination thereof. The directionality of change of gene expression level that is indicative of a high-risk for metastatic melanoma is provided in Table 10, as well as each of Tables 2-5.

[0047] Aspects of the current disclosure are now described with additional details and options as follows: (I) Melanoma Identification and Staging; (II) Representative Definitions of Terms; (III) Statin Treatments; (IV) Gene Expression Profile/Profiling; (V) Optional Additional Clinicopathological Characteristics; (VI) Exemplary Embodiments; (VII) Experimental Examples; and (VIII) Closing Paragraphs. These headings do not limit the interpretation of the disclosure and are provided for organizational purposes only.

I. Melanoma Detection and Staging

[0048] Visual diagnosis of melanomas is the most common method employed by health professionals. Moles that are irregular in color or shape are often treated as candidates of melanoma. The diagnosis of melanoma requires experience, as early stages may look identical to harmless moles or not have any color at all. People with a personal or family history of skin cancer or of dysplastic nevus syndrome (multiple atypical moles) should see a dermatologist at least once a year to be sure they are not developing melanoma. Metastatic melanomas have been detected by X-rays, CT scans, MRIs, PET and PET/CTs, ultrasound, LDH testing, and photoacoustic detection.

[0049] Many melanomas present themselves as lesions smaller than 6 mm in diameter; and all melanomas were malignant on day 1 of growth, which is merely a dot. An astute physician will examine all abnormal moles, including ones less than 6 mm in diameter. Seborrheic keratosis may meet some or all of the identification criteria, and can lead to false alarms among laypeople and sometimes even physicians. An experienced doctor can generally distinguish seborrheic keratosis from melanoma upon examination, or with dermatoscopy.

[0050] Total body photography, which involves photographic documentation of as much body surface as possible, is often used during follow-up of high-risk patients. The technique has been reported to enable early detection and provides a cost-effective approach (being possible with the use of any digital camera), but its efficacy has been questioned due to its inability to detect macroscopic changes. The diagnosis method should be used in conjunction with (and not as a replacement for) dermatoscopic imaging, with a combination of both methods appearing to give extremely high rates of detection.

[0051] Melanoma is divided into the following types: Lentigo maligna melanoma, Superficial spreading melanoma, Acral lentiginous melanoma, Mucosal melanoma, Nodular melanoma,

Polypoid melanoma, Desmoplastic melanoma, Amelanotic melanoma, Soft-tissue melanoma, Melanoma with small nevus-like cells, Melanoma with features of a Spitz nevus Uveal melanoma. Confirmation of a clinical diagnosis is achieved with a skin biopsy. This is usually followed up with a wider excision of the scar or tumor.

[0052] Melanoma stages (see below) depend on the thickness of the tumor, whether cancer has spread to lymph nodes or other parts of the body, and other factors (such as ulceration, . Depending on the stage, a sentinel lymph node biopsy is done.

Stage	Description / Subtypes	5 year survival rate
0	**Melanoma *in situ* (Clark Level I)**	99.9%
I/II	**Invasive melanoma**	85-99%
	T1a: Less than 1.00 mm primary tumor thickness, without ulceration, and mitosis <1/mm^2 T1b: Less than 1.00 mm primary tumor thickness, with ulceration or mitoses ≧1/ mm^2 T2a: 1.00-2.00 mm primary tumor thickness, without ulceration	
II	**High risk melanoma**	40-85%
	T2b: 1.00-2.00 mm primary tumor thickness, with ulceration T3a: 2.00-4.00 mm primary tumor thickness, without ulceration T3b: 2.00-4.00 mm primary tumor thickness, with ulceration T4a: 4.00 mm or greater primary tumor thickness without ulceration T4b: 4.00 mm or greater primary tumor thickness with ulceration	
III	**Regional metastasis**	25-60%
	N1: Single positive lymph node N2: Two to three positive lymph nodes or regional skin/in-transit metastasis N3: Four positive lymph nodes or one lymph node and regional skin/in-transit metastases	
IV	**Distant metastasis**	9-15%
	M1a: Distant skin metastasis, normal LDH M1b: Lung metastasis, normal LDH M1c: Other distant metastasis or any distant metastasis with elevated LDH	

II. Representative Definitions of Terms

[0053] As used herein, the phrase “altered in a predictive manner” means changes in genetic expression profile that predict metastatic risk.

[0054] As used herein, “metastasis” is defined as the recurrence or disease progression that may occur locally (such as local recurrence and in transit disease), regionally (such as nodal micrometastasis or macrometastasis), or distally (such as brain, lung and other tissues).

[0055] The phrase "sequence detection system" refers to any computation method that is used to analyze the results of a PCR or other nucleic acid amplification reaction. Gene expression may be analyzed by direct DNA expression in microarray, Sanger sequencing analysis, Northern blot, Nanostring® technology, serial analysis of gene expression (SAGE), RNA-seq, tissue microarray, or protein expression with immunohistochemistry or western blot technique.

[0056] A "reference training set" as the phrase is used herein is a clinical cohort of cutaneous melanoma tumors with known metastatic outcome and known genetic expression profile used as a reference to compare other cutaneous melanomas and assign them as high or low risk for metastasis. Analysis of genetic expression and comparison to this reference set may be accomplished by any computational method in the art radial basis machine and/or partition tree analysis, LRA, K-nearest neighbor, or other algorithmic approaches.

[0057] As the phrase is used herein, a "high-risk melanoma" is defined by a characteristic gene expression pattern (or profile). This gene expression signature may consist of any combination of the genes listed in Table X. For instance, in embodiments the gene expression panel includes of the genes in Table 2. In another embodiment, the gene expression panel includes the genes in Table 3. In additional embodiments, the gene expression panel includes the genes in Table 4. In additional embodiments, the gene expression panel includes the genes in Table 5. The direction of gene expression (decreased or increased) that would predict a melanoma is high risk is noted in Tables 2-5. The assignment of "high-risk" may be made by comparing the gene expression level of the gene(s) in the melanoma in question to the level of the gene(s) in a reference training set of melanomas. The directionality of gene expression change (increased or decreased) that indicates high-risk for melanoma metastasis is provided.

[0058] The term subject (interchangeable with "individual) is intended to mean a living multicellular vertebrate organism, a category that includes, for example, mammals and birds. A mammal includes human as well as non-human mammals, such as mice. In some instances, a subject is a patient, such as a patient diagnosed with melanoma. In other examples, a subject is a patient yet to be diagnosed.

III. Statin Treatments

[0059] As described herein, it has been discovered that a history of statin treatment can impact or influence the lymph node status, disease progression, likelihood of metastasis, and/or overall prognosis of a subject with primary melanoma. In general, the statin treatment contemplated is conventional for therapeutic inhibition of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCoA reductase, or HMGCR).

[0060] The statin compounds administered to a subject in need thereof may be administered in any appropriate fashion, for instance directed by a medical professional based upon the individual subject's age, weight, medical condition, and other medications or treatments received prior to or concurrent with the statin administrations in question. The subject may receive the statin in question through any route of administration determined by a medical professional. In some embodiments, the statin in question is administered orally.

[0061] For instance, the statin is administered to the subject (*e.g.*, a mammal, such as a human), for instance a human subject or patient, in a "therapeutically effective amount" or "pharmaceutically effective amount," which refers to an amount that is sufficient to effect treatment, as defined herein, when administered to a subject in need of such treatment.

[0062] The terms "treat," "treatment" or "treating," as used herein, refer to an approach for obtaining beneficial or desired results including clinical results. Beneficial or desired clinical results may include one or more of the following: (i) inhibiting the disease or condition (*e.g.*, decreasing one or more symptoms resulting from the disease or condition, such as a high-risk primary melanoma, and/or diminishing the extent of the disease or condition); (ii) slowing or arresting the development of one or more clinical symptoms associated with the disease or condition (*e.g.*, stabilizing the disease or condition, preventing or delaying the worsening or progression of the disease or condition, and/or preventing or delaying the spread (*e.g.*, metastasis) of the disease or condition); and/or (iii) relieving the disease, that is, causing the regression of clinical symptoms (*e.g.*, ameliorating the disease state, providing partial or total remission of the disease or condition, enhancing effect of another medication, delaying the progression of the disease, increasing the quality of life, and/or prolonging survival).

[0063] Based on the discoveries described herein, it is also contemplated that a "beneficial or desired clinical result" may be felt in a subject for a disease or condition that is different from the one for which the statin is or was primarily prescribed. Thus, a subject who is (or was) prescribed a statin in order to lower cholesterol and/or protect against heart attack and/or stroke may also receive from the same statin a beneficial and desirable clinical result including one or more of lowered risk of melanoma metastasis, improved melanoma prognosis, reduced likelihood of spread of the melanoma to a lymph node, lower likelihood of positive SLNB, and so forth.

[0064] Fluvastatin, available commercially under the tradenames LESCOL® and LESCOL XL®, may be administered to a subject in need thereof at a daily dose of from 1 mg to 100 mg. In separate embodiments, the fluvastatin may be administered to the subject at, respectively, 1 mg/day, 5 mg/day, 10 mg/day, 20 mg/day, 25 mg/day, 30 mg/day, 40 mg/day, 50 mg/day, 60 mg/day, 70 mg/day, 80 mg/day, 90 mg/day, and 100 mg/day.

[0065] Pitavastatin, commercially available under the LIVALO® tradename, may be administered to a subject in need thereof at a daily dose of from 0.1 mg to 10 mg. In some

embodiments, the pitavastatin may be administered to the subject in need thereof at a daily dose of from 0.1 mg to 5 mg per day. In other embodiments, pitavastatin may be administered at a dose of from 1 mg/day to 5 mg/day. Individual doses in separate embodiments may be selected from the group of 1 mg/day, 1.5 mg/day, 2 mg/day, 2.5 mg/day, 3 mg/day, 3.5 mg/day, 4 mg/day, 4.5 mg/day, and 5 mg/day.

[0066] Atorvastatin, commercially available as atorvastatin calcium under the LIPITOR® tradename, may be administered to a subject in need thereof at a daily dose of from 1 mg to 100 mg. In separate embodiments, the atorvastatin may be administered to the subject at, respectively, 5 mg/day, 10 mg/day, 20 mg/day, 25 mg/day, 30 mg/day, 40 mg/day, 50 mg/day, 60 mg/day, 70 mg/day, 80 mg/day, 90 mg/day, and 100 mg/day.

[0067] Simvastatin, commercially available under the ZOCOR® tradename, may be administered to a subject in need thereof at a daily dose of from 1 mg to 100 mg. In separate embodiments, the simvastatin may be administered to the subject at, respectively, 5 mg/day, 10 mg/day, 20 mg/day, 25 mg/day, 30 mg/day, 40 mg/day, 50 mg/day, 60 mg/day, 70 mg/day, 80 mg/day, 90 mg/day, and 100 mg/day.

[0068] Lovastatin, commercially available under the MEVACOR® AND ALSOPREV® tradenames, may be administered to a subject in need thereof at a daily dose of from 1 mg to 100 mg. In separate embodiments, the lovastatin is administered to the subject at, respectively, 5 mg/day, 10 mg/day, 20 mg/day, 25 mg/day, 30 mg/day, 40 mg/day, 50 mg/day, 60 mg/day, 70 mg/day, 80 mg/day, 90 mg/day, and 100 mg/day.

[0069] Rosuvastatin, commercially available under the CRESTOR® AND EZALLOR SPRINKLE® tradenames, may be administered to a subject in need at a daily dose of from 1 mg to 100 mg. In separate embodiments, the rosuvastatin may be administered to the subject at, respectively, 5 mg/day, 10 mg/day, 20 mg/day, 25 mg/day, 30 mg/day, 40 mg/day, 50 mg/day, 60 mg/day, 70 mg/day, 80 mg/day, 90 mg/day, and 100 mg/day.

[0070] Pravastatin, commercially available under the PRAVACHOL® tradename, may be administered to a subject in need thereof at a daily dose of from 1 mg to 100 mg. In separate embodiments, the pravastatin may be administered to the subject at, respectively, 5 mg/day, 10 mg/day, 20 mg/day, 25 mg/day, 30 mg/day, 40 mg/day, 50 mg/day.

[0071] Although exemplified herein with subjects who were taking a statin at the time of biopsy of their primary melanoma tumor, it is also believed that prior statin treatment that has ceased before the biopsy is taken would influence (at least for a time) the subject's gene expression. Thus, the statin-related effect continues for a period of time after the subject has stopped taking the statin. It is not required that the subject be actively taking a statin at the time their melanoma is identified or diagnosed, or at the time a tissue biopsy is taken, an analysis of their disease state is carried out, at prognosis, or when any other melanoma-based analysis takes place. Broadly, therefore, the phrase "a subject with history of taking a

statin" is intended to include a subject who is currently taking a statin and has done so for at least a week or more, as well as a subject who was taking a statin for a period of time but who stopped doing so before the selected time point. By way of example, the subject may have taken a statin until a day before the selected time point, or a week before, or two weeks before, or a month before, or two months before, or longer. In exemplary embodiments, a "subject with a history of taking a statin" is one who took statin(s) for at least six months, or at least a year, and is still taking a stating at the time of diagnosis with melanoma.

[0072] The "history of taking a statin" status of an individual can be determined in any conventional way, including: review of the individual's medical history or files; asking the individual; consulting with a physician or other medical professional who has treated or is treating the individual (for instance, a medical professional who is treating the individual for their general health, or their cardiac or cardiovascular health, even if that professional is not treating the individual for melanoma); and analyzing blood or another sample from the individual for alterations caused by having taken statins (e.g., changes in cholesterol and/or triglycerides), or the presence of a statin or statin degradation product.

IV. Gene Expression Profile/Profiling

[0073] In various embodiments, determination of whether a subject has a history of taking a statin is combined with the results from a gene expression profile (GEP) from the subject's primary melanoma tumor, in order to carry out an analysis or provide a classification of the subject.

[0074] There are art-recognized systems and methods, useful with the current disclosure, for obtaining cancer tissue samples (*e.g.*, tissue form a primary melanoma tumor, such as a primary cutaneous melanoma), analyzing such samples for the expression level of target gene(s), and/or the methylation level of target gene(s) (as a stand-in or indirect measure of gene expression levels, or a supplement thereto), assembling or producing gene expression profile(s) that contain or include the level of expression (or of methylation) of two or more genes expressed from the melanoma tissue sample, preparing reference sample sets and reference set gene expression profiles, and related methods. See, for instance, U.S. Patents No. 9,410,205, 10,577,660 and 10,233,502; U.S. Application Publication No. US20200362419A1 and International Patent Publication WO2020022895A2. Representative methods are also described herein.

[0075] Genetic expression can refer to whether a cell (i) has a particular sequence of a gene, and/or (ii) whether the cell is expressing the gene and/or (iii) whether the protein produced is maintained/stable in the cell or system. Through the process of transcription, a cell expressing a gene generates RNA sequences corresponding to that gene. Accordingly, in various embodiments, expression of a particular gene from a tumor is identified based on

the presence and/or amount of RNA sequence(s) in the tumor or a sample from the tumor. Particular embodiments of the present disclosure include identifying the presence and/or amount of particular RNA sequence(s) corresponding to the set of genetic biomarkers. Various methods and systems described herein involve RNA (transcriptome) analysis. It will be understood that the isolation, detection, and quantification of RNA, including specific RNA corresponding to a specific target gene (such as any or all of the genes that are included in a detection model provided herein), can be carried out using any art-recognized methods. These include for instance, array-based detection methods as well as sequencing.

[0076] Methods for analyzing gene expression include methods based on hybridization analysis of polynucleotides, sequencing of polynucleotides, and analysis of protein expression (e.g., proteomics-based methods). Commonly used methods for the quantification of mRNA expression in a sample include northern blotting and in situ hybridization (Parker & Barnes, *Meth Mol Biol* 106:247-283, 1999); RNAse protection assays (Hod, *Biotechniques* 13:852 854, 1992); and PCR-based methods, such as reverse transcription polymerase chain reaction (RT-PCR) (Weis et al., *Trends in Genetics* 8:263 264, 1992). Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. Representative methods for sequencing-based gene expression analysis include Serial Analysis of Gene Expression (SAGE), and gene expression analysis by massively parallel signature sequencing (MPSS).

[0077] Evaluating gene expression of a melanoma sample can be performed with microarrays. Microarrays permit simultaneous analysis of a large number of gene expression products. Typically, polynucleotides of interest are plated, or arrayed, on a microchip substrate. The arrayed sequences are then hybridized with nucleic acids (e.g., DNA or RNA) from cells or tissues of interest (e.g., cutaneous tissue samples). The source of mRNA typically is total RNA (e.g., total RNA isolated from human melanoma samples, and normal skin samples). If the source of mRNA is a primary tumor, mRNA can be extracted, for example, from frozen or archived paraffin-embedded and fixed (e.g. formalin-fixed) tissue samples.

[0078] In various embodiments of the microarray technique, probes to (e.g., specific for) at least 2, 6, 10, 25, 50, 100, 150, 200, or more genes are immobilized on an array substrate (e.g., a porous or nonporous solid support, such as a glass, plastic, or gel surface). The probes can include DNA, RNA, copolymer sequences of DNA and RNA, DNA and/or RNA analogues, or combinations thereof.

[0079] In some embodiments, a microarray includes a support with an ordered array of binding (e.g., hybridization) sites for each individual gene. The microarrays can be addressable arrays, for instance positionally addressable arrays, i.e., each probe of the array

is located at a known, predetermined position on the solid support such that the identity (i.e., the sequence) of each probe can be determined from its position in the array.

[0080] Each probe on the microarray can be between 10-50,000 nucleotides, e.g., between 300-1,000 nucleotides, in length. The probes of the microarray can consist of nucleotide sequences with lengths: less than 1,000 nucleotides, e.g., sequences 10-1,000, or 10-500, or 10-200 nucleotides in length. An array can include positive control probes, e.g., probes known to be complementary and hybridizable to sequences in the test sample; and negative control probes, e.g., probes known to not be complementary and hybridizable to sequences in the test sample.

[0081] Methods for attaching nucleic acids to a surface are known. Methods for immobilizing nucleic acids on glass have been described (Schena et al, *Science* 270:467-470, 1995; DeRisi et al, *Nature Genetics* 14:457-460, 1996; Shalon et al., *Genome Res.* 6:639-645, 1996; and Schena et al., *Proc. Natl. Acad. Sci. U.S.A.* 93:10539-11286, 1995). Techniques are known for producing arrays with thousands of oligonucleotides at defined locations using photolithographic techniques are described by Fodor et al. (*Science* 251:767-773, 1991), Pease et al. (*Proc. Natl. Acad. Sci. U.S.A.* 91:5022-5026, 1994), Lockhart et al. (*Nature Biotechnology* 14:1675, 1996), and in U.S. Patents No. 5,578,832; 5,556,752; and 5,510,270. Other methods for making microarrays have been described. See, e.g., Maskos and Southern, *Nuc. Acids. Res.* 20:1679-1, 684, 1992. In principle, any type of array, for example, dot blots on a nylon hybridization membrane (see Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd Ed., Vols. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989)) could be used.

[0082] The polynucleotide molecules to be analyzed using a microarray may be from any clinically relevant source (such as from a portion of a melanoma tissue biopsy), and are expressed RNA or a nucleic acid derived therefrom (e.g., cDNA or amplified RNA derived from cDNA that incorporates an RNA polymerase promoter), including naturally occurring nucleic acid molecules, as well as synthetic nucleic acid molecules. For example, the test polynucleotide molecules include total cellular RNA, poly(A)+ messenger RNA (mRNA), or fraction thereof, cytoplasmic mRNA, or RNA transcribed from cDNA (i.e., cRNA). Methods for preparing RNA are known and are described, e.g., in Sambrook et al., Molecular Cloning, A Laboratory Manual (2Supnd /SupEd.), Vols. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989. RNA can be fragmented by methods known in the art, e.g., by incubation with $ZnCl_2$, to generate fragments of RNA.

[0083] Test polynucleotide molecules that are poorly expressed in particular cells can be enriched using normalization techniques (Bonaldo et al., *Genome Res.* 6:791-806, 1996).

[0084] The test polynucleotides may be detectably labeled at one or more nucleotides. Any method known in the art may be used to detectably label the polynucleotides.

[0085] Nucleic acid hybridization and wash conditions are chosen so that the test polynucleotide molecules specifically bind or specifically hybridize to the complementary polynucleotide sequences of the array, preferably to a specific array site, wherein its complementary nucleic acid is located. General parameters for specific (i.e., stringent) hybridization conditions for nucleic acids are described in Sambrook et al., supra, and in Ausubel et al., Current Protocols in Molecular Biology, vol. 2, Current Protocols Publishing, New York, 1994. Typically, stringent conditions for short probes (e.g., 10 to 50 nucleotide bases) will be those in which the salt concentration is at least about 0.01 to 1.0 M at pH 7.0 to 8.3 and the temperature is at least about 30° C. Stringent conditions can also be achieved with the addition of destabilizing agents such as formamide. When fluorescently labeled probes are used, the fluorescence emissions at each site of a microarray can be detected by scanning confocal laser microscopy or other methods (see Shalon et al., *Genome Research* 6:639-645, 1996; Schena et al., *Genome Res.* 6:639-645, 1996; and Ferguson et al., N*ature Biotech.* 14:1681-1684, 1996). Signals are recorded and typically analyzed by computer. Methods for evaluating microarray data and classifying samples are described in U.S. Pat. No. 7,171,311.

[0086] Gene expression profiles can also be determined using PCR. PCR is useful to amplify and detect transcripts from a melanoma sample. Various PCR methodologies are useful for gene expression analyses.

[0087] *Reverse Transcriptase PCR (RT-PCR):* RT-PCR is a sensitive quantitative method that can be used to compare mRNA levels in different samples to examine gene expression signatures. To perform RT-PCR, mRNA is isolated from a. mRNA can be extracted, for example, from frozen or archived paraffin-embedded and fixed (e.g. formalin-fixed) tissue samples. Methods for mRNA extraction are known in the art. See, e.g., Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, 1997. Methods for RNA extraction from paraffin embedded tissues are disclosed, for example, in Rupp and Locker, *Lab Invest.* 56:A67, 1987, and De Andres et al., *BioTechniques* 18:42044, 1995. Purification kits for RNA isolation from commercial manufacturers, such as Qiagen, can be used. For example, total RNA from a sample can be isolated using Qiagen RNeasy mini-columns. Other commercially available RNA isolation kits include MasterPure™. Complete DNA and RNA Purification Kit (EPICENTRE™, Madison, Wis.), and, Paraffin Block RNA Isolation Kit (Ambion, Inc.). Total RNA from tissue samples can be also isolated using RNA Stat-60 (Tel-Test) or by cesium chloride density gradient centrifugation.

[0088] Isolated RNA is reverse transcribed into cDNA. The cDNA is amplified in a PCR reaction. Two commonly used reverse transcriptases are avian myeloblastosis virus reverse transcriptase (AMV-RT) and Moloney murine leukemia virus reverse transcriptase (MMLV-RT). The reverse transcription step is typically primed using specific primers, random

hexamers, or oligo-dT primers, depending on the conditions and desired readout. For example, extracted RNA can be reverse-transcribed using a GeneAmp RNA PCR kit (Perkin Elmer, Calif., USA), following the manufacturer's instructions. The derived cDNA can then be used as a template in the subsequent PCR reaction. The PCR reaction typically employs the Taq DNA polymerase, which has a 5′-3′ nuclease activity but lacks a 3′-5′ proofreading endonuclease-activity. Two oligonucleotide primers are used to generate an amplicon in the PCR reaction.

[0089] Guidelines for PCR primer and probe design are described, e.g., in Dieffenbach et al., “General Concepts for PCR Primer Design” in: PCR Primer, A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 133-155, 1995; Innis and Gelfand, “Optimization of PCRs” in: PCR Protocols, A Guide to Methods and Applications, CRC Press, London, 5-11, 1994; and Plasterer, T. N. Primerselect: Primer and probe design. Methods Mol. Biol. 70:520-527, 1997. Factors considered in PCR primer design include primer length, melting temperature (T_m), and G/C content, specificity, complementary primer sequences, and 3′-end sequence. PCR primers are generally 17-30 bases in length, and Tm's between 50-80° C., e.g. about 50 to 70° C. are typically preferred.

[0090] For quantitative PCR, a third oligonucleotide, or probe, is used to detect nucleotide sequence located between the two PCR primers. The probe is non-extendible by Taq DNA polymerase enzyme, and typically is labeled with a reporter fluorescent dye and a quencher fluorescent dye. Any laser-induced emission from the reporter dye is quenched by the quenching dye when the two dyes are located close together as they are on the probe. During the amplification reaction, the Taq DNA polymerase enzyme cleaves the probe in a template-dependent manner. The resultant probe fragments disassociate in solution, and signal from the released reporter dye is free from the quenching effect of the second fluorophore. One molecule of reporter dye is liberated for each new molecule synthesized, and detection of the unquenched reporter dye provides the basis for quantitative analysis.

[0091] RT-PCR can be performed using commercially available equipment, such as an ABI PRISM 7700™ Sequence Detection System (Perkin-Elmer-Applied Biosystems, Foster City, Calif., USA), or Lightcycler®. (Roche Molecular Biochemicals, Mannheim, Germany). Samples can be analyzed using a real-time quantitative PCR device such as the ABI PRISM 7700™ Sequence Detection System™.

[0092] To minimize errors and the effect of sample-to-sample variation, RT-PCR is usually performed using an internal standard. A suitable internal standard is expressed at a constant level among different tissues, and is unaffected by the experimental variable. RNAs frequently used to normalize patterns of gene expression are mRNAs for the housekeeping genes glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and β-actin.

[0093] A variation of the RT-PCR technique is real time quantitative PCR, which measures PCR product accumulation through a dual-labeled fluorogenic probe (i.e., TaqMan™ probe). Real time PCR is compatible both with quantitative competitive PCR, where internal competitor for each target sequence is used for normalization, and with quantitative comparative PCR using a normalization gene contained within the sample, or a housekeeping gene for RT-PCR. For further details see, e.g. Held et al., *Genome Res.* 6:986-994, 1996.

[0094] Gene expression can be examined using fixed, paraffin-embedded tissues as the RNA source. Briefly, in one exemplary method, sections of paraffin-embedded melanoma tumor tissue samples are cut (~10 µm thick). RNA is extracted, and protein and DNA are removed. After analysis of the RNA concentration, RNA repair and/or amplification steps may be performed, if necessary, and RNA is reverse transcribed using gene specific promoters followed by RT-PCR. Methods of examining expression in fixed, paraffin-embedded tissues, are described, for example, in Godfrey et al. (*J Molec. Diagn*. 2: 84-91, 2000) and Specht et. al. (*Am. J. Pathol*. 158: 419-29, 2001).

[0095] Another approach for gene expression analysis employs competitive PCR design and automated, high-throughput matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) detection and quantification of oligonucleotides. This method is described by Ding and Cantor (*Proc. Natl. Acad. Sci. U.S.A.* 100:3059-3064, 2003). See also the MassARRAY-based gene expression profiling method, developed by Sequenom, Inc. (San Diego, Calif.).

[0096] Additional PCR-based techniques for gene expression analysis include, e.g., differential display (Liang and Pardee, *Science* 257:967-971, 1992); amplified fragment length polymorphism (iAFLP) (Kawamoto et al., *Genome Res*. 12:1305-1312, 1999); BeadArray™ technology (Illumina, San Diego, Calif.; Oliphant et al., Discovery of Markers for Disease (Supplement to Biotechniques), June 2002; Ferguson et al., A*nalytical Chemistry* 72:5618, 2000); BeadsArray for Detection of Gene Expression (BADGE), using the commercially available Luminex100 LabMAP system and multiple color-coded microspheres (Luminex Corp., Austin, Tex.) in a rapid assay for gene expression (Yang et al., *Genome Res.* 11:1888-1898, 2001); and high coverage expression profiling (HiCEP) analysis (Fukumura et al*., Nucl. Acids. Res.* 31(16) e94, 2003).

[0097] *Serial Analysis of Gene Expression (SAGE):* Gene expression in melanoma samples can also be determined by serial analysis of gene expression (SAGE), which is a method that allows the simultaneous and quantitative analysis of a large number of gene transcripts, without the need of providing an individual hybridization probe for each transcript (see, e.g. Velculescu et al., *Science*. 270:484-487, 1995; and Velculescu et al., *Cell* 88:243-51, 1997). Briefly, a short sequence tag (about 10-14 nucleotides) is generated that contains sufficient

information to uniquely identify a transcript, provided that the tag is obtained from a unique position within each transcript. Many transcripts are then linked together to form long serial molecules that can be sequenced, revealing the identity of the multiple tags simultaneously. The expression pattern of a population of transcripts can be quantitatively evaluated by determining the abundance of individual tags, and identifying the gene corresponding to each tag.

[0098] Gene expression assays include measures to correct for differences in RNA variability and quality. For example, an assay typically measures and incorporates the expression of certain normalizing genes, such known housekeeping genes, e.g., GAPDH, β-actin, and Cyp1. Alternatively, normalization can be based on the mean or median signal (Ct) of all of the assayed genes or a large subset thereof (global normalization approach). In some embodiments, a normalized test RNA (e.g., from a patient sample) is compared to the amount found in a metastatic melanoma, non-metastatic melanoma, and/or normal skin sample reference set. The level of expression measured in a particular test sample can be determined to fall at some percentile within a range observed in reference sets.

[0099] *Protein Detection Methodologies*: Immunohistochemical methods are also suitable for detecting the expression of melanoma signature genes such as those described herein. Antibodies, most preferably monoclonal antibodies, specific for a gene product are used to detect expression. The antibodies can be detected by direct labeling of the antibodies themselves, for example, with radioactive labels, fluorescent labels, hapten labels such as, biotin, or an enzyme such as horse radish peroxidase or alkaline phosphatase. Alternatively, unlabeled primary antibody is used in conjunction with a labeled secondary antibody, comprising antisera, polyclonal antisera or a monoclonal antibody specific for the primary antibody. Immunohistochemistry protocols and kits are well known in the art and are commercially available.

[0100] Proteomic methods can allow examination of global changes in protein expression in a sample. Proteomic analysis typically involves separation of individual proteins in a sample by 2-D gel electrophoresis (2-D PAGE), and identification of individual proteins recovered from the gel, e.g. my mass spectrometry or N-terminal sequencing, and analysis of the data using bioinformatics. Proteomics methods can be used alone or in combination with other methods for evaluating gene expression.

[0101] In various aspects, the expression of certain genes in a cutaneous sample is detected to provide clinical information (e.g., prognostic information, classification of the melanoma tumor from which the sample is derived, as a melanoma associated with prolonged or truncated longevity).

[0102] The following Tables provide representative subsets of the genes listed in Table 10, which subsets optionally may be used in gene expression profiles as described herein. The direction of expression change (expression delta, compared to expression in a reference group of melanomas) that indicates high (or relatively higher) risk of melanoma metastasis is indicated. Other subsets are also contemplated, including any five, any eight, any 10, any 15, any 20, any 25, any 30, or more of the genes listed in Table 10, or in any of Tables 2-5 or combinations thereof.

[0103] Table 2.

Gene Symbol	Gene Title	Direction of Expression in High risk Melanoma
BAP1	BRCA1-associated protein-1	Decreased
MGP	Matrix Gla protein	Decreased
SPP1	Secreted phosphoprotein 1	Increased
CXCL14	Chemokine (C-X-C motif) ligand 14	Decreased
CLCA2	Chloride channel accessory 2	Decreased
S100A8	S100 calcium-binding protein A8	Decreased
BTG1	B-cell translocation gene 1, antiproliferative	Decreased
SAP130	Sin3A-associated protein, 130 kDa	Decreased
ARG1	Arginase 1	Decreased
KRT6B	Keratin 6B	Increased
GJA1	Gap junction protein, alpha 1, 43 kDa	Decreased
ID2	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	Decreased
EIF1B	Eukaryotic translation initiation factor 1B	Increased
S100A9	S100 calcium-binding protein A9	Decreased
CRABP2	Cellular retinoic acid binding protein 2	Decreased
KRT14	Keratin 14	Decreased
ROBO1	Roundabout, axon guidance receptor, homolog 1	Decreased
RBM23	RNA-binding motif protein 23	Decreased
TACSTD2	Tumor-associated calcium signal transducer 2	Decreased
DSC1	Desmocollin 1	Decreased
SPRR1B	Small proline-rich protein 1B	Decreased
TRIM29	Tripartite motif containing 29	Decreased
AQP3	Aquaporin 3 (Gill blood group)	Decreased
TYRP1	Tyrosinase-related protein 1	Decreased
PPL	Periplakin	Decreased
LTA4H	Leukotriene A4 hydrolase	Decreased
CST6	Cystatin E/M	Decreased

[0104] Table 3.

Gene Symbol	Gene Title	Direction of Expression in High risk Melanoma
CXCL8	Interleukin 8	Increased
ITGB3	Integrin beta 3	Increased
LAMB1	laminin B1 chain	Increased
PLAT	tissue-type plasminogen activator	Increased
TP53	Cellular tumor antigen p53	Decreased

[0105] Table 4.

Gene Symbol	Gene Title	Direction of Expression in High risk Melanoma
ANGPT1	Angiopoietin 1	Increased
ANGPT2	Angiopoietin 2	Increased
BMP2	Bone morphogenic protein 2	Decreased
FGF2	Fibroblast Growth Factor 2	Increased
S1PR1	Sphingosine 1-phosphate	Increased
TGFB1	Transforming growth factor beta	Decreased
VEGFA	Vascular Endothelial Growth Factor A	Increased
VEGFC	Vascular Endothelial Growth Factor C	Increased
VEGFD	Vascular Endothelial Growth Factor D	Increased
IFNG	Interferon-gamma	Decreased

[0106] Table 5.

Gene Symbol	Gene Title	Direction of Expression in High risk Melanoma
GDF15	Growth Differentiation Factor 15	Increased
MLANA	Melan-A	Increased
PLAT	tissue-type plasminogen activator	Increased
CXCL8 / IL8	Interleukin 8	Increased
ITGB3	Integrin beta 3	Increased
LOXL4	Lysyl oxidase like 4	Increased
PRKCB	protein kinase C-β	Increased
SERPINE2	glia-derived nexin	Increased
ADAM12	ADAM metallopeptidase domain 12	Increased
LGALSl	galectin 1	Increased
TGFBRl	transforming growth factor-β (TGF-β) receptor 1	Increased

V. Optional Additional Clinicopathological Characteristics

[0107] It will be recognized that the improvement in classification of a subject that arises by determining whether they have a history of taking a statin can be combined with additional clinicopathological characterization of the individual, to further refine classification of the

individual, their prognosis, and so forth. Any art-recognized methods for diagnosis, staging, or other characterization of a melanoma patient can therefore be improved by also considering the "history of taking a statin" status of the patient. Thus, this factor can be added to the possible factors that may predispose a subject to metastasis or severe disease, or poor prognosis.

[0108] Additional factors/characteristics may include, but are not limited to, the following: age of the subject, skin color, freckling, family history of cancer (such as skin cancer, and particularly melanoma), history of sun or other UV light exposure (including type and pattern of exposure), history (or family history) of atypical moles (dysplastic nevi), depth of primary melanoma, type or location of primary melanoma, size/surface area of primary melanoma, ulceration, dermal mitotic rate, and genetic analysis for mutations and/or expression changes linked to cancer, melanoma, or metastasis (even if not described herein).

[0109] The Exemplary Embodiments and Experimental Examples below are included to demonstrate particular embodiments of the disclosure. Those of ordinary skill in the art will recognize in light of the present disclosure that many changes can be made to the specific embodiments disclosed herein and still obtain a like or similar result without departing from the spirit and scope of the disclosure.

VI. Exemplary Embodiments

[0110] 1. A method, including: determining whether a subject with a primary melanoma has taken a statin, and classifying the subject as: having a relatively lower risk of sentinel lymph node metastasis and/or a relatively positive prognosis if the subject has taken a statin; and having a relatively higher risk of sentinel lymph node metastasis and/or a relatively negative prognosis if the subject has not taken a statin.

[0111] 2. The method of embodiment 1, further including factoring in Breslow depth and ulceration status of the primary melanoma tumor of the subject in classifying the subject.

[0112] 3. The method of embodiment 1, further including analyzing a genetic expression profile (GEP) of the primary melanoma in classifying the subject.

[0113] 4. The method of embodiment 3, wherein the GEP includes measurement of the expression level of one or more genes selected from: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0114] 5. The method of embodiment 4, wherein the GEP includes measurement of the expression level of: at least two, at least three, at least four, at least five, at least six, at least

seven, at least eight, at least 10, at least 15, at least 20, at least 25, or at least 30 genes selected from GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0115] 6. The method of embodiment 4, wherein the GEP includes measurement of the expression level of all of the following genes: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0116] 7. The method of embodiment 4, wherein the GEP includes measurement of the expression level of any one or more of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI.

[0117] 8. The method of embodiment 7, wherein the GEP includes measurement of the expression level of each of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI.

[0118] 9. The method of embodiment 4, wherein the GEP includes measurement of the expression level of any or more of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0119] 10. The method of embodiment 9, wherein the GEP includes measurement of the expression level of each of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0120] 11. The method of embodiment 1, further including factoring in Breslow depth, ulceration status of the primary melanoma tumor, and a genetic expression profile (GEP) of the primary melanoma tumor in classifying the subject.

[0121] 12. The method of embodiment 11, wherein the GEP includes measurement of the expression level of one or more genes selected from: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0122] 13. The method of embodiment 12, wherein the GEP includes measurement of the expression level of: at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least 10, at least 15, at least 20, at least 25, or at least 30 genes selected from GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0123] 14. The method of embodiment 12, wherein the GEP includes measurement of the expression level of all of the following genes: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0124] 15. The method of embodiment 12, wherein the GEP includes measurement of the expression level of any one or more of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI.

[0125] 16. The method of embodiment 15, wherein the GEP includes measurement of the expression level of each of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI.

[0126] 17. The method of embodiment 12, wherein the GEP includes measurement of the expression level of any or more of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0127] 18. The method of embodiment 17, wherein the GEP includes measurement of the expression level of each of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0128] 19. The method of embodiment 1, wherein classifying the subject is used to determining a treatment and/or diagnostic work-up schedule for the individual.

[0129] 20. The method of embodiment 1, wherein the statin includes at least one of fluvastatin, pitavastatin, atorvastatin, simvastatin, lovastatin, rosuvastatin, or pravastatin.

[0130] 21. The method of embodiment 1, further including considering at least one additional measure or marker of melanoma disease state, disease severity, or disease progression in classifying the subject.

[0131] 22. The method of embodiment 1, further including considering the age of the subject in classifying the subject.

[0132] 23. The method of any one of embodiments 4-10 or embodiment 12-18, wherein the gene expression levels are measured using one or more of polymerase chain reaction (PCR), real-rime polymerase chain reaction (RT-PCR), direct DNA expression in microarray, Sanger sequencing analysis, Northern blot, direct RNA expression detection, serial analysis of gene expression (SAGE), or next-generation RNA-sequencing.

[0133] 24. The method of embodiment 23, wherein the gene expression levels are measured using RT-PCR.

[0134] 25. A method for assessing pre-test probability of a positive sentinel lymph node biopsy (SLNB) for a subject with cutaneous melanoma, including: determining whether a subject has taken a statin, and classifying the subject as: having a relatively lower risk of a positive sentinel lymph node biopsy if the subject has taken a statin; and having a relatively higher risk of positive sentinel lymph node biopsy if the subject has not taken a statin.

[0135] 26. The method of embodiment 25, further including considering at least one additional measure or marker of melanoma disease state, disease severity, or disease progression in classifying the subject.

[0136] 27. The method of embodiment 25, further including factoring in Breslow depth and ulceration status of the primary melanoma tumor of the subject in classifying the subject.

[0137] 28. The method of embodiment 25, further including analyzing a genetic expression profile (GEP) of the primary melanoma in classifying the subject.

[0138] 29. The method of embodiment 28, wherein the GEP includes measurement of the expression level of one or more genes selected from: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0139] 30. The method of embodiment 28, wherein the GEP includes measurement of the expression level of: at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least 10, at least 15, at least 20, at least 25, or at least 30 genes selected from GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0140] 31. The method of embodiment 28, wherein the GEP includes measurement of the expression level of all of the following genes: GDF15, MLANA, PLAT, IL8, ITGB3,

LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0141] 32. The method of embodiment 28, wherein the GEP includes measurement of the expression level of any one or more of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI.

[0142] 33. The method of embodiment 32, wherein the GEP includes measurement of the expression level of each of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI.

[0143] 34. The method of embodiment 28, wherein the GEP includes measurement of the expression level of any or more of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0144] 35. The method of embodiment 34, wherein the GEP includes measurement of the expression level of each of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0145] 36. The method of embodiment 25, further including factoring in Breslow depth, ulceration status of the primary melanoma tumor, and a genetic expression profile (GEP) of the primary melanoma tumor in classifying the subject.

[0146] 37. The method of embodiment 36, wherein the GEP includes measurement of the expression level of one or more genes selected from: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0147] 38. The method of embodiment 37, wherein the GEP includes measurement of the expression level of: at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least 10, at least 15, at least 20, at least 25, or at least 30 genes selected from GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0148] 39. The method of embodiment 37, wherein the GEP includes measurement of the expression level of all of the following genes: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0149] 40. The method of embodiment 37, wherein the GEP includes measurement of the expression level of any one or more of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI.

[0150] 41. The method of embodiment 41, wherein the GEP includes measurement of the expression level of each of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI.

[0151] 42. The method of embodiment 41, wherein the GEP includes measurement of the expression level of any or more of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0152] 43. The method of embodiment 42, wherein the GEP includes measurement of the expression level of each of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

[0153] 44. The method of embodiment 25, wherein classifying the subject is used to determining a treatment and/or diagnostic work-up schedule for the individual.

[0154] 45. The method of embodiment 25, wherein the statin includes at least one of fluvastatin, pitavastatin, atorvastatin, simvastatin, lovastatin, rosuvastatin, or pravastatin.

[0155] 46. The method of embodiment 25, further including considering the age of the subject in classifying the subject.

[0156] 47. The method of any one of embodiments 25-35 or embodiment 37-43, wherein the gene expression levels are measured using one or more of polymerase chain reaction (PCR), real-rime polymerase chain reaction (RT-PCR), direct DNA expression in microarray, Sanger sequencing analysis, Northern blot, direct RNA expression detection, serial analysis of gene expression (SAGE), or next-generation RNA-sequencing.

[0157] 48. The method of embodiment 47, wherein the gene expression levels are measured using RT-PCR.

[0158] 49. An improved method of classifying a subject with melanoma, the improvement including taking into account in the classifying whether the subject has a

history of taking a statin, wherein: a history of taking a statin classifies the subject as having one or more of: a relatively lower risk of a positive sentinel lymph node biopsy, a relatively low risk of sentinel lymph node metastasis, and a relatively good prognosis; and no history of taking a statin classifies the subject as having one or more of: a relatively higher risk of positive sentinel lymph node biopsy, a relatively high risk of sentinel lymph node metastasis, and a relatively poor prognosis.

VII. Experimental Examples

Example 1: Computational Drug Repositioning Identifies Statins as Modifiers of Prognostic Genetic Expression Signatures and Metastatic Behavior in Melanoma

[0159] This example describes the discovery that statin use causes differential expression in genes associated with melanoma metastasis, and that these changes in expression can be used clinically. At least some of the material described in this example was published in Yu et al. (*J Invest. Dermatol*. 141:1802-18-9, 2021; doi:10.1061/j.jid.2020.12.015; published online January 6, 2021).

[0160] Despite advances in melanoma treatment, more than 70% of patients with distant metastasis die within 5 years. Proactive treatment of early melanoma to prevent metastasis could save lives and reduce overall healthcare costs. Currently, there are no treatments specifically designed to prevent early melanoma from progressing to metastasis. The Connectivity Map was used to conduct an in silico drug screen and identified 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitors (statins) as a drug class that might prevent melanoma metastasis. To confirm the *in vitro* effect of statins, RNA sequencing was completed on A375 cells after treatment with fluvastatin to describe changes in the melanoma transcriptome. Statins induced differential expression in genes associated with metastasis and are used in commercially available prognostic tests for melanoma metastasis. Finally, a chart review of 475 patients with melanoma was completed. Patients taking statins were less likely to have metastasis at the time of melanoma diagnosis in both univariate and multivariate analyses (24.7% taking statins vs. 37.6% not taking statins, absolute risk reduction = 12.9%, P = 0.038). These findings suggest that statins might be useful as a treatment to prevent melanoma metastasis. Prospective trials are required to verify these findings and to determine the mechanism of metastasis prevention.

[0161] Metastasis is the primary driver of cancer mortality (Dillekås et al., *Cancer Med* 2019;8:5574e69; Zbytek et al., *Expert Rev Dermatol* 2008;3: 569e85). Despite recent advances in treatment, patients with metastatic melanoma survive on average <2 years after diagnosis (Kandel et al., *Eur J Cancer* 2018;105:33e408; Larkin et al., *N Engl J Med* 2019;381:1535e; Robert et al., *N Engl J Med* 2019;381:626e36). In addition, the cost of treating metastatic disease has increased significantly (Kandel et al., *Eur J Cancer*

2018;105:33). Preventing early cutaneous melanomas from progressing to metastasis may decrease healthcare costs and save lives.

[0162] Melanomas at high risk of metastasis can be identified by their gene expression signature (Gerami et al., *J Am Acad Dermatol* 2015a;72:780e5.e3; Gerami et al., *Clin Cancer Res* 2015b;21:175e83; Kashani-Sabet et al., *Clin Cancer Res* 2017;23:6888e92; Kashani-Sabet et al., *Clin Cancer Res* 2009;15:6987e92; Zager et al., *BMC Cancer* 2018;18:130). Retrospective and prospective studies have identified a 28-gene expression signature as an independent predictor of metastasis (Gerami et al., *J Am Acad Dermatol* 2015a;72:780e5.e3; Gerami et al., *Clin Cancer Res* 2015b;21:175e; Greenhaw et al., *J Am Acad Dermatol* 2020;83:745e53; Zager et al., *BMC Cancer* 2018;18:130). The immediate clinical utility of these tests is controversial, and the functional role of these genes in metastasis remains elusive. However, the fact that these gene signatures identify melanomas with up to 22-fold higher odds of recurrence or metastasis means that they might yield insights into the metastatic process and could even lead to potential therapies (Chan & Tsao, *JAMA Dermatol* 2020;156:949e51; Grossman et al., *JAMA Dermatol* 2020;156:1004e11).

[0163] The Connectivity Map (cMap) is a publicly available database maintained by the Broad Institute that contains microarray gene expression measurements from over 27,000 pharmaceutical compounds (Lamb, *Nat Rev Cancer* 2007;7:54e60; Lamb et al., *Science* 2006;313:1929e35). This database can be queried to identify drugs that induce expression signatures either similar to or opposed to a specified profile. By screening the database for compounds that induce genetic expression patterns directly opposed to a disease signature, the database has been used successfully to identify drugs for computational drug repurposing (the process of discovering new indications for existing drugs) (Chen et al., *Nat Commun* 2017;8:16022; Sirota et al., *Sci Transl Med* 2011;3:96ra77).

[0164] The cMap was used to screen for drugs that reverse a high-risk gene expression profile of melanoma that has been validated in clinical samples (Gerami et al., *J Am Acad Dermatol* 2015a;72:780e5.e3; Greenhaw et al., *Dermatol Surg* 2018;44:1494e500; Zager et al., *BMC Cancer* 2018;18:130). 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) were identified as candidate agents to oppose the high-risk melanoma gene expression profile. Previous studies on statins in melanoma have focused on initiation or primary prevention and have had mixed results. Two large cardiovascular trials demonstrated a reduction in melanoma incidence with statin use, but this effect was not observed in the Women's Health Initiative or two Dutch epidemiologic studies (Jagtap et al., *Cancer* 2012;118:5124e31; Rubins et al., *N Engl J Med* 1999;341:410e8; Splichal et al., *Semin Thromb Hemost* 2003;29: 259e74). Two meta-analyses also demonstrated no reduction in melanoma incidence with statin use, and a randomized controlled trial of

lovastatin for melanoma prevention did not identify any significant decreases in melanocytic atypia or other melanoma initiation markers (Bonovas et al., *Eur J Epidemiol* 2010;25:29e35; Freeman et al., *J Natl Cancer Inst* 2006;98:1538e46; Linden et al., *Cancer Prev Res (Phila)* 2014;7:496e504). Recently, a Mendelian randomization analysis using the UK Biobank demonstrated that individuals with variants in the 3-hydroxy-3-methylglutaryl-coenzyme A reductase region, which represent proxies for statin use, had decreased overall cancer risk but did not reach statistical significance for any site-specific cancers (Carter et al., Elife 2020;9:e57191).

[0165] Although results have been equivocal in melanoma initiation, there is more consistent evidence that statins may prevent melanoma progression and metastasis. Both in vitro and animal models have demonstrated potential mechanisms by which statins could prevent melanoma metastasis by decreasing tumor cell migration, decreasing cell adhesion, and increasing immunogenicity (Collisson et al., *Mol Cancer Ther* 2003;2:941e8; Kidera et al., *J Exp Clin Cancer Res* 2010;29:127; Pich et al., *Front Immunol* 2013;4:62; Zanfardino et al., *Int J Oncol* 2013;43:1763e70). One study observed decreased Breslow depth and metastasis rate with statin use, but the decrease in metastasis was not statistically significant as observed when multivariable analysis was conducted (Koomen et al., *Eur J Cancer* 2007;43:2580e9). Another population-based study on all-cause mortality in patients with melanoma found a trend toward decreased hazard of death, particularly in men, but did not reach statistical significance (Livingstone et al., *Cancer Med* 2014;3:1284e93).

[0166] In this study, an *in silico* drug screen is presented that suggests that statins might modify gene expression correlated with metastasis. Next-generation RNA sequencing (RNA-seq) was then conducted to characterize the direct effects of clinically relevant doses of fluvastatin on the melanoma transcriptome in vitro. Finally, the association of statin use with metastasis was explored in a retrospective cohort of cutaneous melanoma.

RESULTS

[0167] *Identification of statins as a potential treatment for metastasis prevention*

[0168] It was hypothesized that compounds that induced gene expression signatures opposite to that of metastatic melanoma would prevent metastasis. A search of the cMap database identified piroxicam, sotalol, acyclovir, zalcitabine, and simvastatin as potential therapeutic agents (Table 6). The ability of a drug to shift a gene expression signature is measured by its connectivity score (tau), which is a standardized measure ranging from –100 to 100. For each drug in the list of query results, the score corresponds to the fraction of reference gene sets that affect the 28 genes more strongly. The reference gene sets are generated from all reference signatures of drugs in the cMap database. A score of 90 indicates that only 10% of the reference gene sets showed stronger effects. In general, tau

2:90 is considered strong and should be considered a hypothesis for further study. Simvastatin had a score of 91. The score of all statins combined was also checked to ensure that these drugs as a class had a consistent effect. The statin class score was 84.9, which is strong for a class of drugs averaged together. Statins were selected for further study because of their proven long-term tolerability, benign side effect profile suitable for the intended clinical use as preventive drugs, and possible melanoma chemopreventive effects published in the literature.

Table 6. Candidate Drugs

Drug	*Tau Score*	*Drug Class*	*Class Score*
Piroxicam	99.93	Cyclooxygenase inhibitor	–33.86
Sotalol	99.47	β-Adrenergic receptor antagonist	–13.36
Acyclovir	99.44	DNA polymerase inhibitor	23.83
Zalcitabine	99.12	Nucleoside reverse transcriptase inhibitor	97.48
Simvastatin	91.05	HMGCR inhibitors (statins)	84.94

Abbreviation: HMGCR, 3-hydroxy-3-methyl glutaryl-coenzyme A reductase.

[0169] *Fluvastatin alters the gene expression profile of melanoma*

[0170] The cMap data are derived from the treatment of cell lines with statins at 10 mM concentration, which is above the maximal tolerated human dose (López-Aguilar et al., *Arch Med Res* 1999;30:128e31; Tse et al., *J Clin Pharmacol* 1992;32:630e8). Thus, the effect of statins on the melanoma transcriptome at clinically tolerable doses was characterized. RNA-seq was used to measure the gene expression of A375 melanoma cells before and after treatment with fluvastatin. The A375 cell line was specifically chosen because it has moderate metastatic potential and has been used in previous mechanistic studies of statins. Fluvastatin was chosen because of its lipophilicity (allowing extrahepatic distribution), benign side effect profile, and excellent bioavailability. 2,615 differentially expressed genes were identified (FIG. 1).

[0171] Fluvastatin significantly affected the expression of genes previously shown to be involved in metastasis, including MAGEA1, MAGEA3, MAGEA4, MAGEA6, MAGED1, SOX4, BUB1, and KIFC1 (FIG. 2) (Brasseur et al., *Int J Cancer* 1995;63:375e80; Jafarnejad et al., *Oncogene* 2013;32:2131e9; Li et al., *Pigment Cell Melanoma Res* 2015;28:453e63; Riker et al., *BMC Med Genomics* 2008;1:13; Weon and Potts, *Curr Opin Cell Biol* 2015;37:1e8). Genes that drive lymph angiogenesis were also found to be significantly decreased by fluvastatin treatment, including FGF2, S1PR5, and TGFBRAP1 (Table 7) (Cao et al., *Proc Natl Acad Sci USA* 2012;109:15894e9; Huang et al., *Biomolecules* 2013;3:408e34; James et al., *Development* 2013;140:3903e14).

Table 7. Genes of Interest Differentially Expressed after Fluvastatin Treatment

Gene Symbol	Log_2 Fold Change	q-Value
MAGEA1	–0.4357	0.0018
MAGEA3	–0.4574	0.0018
MAGEA4	–0.2919	0.0407
MAGEA6	–0.4529	0.0018
MAGED1	0.7222	0.0018
SOX4	–0.4139	0.0018
BUB1	–0.7319	0.0018
KIFC1	–0.4213	0.0355
FGF2	–0.5791	0.0018
S1PR5	–1.3212	0.0292
TGFBRAP1	–0.3389	0.0381
CDKN1A	1.3053	0.0018
CDKN1C	3.2643	0.0018
CUL1	–0.3847	0.0062
CCND3	–0.7194	0.0089
ITGB3[1]	0.5304	0.027
AQP3[2]	3.2162	0.0018
K14[2]	2.8530	0.0018
PLAT[1]	1.7389	0.0018
CXCL8[1]	0.8218	0.0018

Abbreviation: K, keratin.
[1]SkylineDx profile.
[2]Castle Biosciences profile.

[0172] Consistent with the prediction that statins affect melanoma metastasis rather than initiation, fluvastatin significantly altered the expression of genes included in melanoma prognostic tests (DecisionDx-Melanoma, Castle Biosciences, Friendswood, TX; Merlin Assay, SkylineDx, Rotterdam, The Netherlands) but did not alter the expression of any genes used in a diagnostic test that distinguishes melanoma from nevi (myPath Melanoma, Myriad Genetics, Salt Lake City, UT) (Clarke et al., *J Cutan Pathol* 2015;42:244e52). Genes included in these assays that were significantly shifted are presented in Table 7 (ITGB3; PLAT; CXCL; AQP3; and keratin 14 gene, K14). This suggests that the effect of statins is specific to progression and metastasis rather than to tumor initiation.

[0173] Gene ontology analysis of differentially expressed genes suggested significant enrichment of genes involved in the biological processes of cell proliferation, regulation of cell proliferation, tissue development, response to stimulus, and cell communication (all $P < 0.05$). The molecular functions represented included signaling receptor activity, molecular transducer activity, receptor-ligand activity, receptor regulator activity, and transmembrane RSK binding (all $P < 0.05$). Finally, the cellular components represented included plasma membrane, cell periphery, extracellular matrix, plasma membrane part, and extracellular region (all $P < 0.05$).

[0174] *Patients taking statins have a significantly lower incidence of metastasis at diagnosis*

[0175] To evaluate the clinical impact of statin use on melanoma metastasis, a retrospective cohort of 475 patients with melanoma were reviewed, of which 311 patients met the inclusion criteria. The mean age was 64.7 years. The mean Breslow depth in patients taking statins was 3.32 mm compared with 2.48 mm in those not taking statins (P = 0.038) (Table 8). Regional or distant metastasis (defi as a positive completion of lymph node dissection or distant metastasis detected on imaging) was identified at diagnosis in 24.7% of patients taking statins and 37.6% of patients not taking statins (P = 0.038). This result was significant in multivariate analysis after controlling for age, Breslow depth, ulceration, and mitotic rate (P = 0.016) (Table 9).

[0176] Table 8. Demographics of Patients Taking and Not Taking Statin at the Time of Biopsy

Characteristics	Taking Statin (n = 77)	Not Taking Statin (n = 234)	*P*-Value
Mean age (y), n	72.9	61.5	<0.001
Mean Breslow depth (mm), n	3.32	2.48	0.038
Ulceration, n (%)			
Yes	26 (33.8)	79 (33.8)	
No	51 (66.2)	155 (66.2)	0.999
Mitotic rate (mitoses/mm^2), n (%)			
0	9 (11.7)	28 (11.9)	
1–5	56 (72.7)	181 (77.4)	
6–10	5 (6.5)	21 (9.0)	
>10	7 (9.1)	4 (1.7)	0.015
Metastasis detected during initial workup, n (%)			
Yes	19 (24.7)	88 (37.6)	
No	58 (75.3)	146 (62.4)	0.038

Table 9. Multivariate Analysis for Factors that Predispose to Metastasis at Initial Workup

Factors	OR (95% CI)	*P*-Value
Age (y)	0.98 (0.97e1.00)	0.01
Depth (mm)	1.12 (1.00e1.24)	0.04
Ulceration present	1.44 (0.81e2.55)	0.22
Dermal mitoses (per mm^2)	1.02 (0.95e1.10)	0.61
Histologic type		
Nodular	1	—
Superficial spreading	0.93 (0.47e1.81)	0.82
Lentigo maligna	0.27 (0.08e0.88)	0.03
Other	0.81 (0.39e1.67)	0.57
Taking a statin	0.48 (0.25e0.94)	0.03

Abbreviation: CI, confidence interval.

DISCUSSION

[0177] Computational prediction has been successful in the past for identifying repurposing opportunities (Chen et al., *Nat Commun* 2017;8:16022; Menden et al., *Nat Commun* 2019;10:2674; Sirota et al., *Sci Transl Med* 2011;3:96ra77). In this study, an *in silico* screen was used to identify Food and Drug Administration-approved drugs that induce a genetic profile opposed to a validated gene expression profile that predicts melanoma metastasis. Statins were selected for further investigation on the basis of their long record of safety, their benign side effect profile consistent with their use as a preventive drug, and the literature suggestive of their potential activity in melanoma chemoprevention. Because the *in silico* screen uses data from experiments at doses above the maximal tolerated human serum concentration, the efficacy of statins in appropriately shifting the melanoma transcriptome at clinically achievable doses (3 mM) was verified.

[0178] It was found that fluvastatin caused significant changes in the melanoma transcriptome and affected the genes specific to melanoma metastasis at doses below the maximal tolerated dose (López-Aguilar et al., *Arch Med Res* 1999;30:128e31; Tse et al., *J Clin Pharmacol* 1992;32:630e8). At these doses, fluvastatin did not affect the expression of genes that are used to differentiate nevi from melanoma, consistent with previous clinical trial results demonstrating no effect of statins on the progression of dysplastic nevi to melanoma (Linden et al., *Cancer Prev Res (Phila)* 2014;7:496e504). However, fluvastatin influenced the expression of genes used to measure the risk of metastasis in commercially available tests, suggesting that the effect of statins is specific to melanoma progression and metastasis rather than to melanoma initiation. These data also imply that the history of statin use may be an important factor in interpreting the results of these prognostic tests. Because the drug concentrations used were lower than those in cMap (therapeutic rather than supratherapeutic) and because RNA-seq was used rather than microarray, there were fewer changes in the 28-gene expression profile than initially predicted by cMap. It was found that the RNA-seq validation experiments also revealed changes in genes outside of commercial tests that are known to influence metastatic potential and melanoma development.

[0179] These results suggest potential mechanisms for the effect of statins on melanoma metastasis. Regulation of the G1/S transition appeared to be affected by statin treatment. CDKN1A (p21) and CDKN1C (KIP2), which inhibit cell cycle progression in G1 and S phases, are typically suppressed in melanoma but had significantly increased expression after fluvastatin exposure (Jalili et al., *J Natl Cancer Inst* 2012;104:1673e9; Yang et al., *Cancer Cell Int* 2020;20: 32). The expression of CUL1 (Cullin 1), which promotes G1 to S phase transition and drives melanoma proliferation, was significantly decreased after fluvastatin treatment (Chen & Li, *Int J Oncol* 2010;37: 1339e44). In addition, increased

expression of CCND3 (Cyclin D3) has been shown to decrease survival and promote early relapse in melanoma (Flørenes et al., *Clin Cancer Res* 2000;6:3614e20). In this study, it was found that CCND3 expression was significantly decreased after fluvastatin treatment. KIFC1, a gene important in centrosome clustering, is overexpressed in primary and uveal melanoma cell lines as well as in breast and lung cancers (Pannu et al., *Oncotarget* 2015;6: 6076e91). This study demonstrated that fluvastatin decreased the expression of KIFC1. Previous studies have demonstrated that metabolic differences in melanoma cells result in differences in metastatic potential (Tasdogan et al., *Nature* 2020;577:115e20). SLC16A1 (MCT1), which has metabolic functions in lactate transport, has been shown to be an oncogene in malignant melanomas and neuroblastomas and to drive melanoma metastasis (Avitabile et al., *Carcinogenesis* 2020;41:284e95). SLC16A1 downregulation was observed after fluvastatin exposure but did not achieve statistical significance after correction for multiple hypothesis testing (fold change = 0.797, q = 0.062). Lymphangiogenesis is thought to be involved in both metastasis and immune regulation of the tumor microenvironment (Lane et al., *J Exp Med* 2018;215:3; Lund et al., *Cell Rep* 2012;1:191e9; Lund et al., *Cancer Discov* 2016a;6: 22e35; Lund et al., *J Clin Invest* 2016b;126:3389e402). FGF2, S1PR5, and TGFBRAP1 are all involved in lymphangiogenesis and were downregulated by statin treatment. Finally, the MAGE gene family has been demonstrated to be expressed in a wide variety of malignancies, including melanoma, and is associated with increased invasion and metastasis (Barrow et al., *Clin Cancer Res* 2006;12:764e71; Brasseur et al., *Int J Cancer* 1995;63:375e80). Decreased expression of MAGEA1, MAGEA3, MAGEA4, and MAGEA6 was observed with fluvastatin treatment.

[0180] A previous study found that atorvastatin decreases isoprenylation of RhoC, thereby decreasing migration and invasion in a Matrigel transwell assay of A375 cells and metastasis in a mouse model (Collisson et al., *Mol Cancer Ther* 2003;2:941e8). The A375 cell line was chosen to build on this previous literature, and the data suggest that statins may also affect lymphangiogenesis, cell cycle regulation, and metabolism to reduce metastasis. The effect on cell cycle regulation identified in this study may be particularly relevant for the treatment of familial melanomas induced by CDKN2A mutations (Aspinwall et al., *Cancer Epidemiol Biomarkers Prev* 2008;17: 1510e9; Goldstein et al., *Cancer Res* 2006;66:9818e28; Goldstein et al., *J Med Genet* 2007;44:99e106; Leachman et al., *J Am Acad Dermatol* 2009;61:677.e1e14).

[0181] It was considered that statins might induce gene expression changes that are correlated with metastasis but are not causative of metastasis. If this were true, statin use should not be correlated with the risk of metastasis. Thus, the association between statin use and metastasis was investigated in a retrospective cohort of patients with melanoma Patients taking statins at the time of biopsy were found to be significantly less likely to have

metastasis at the time of melanoma diagnosis than those not taking statins, thereby suggesting that statins may be protective against melanoma metastasis. Statin use remained the strongest independent predictor of metastasis after correction for other prognostic factors, including depth, ulceration, mitoses, and age. The possibility was considered that statins may simply be a marker of better access to health care resulting in earlier melanoma diagnosis. However, the statin group in the described cohort actually had thicker primary melanomas with a higher mitotic count, indicating later diagnosis. The fact that these patients still had fewer metastases despite significantly worse primary tumors is remarkable.

[0182] The data in this stud are retrospective and correlative; despite controlling for all the prognostic factors available, it is possible that there are confounding variables beyond the researchers' knowledge, such as differences in patient behavior or access to health care. There is not enough follow-up data to determine for certain whether patients on statins have better future outcomes, although the reduction in metastases at diagnosis is promising. In addition, the study cohort is from a single tertiary referral center and thus may be biased toward a larger effect size than might be seen in a population-based study.

[0183] By leveraging a validated prognostic gene expression signature, statins were identified as a potential preventive therapy for melanoma metastasis, describe the effects of fluvastatin on the melanoma transcriptome, and demonstrate clinical activity in a retrospective cohort. Because the discovery of statins as potential prevention for metastasis was based on an existing commercial test, future clinical trials may be able to elegantly select the specific subset of patients who are most likely to benefit. Finally, as other genetic profiles are discovered, this tailored approach may identify additional drugs for the prevention or treatment of metastasis.

METHODS

[0184] *In silico selection of candidates for metastasis prevention*

[0185] The cMap Query Tool (https://clue.io/query) was used to conduct an in silico drug screen. The input query consisted of the 28 gene expression profiles from a commercially available prognostic test that predicts melanoma metastasis annotated by the desired change in expression (up or down) (Gerami et al., *Clin Cancer Res* 2015b;21:175e83). Each compound and the corresponding drug class were scored for their ability to oppose the high-risk melanoma gene expression profile using the cMap connectivity score (tau), a standardized measure ranging from –100 to 100. The top 10% of drug candidates were then evaluated for Food and Drug Administration approval status and overall safety profile.

[0186] Characterization of the transcriptome in human melanoma cells exposed to fluvastatin The effect of statins on the melanoma transcriptome was characterized using a

well-established melanoma cell line. Human melanoma cell line A375 (a generous gift of Dr John Letterio, Case Western Reserve University, Cleveland, OH) were maintained in standard growth media consisting of RPMI 1640 (Thermo Fisher Scientific, Waltham, MA) þ 10% fetal bovine serum þ 2 mM glutamine and grown in a 5% carbon dioxide–humidified atmosphere at 37 ºC. Cells were tested biannually and shown to be negative for mycoplasma contamination using the Mycoplasma Detection kit (MycoAlert, Lonza, Basel, Switzerland). For gene expression studies, A375 cells were seeded at 2.5E6 per 10 cm^2 dish and allowed to adhere overnight. Test samples (in triplicate) were treated with fluvastatin (purchased from Millipore Sigma, St. Louis, MO) at 3 mM concentration for 24 hours and then harvested for RNA extraction using the RNeasy Plus Mini Kit (catalog #74134; Qiagen, Germantown, MD) as per manufacturer's instructions. Untreated control cells grown side by side were used as the reference control for differential expression analysis. RNA was quantified using the Qubit Broad Range RNA kit (catalog #Q10210; Thermo Fisher Scientific) and diluted to 50 ng/ml for RNA-seq.

[0187] Sequencing reads generated from the Illumina HiSeq platform (Illumina, San Diego, CA) were assessed for quality and trimmed for adapter sequences using TrimGalore!, version 0.4.2 (Babraham Bioinformatics, Cambridge, United Kingdom), a wrapper script for FastQC and cutadapt. Reads that passed quality control were subsequently aligned to the human reference genome (GRCh38) using STAR aligner, version 2.5.1. Sequence alignment was guided using the GENCODE annotation for hg38. The aligned reads were analyzed for differential expression using Cufflinks, version 2.2.1, an RNA-seq analysis package that reports the fragments per kilobase of exon per million mapped for each gene. A differential analysis report was generated using the cuffdiff command performed in a pairwise manner for each group. Differential genes were identified using a significance cutoff of $q < 0.05$. The differential expression profiles were then used as input in iPathwayGuide (Advaita Bioinformatics, Ann Arbor, MI) for pathway analysis.

[0188] *Multivariate analysis of the effect of statin use on metastasis incidence*

[0189] To further understand how statins affect melanoma metastasis rates in the clinical setting, a retrospective chart review was performed of patients diagnosed with melanoma in the dermatopathology archive at the tertiary medical center from 1 January 2007 through 31 December 2017. This study was approved by Institutional Review Board. Patients with a histopathological diagnosis of melanoma with Breslow depth >0.8 mm or with ulceration were included. Patients with >3 primary melanomas were excluded to avoid confounding by patients with a germline predisposition to melanoma. Data collected included age at diagnosis of primary melanoma, sex, race, immunosuppression status (has a transplant, is HIV positive, has hematologic malignancy), statin use at the time of biopsy, histologic type, Breslow depth, ulceration, mitotic rate, tumor-infiltrating lymphocytes, regression, sentinel

lymph node biopsy results, complete lymph node dissection results, and presence of metastasis at diagnosis. Univariate and multivariate analyses using logistic regression were performed to determine the relationship of statin use with the presence of metastasis at diagnosis, controlled for Breslow depth, ulceration status, and mitotic rate (glm function, R, version 4.0.2). A P-value of at least 0.05 was considered significant.

[0190] *Data availability statement*

[0191] Datasets related to this article can be found online at ncbi.nlm. nih.gov/geo/, hosted at the National Center for Biotechnology Information Gene Expression Omnibus.

Example 2. Representative Clinical Uses.

[0192] This Example provides a description of possible clinical uses that are enabled based on the teachings in this disclosure.

[0193] A patient is identified as having melanoma, such as early stage melanoma (AJCC Stage 1-2). Whether the patient has a history of taking a statin is determined. If the patient does have a history (prior or concurrent with their diagnosis with melanoma) of taking a statin, the patient is identified as having one or more of: a relatively lower risk of a positive sentinel lymph node biopsy, a relatively lower risk of sentinel lymph node metastasis, and a relatively good prognosis. Thus, a history of taking a statin (or not) is used in classifying the patient.

[0194] Optionally, use of the history of taking a statin (or not) to classify the subject is combined with one or more additional clinicopathological analyses or determinations. For instance, the age of the patient, Breslow depth, ulceration status of the primary melanoma tumor, and/or a genetic expression profile (GEP) of the primary melanoma tumor may also be considered in classifying the subject, or in refining the classification.

[0195] For instance, in some instances such as where the diagnosis of melanoma includes analysis of a skin biopsy from the primary melanoma, that melanoma specimen is tested by gene expression profiling (GEP) to determine whether it is a high-risk cancer (as described herein; specifically high risk for recurrence or metastasis).

[0196] Table 10: Gene List with Direction of Expression change in High-risk Melanoma

Gene Symbol	Entrez Gene ID	Gene Title / Name	Direction
ABCC12	94160	Multidrug resistance-associated protein 9	Decreased
ACOT1	641371	Acyl-coenzyme A thioesterase 1	Decreased
ADAM12	8038	ADAM metallopeptidase domain 12	Increased
AGBL4	84871	Cytosolic carboxypeptidase 6	Increased
AGXT	189	Alanine-glyoxylate aminotransferase	Decreased
AIM2	9447	Interferon-inducible protein AIM2	Decreased
ALK	238	ALK tyrosine kinase receptor	Decreased
ANGPT1	284	Angiopoietin 1	Increased
ANGPT2	285	Angiopoietin 2	Increased
ANGPTL7	10218	Angiopoietin-related protein 7	Increased
ANK1	286	Ankyrin-1	Decreased
AQP3	360	Aquaporin 3 (Gill blood group)	Decreased
ARG1	383	Arginase 1	Decreased
ARRDC1	92714	Arrestin domain-containing protein 1	Decreased
ART1	417	GPI-linked NAD(P)(+)-arginine ADP-ribosyltransferase 1	Decreased
BAP1	8314	BRCA1-associated protein-1	Decreased
BIN1	274	Myc box-dependent-interacting protein 1	Decreased
BMP2	650	Bone morphogenic protein 2	Decreased
BMX	660	Cytoplasmic tyrosine-protein kinase BMX	Decreased
BTG1	694	B-cell translocation gene 1, antiproliferative	Decreased
C8G	733	Complement component C8 gamma chain	Decreased
CACNG4	27092	Voltage-dependent calcium channel γ-4 subunit	Increased
CAMK2B	816	Calcium/calmodulin-dependent protein kinase type II subunit beta	Decreased
CASQ1	844	Calsequestrin-1	Decreased
CCR3	1232	C-C chemokine receptor type 3	Decreased
CCR5	1234	C-C chemokine receptor type 5	Decreased
CDC5L	988	Cell division cycle 5-like protein	Increased
CENPQ	55166	Centromere protein Q	Increased
CETP	1071	Cholesteryl ester transfer protein	Decreased
CLCA2	9635	Chloride channel accessory 2	Decreased
CLIC5	53405	Chloride intracellular channel protein 5	Decreased
COL24A1	255631	Collagen alpha-1(XXIV) chain	Increased
CPN2	1370	Carboxypeptidase N subunit 2	Increased
CRABP2	1382	Cellular retinoic acid binding protein 2	Decreased
CST6	1474	Cystantin E/M	Decreased
CTAGE1	64693	cTAGE family member 2	Decreased
CTCFL	140690	Transcriptional repressor CTCFL	Increased
CUL7	9820	Cullin-7	Increased
CXCL14	9547	Chemokine (C-X-C motif) ligand 14	Decreased
CXCL8 / IL8	3576	Interleukin 8	Increased

Gene Symbol	Entrez Gene ID	Gene Title / Name	Direction
DIO3OS	64150	DIO3 opposite strand upstream RNA [Source:HGNC Symbol;Acc:HGNC:20348]	Decreased
DMAP1	55929	DNA methyltransferase 1-associated protein 1	Increased
DOCK3	1795	Dedicator of cytokinesis protein 3	Decreased
DPEP3	64180	Dipeptidase 3	Increased
DSC1	1823	Desmocollin 1	Decreased
DYSF	8291	Dysferlin	Decreased
EEF1A2	1917	Elongation factor 1-alpha 2	Decreased
EHBP1L1	254102	EH domain-binding protein 1-like protein 1	Decreased
EIF1B	10289	Eukaryotic translation initiation factor 1B	Increased
ERGIC2	51290	Endoplasmic reticulum-Golgi intermediate compartment protein 2	Increased
F7	2155	Coagulation factor VII	Decreased
FASLG	356	Tumor necrosis factor ligand superfamily member 6	Decreased
FGF2	2247	Fibroblast Growth Factor 2	Increased
FLOT1	10211	Flotillin-1	Increased
FLVCR2	55640	Feline leukemia virus subgroup C receptor-related protein 2	Decreased
FNBP1L	54874	Formin-binding protein 1-like	Increased
FREM2	341640	FRAS1-related extracellular matrix protein 2	Decreased
GABBR2	9568	Gamma-aminobutyric acid type B receptor subunit 2	Decreased
GBP5	115362	Guanylate-binding protein 5	Decreased
GCH1	2643	GTP cyclohydrolase 1	Decreased
GDF15	9518	Growth Differentiation Factor 15	Increased
GFRA1	2674	GDNF family receptor alpha-1	Decreased
GJA1	2697	Gap junction protein, alpha 1, 43 kDa	Decreased
GLDC	2731	Glycine dehydrogenase (decarboxylating), mitochondrial	Decreased
GPR39	2863	G-protein coupled receptor 39	Decreased
GPR63	81491	Probable G-protein coupled receptor 63	Decreased
GRAMD1B	57476	GRAM domain containing 1B	Decreased
HEPACAM	220296	Hepatocyte cell adhesion molecule	Decreased
HHATL	57467	Protein-cysteine N-palmitoyltransferase HHAT-like protein	Increased
HMGCR	3156	3-hydroxy-3-methylglutaryl-CoA reductase	Increased
HNF4A	3172	Hepatocyte nuclear factor 4-alpha	Increased
HTR3B	9177	5-hydroxytryptamine receptor 3B	Decreased
ID2	3398	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	Decreased
IFNG	3458	Interferon-gamma	Decreased
ITGB3	3690	Integrin beta 3	Increased
JARID2	3720	Protein Jumonji	Increased
JPH3	57338	Junctophilin-3	Decreased

Gene Symbol	Entrez Gene ID	Gene Title / Name	Direction
KCNT1	57582	Potassium channel subfamily T member 1	Decreased
KIF19	124602	Kinesin-like protein KIF19	Decreased
KLC4	89953	Kinesin light chain 4	Increased
KLHDC8A	55220	Kelch domain containing 8A	Decreased
KNDC1	85442	Protein very KIND	Decreased
KRT14	3861	Keratin 14	Decreased
KRT6B	3854	Keratin 6B	Increased
KRTAP19-6	337973	Keratin-associated protein 19-6	Increased
KSR2	283455	Kinase suppressor of Ras 2	Decreased
KYNU	8942	Kynureninase	Decreased
LAG3	3902	Lymphocyte activation gene 3 protein	Decreased
LAMB1	3912	laminin B1 chain	Increased
LGALSl	3956	galectin 1	Increased
LGI4	163175	Leucine rich repeat LGI family member 4	Increased
LRRC31	79782	Leucine-rich repeat-containing protein 31	Decreased
LTA4H	4048	Leukotriene A4 hydrolase	Decreased
LOXL4	84171	Lysyl oxidase like 4	Increased
MCF2	4168	Proto-oncogene DBL	Decreased
ME3	10873	NADP-dependent malic enzyme, mitochondrial	Decreased
MFSD6L	162387	Major facilitator superfamily domain containing 6 like	Decreased
MGP	4256	Matrix Gla protein	Decreased
MIR222	407007	microRNA 222 [Source:HGNC Symbol;Acc:HGNC:31602]	Decreased
MLANA	2315	Melan-A	Increased
MLF1	4291	Myeloid leukemia factor 1	Increased
MTUS2	23281	Microtubule-associated tumor suppressor candidate 2	Decreased
NFYA	4800	Nuclear transcription factor Y subunit alpha	Increased
NPPC	4880	C-type natriuretic peptide	Decreased
NTRK1	4914	High affinity nerve growth factor receptor	Decreased
NXPH1	30010	Neurexophilin-1	Increased
OCIAD2	132299	OCIA domain containing 2	Decreased
OR52K1	390036	Olfactory receptor 52K1	Decreased
OR5AK2	390181	Olfactory receptor 5AK2	Decreased
P2RY14	9934	P2Y purinoceptor 14	Decreased
PARP11	57097	Poly [ADP-ribose] polymerase 11	Decreased
PHEX	5251	Phosphate-regulating neutral endopeptidase	Decreased
PIK3R6	146850	Phosphoinositide 3-kinase regulatory subunit 6	Decreased
PLA2G2D	26279	Group IID secretory phospholipase A2	Decreased
PLAT	5327	tissue-type plasminogen activator	Increased
PLCZ1	89869	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase zeta-1	Decreased
PPL	5493	Periplakin	Decreased
PRF1	5551	Perforin-1	Decreased

Gene Symbol	Entrez Gene ID	Gene Title / Name	Direction
PRKCB	5579	protein kinase C-β	Increased
PROCA1	147011	Protein interacting with cyclin A1	Decreased
PTPRG	5793	Receptor-type tyrosine-protein phosphatase γ	Increased
RAB15	376267	Ras-related protein Rab-15	Decreased
RBBP4	5928	Histone-binding protein RBBP4	Increased
RBM23	55147	RNA-binding motif protein 23	Decreased
RGS7	6000	Regulator of G-protein signaling 7	Decreased
RNF213	57674	E3 ubiquitin-protein ligase RNF213	Decreased
ROBO1	6091	Roundabout, axon guidance receptor, homolog 1	Decreased
RP1	6101	Oxygen-regulated protein 1	Increased
RPL7L1	285855	Ribosomal protein L7 like 1	Increased
S100A8	6279	S100 calcium-binding protein A8	Decreased
S100A9	6280	S100 calcium-binding protein A9	Decreased
S1PR1	1901	Sphingosine 1-phosphate	Increased
SAP130	79595	Sin3A-associated protein, 130 kDa	Decreased
SERPINE2	5270	glia-derived nexin	Increased
SFMBT2	57713	Scm-like with four MBT domains protein 2	Decreased
SIGLEC12	89858	Sialic acid-binding Ig-like lectin 12	Decreased
SLC17A3	10786	Sodium-dependent phosphate transport protein 4	Increased
SLC29A1	2030	Equilibrative nucleoside transporter 1	Increased
SLCO5A1	81796	Solute carrier organic anion transporter family member 5A1	Decreased
SLIT1	6585	Slit homolog 1 protein	Decreased
SOCS1	8651	Suppressor of cytokine signaling 1	Decreased
SOX4	6659	Transcription factor SOX-4	Increased
SPAG6	9576	Sperm-associated antigen 6	Decreased
SPP1	6696	Secreted phosphoprotein 1	Increased
SPRR1B	6699	Small proline-rich protein 1B	Decreased
STMN1	3925	Stathmin	Increased
TACSTD2	4070	Tumor-associated calcium signal transducer 2	Decreased
TAS2R60	338398	Taste receptor type 2 member 60	Decreased
TDRD12	91646	Putative ATP-dependent RNA helicase TDRD12	Increased
TFF2	7032	Trefoil factor 2	Decreased
TGFB1	7040	Transforming growth factor beta	Decreased
TGFBRI	7046	transforming growth factor-β (TGF-β) receptor 1	Increased
TLR4	7099	Toll-like receptor 4	Decreased
TMCC3	57458	Transmembrane and coiled-coil domain containing	Decreased
TNFRSF10A	8797	Tumor necrosis factor receptor superfamily member 10A	Decreased
TP53	7157	Cellular tumor antigen p53	Decreased
TRIM22	10346	E3 ubiquitin-protein ligase TRIM22	Decreased
TRIM29	23650	Tripartite motif containing 29	Decreased
TRNAU1AP	54952	tRNA selenocysteine 1-associated protein 1	Increased

Gene Symbol	Entrez Gene ID	Gene Title / Name	Direction
TTN	7273	Titin	Decreased
TUBB	203068	Tubulin beta chain	Increased
TYRP1	7306	Tyrosinase-related protein 1	Decreased
UTS2	10911	Urotensin 2	Decreased
VAMP5	10791	Vesicle-associated membrane protein 5	Decreased
VEGFA	7422	Vascular Endothelial Growth Factor A	Increased
VEGFC	7424	Vascular Endothelial Growth Factor C	Increased
VEGFD	2277	Vascular Endothelial Growth Factor D	Increased
WDR49	151790	WD repeat-containing protein 49	Decreased
WIF1	11197	Wnt inhibitory factor 1	Decreased
ZIM2	23619	Zinc finger imprinted 2	Increased
ZNF560	147741	Zinc finger protein 560	Increased
ZNF697	90874	Zinc finger protein 697	Increased

VIII. Closing Paragraphs

[0197] As will be understood by one of ordinary skill in the art, each embodiment disclosed herein can comprise, consist essentially of or consist of its particular stated element, step, ingredient or component. Thus, the terms “include” or “including” should be interpreted to recite: “comprise, consist of, or consist essentially of.” The transition term “comprise” or “comprises” means has, but is not limited to, and allows for the inclusion of unspecified elements, steps, ingredients, or components, even in major amounts. The transitional phrase “consisting of” excludes any element, step, ingredient or component not specified. The transition phrase “consisting essentially of” limits the scope of the embodiment to the specified elements, steps, ingredients or components and to those that do not materially affect the embodiment. A material effect would cause a statistically significant change in measurement of a gene expression level (*e.g.*, in a gene expression profile), and/or in identification of a subject as having a “high risk melanoma”, and/or in the success of treatment using a statin to reduce the risk of melanoma metastasis.

[0198] Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term “about.” Accordingly, unless indicated to the contrary, the numerical parameters set forth in the specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. When further clarity is

required, the term "about" has the meaning reasonably ascribed to it by a person skilled in the art when used in conjunction with a stated numerical value or range, *i.e.* denoting somewhat more or somewhat less than the stated value or range, to within a range of ±20% of the stated value; ±19% of the stated value; ±18% of the stated value; ±17% of the stated value; ±16% of the stated value; ±15% of the stated value; ±14% of the stated value; ±13% of the stated value; ±12% of the stated value; ±11% of the stated value; ±10% of the stated value; ±9% of the stated value; ±8% of the stated value; ±7% of the stated value; ±6% of the stated value; ±5% of the stated value; ±4% of the stated value; ±3% of the stated value; ±2% of the stated value; or ±1% of the stated value.

[0199] Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

[0200] The terms "a," "an," "the" and similar referents used in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. Recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (*e.g.*, "such as") provided herein is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention otherwise claimed. No language in the specification should be construed as indicating any non-claimed element essential to the practice of the invention.

[0201] Groupings of alternative elements or embodiments of the invention disclosed herein are not to be construed as limitations. Each group member may be referred to and claimed individually or in any combination with other members of the group or other elements found herein. It is anticipated that one or more members of a group may be included in, or deleted from, a group for reasons of convenience and/or patentability. When any such inclusion or deletion occurs, the specification is deemed to contain the group as modified thus fulfilling the written description of all Markush groups used in the appended claims.

[0202] Certain embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Of course, variations on these described embodiments will become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventor expects skilled artisans to employ such

variations as appropriate, and the inventors intend for the invention to be practiced otherwise than specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

[0203] Furthermore, numerous references have been made to patents, printed publications, journal articles, other written text, and web site content throughout this specification (referenced materials herein). Each of the referenced materials are individually incorporated herein by reference in their entirety for their referenced teaching(s), as of the filing date of the first application in the priority chain in which the specific reference was included. For instance, with regard to chemical compounds, nucleic acid, and amino acids sequences referenced herein that are available in a public database, the information in the database entry is incorporated herein by reference as of the date of an application in the priority chain in which the database identifier for that compound or sequence was first included in the text.

[0204] It is to be understood that the embodiments of the invention disclosed herein are illustrative of the principles of the present invention. Other modifications that may be employed are within the scope of the invention. Thus, by way of example, but not of limitation, alternative configurations of the present invention may be utilized in accordance with the teachings herein. Accordingly, the present invention is not limited to that precisely as shown and described.

[0205] The particulars shown herein are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of various embodiments of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for the fundamental understanding of the invention, the description taken with the drawings and/or examples making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

[0206] Definitions and explanations used in the present disclosure are meant and intended to be controlling in any future construction unless clearly and unambiguously modified in the example(s) or when application of the meaning renders any construction meaningless or essentially meaningless. In cases where the construction of the term would render it meaningless or essentially meaningless, the definition should be taken from Webster's Dictionary, 11th Edition or a dictionary known to those of ordinary skill in the art, such as the Oxford Dictionary of Biochemistry and Molecular Biology, 2nd Edition (Ed. Anthony Smith,

Oxford University Press, Oxford, 2006), and/or A Dictionary of Chemistry, 8th Edition (Ed. J. Law & R. Rennie, Oxford University Press, 2020).

LISTING OF CLAIMS

1. A method, comprising:

determining whether a subject with a primary melanoma has taken inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (a statin), and

classifying the subject as:

having a relatively lower risk of sentinel lymph node metastasis and/or a relatively positive prognosis if the subject has taken a statin; and

having a relatively higher risk of sentinel lymph node metastasis and/or a relatively negative prognosis if the subject has not taken a statin.

2. The method of claim 1, further comprising factoring in Breslow depth and ulceration status of the primary melanoma tumor of the subject in classifying the subject.

3. The method of claim 1, further comprising analyzing a genetic expression profile (GEP) of the primary melanoma in classifying the subject.

4. The method of claim 3, wherein the GEP comprises measurement of the expression level of one or more genes selected from: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

5. The method of claim 4, wherein the GEP comprises measurement of the expression level of:

at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least 10, at least 15, at least 20, at least 25, or at least 30 genes selected from GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

6. The method of claim 4, wherein the GEP comprises measurement of the expression level of all of the following genes: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2,

KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

7. The method of claim 4, wherein the GEP comprises measurement of the expression level of any one or more of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI.

8. The method of claim 7, wherein the GEP comprises measurement of the expression level of each of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI.

9. The method of claim 4, wherein the GEP comprises measurement of the expression level of any or more of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

10. The method of claim 9, wherein the GEP comprises measurement of the expression level of each of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

11. The method of claim 1, further comprising factoring in Breslow depth, ulceration status of the primary melanoma tumor, and a genetic expression profile (GEP) of the primary melanoma tumor in classifying the subject.

12. The method of claim 11, wherein the GEP comprises measurement of the expression level of one or more genes selected from: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

13. The method of claim 12, wherein the GEP comprises measurement of the expression level of:

at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least 10, at least 15, at least 20, at least 25, or at least 30 genes selected from GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI,

TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

14. The method of claim 12, wherein the GEP comprises measurement of the expression level of all of the following genes: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

15. The method of claim 12, wherein the GEP comprises measurement of the expression level of any one or more of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI.

16. The method of claim 15, wherein the GEP comprises measurement of the expression level of each of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI.

17. The method of claim 12, wherein the GEP comprises measurement of the expression level of any or more of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

18. The method of claim 17, wherein the GEP comprises measurement of the expression level of each of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

19. The method of claim 1, wherein classifying the subject is used to determining a treatment and/or diagnostic work-up schedule for the subject.

20. The method of claim 1, wherein the statin comprises at least one of fluvastatin, pitavastatin, atorvastatin, simvastatin, lovastatin, rosuvastatin, or pravastatin.

21. The method of claim 1, further comprising considering at least one additional measure or marker of melanoma disease state, disease severity, or disease progression in classifying the subject.

22. The method of claim 1, further comprising considering the age of the subject in classifying the subject.

23. The method of any one of claims 4-10 or claim 12-18, wherein the gene expression levels are measured using one or more of polymerase chain reaction (PCR), real-rime polymerase chain reaction (RT-PCR), direct DNA expression in microarray, Sanger sequencing analysis, Northern blot, direct RNA expression detection, serial analysis of gene expression (SAGE), or next-generation RNA-sequencing.

24. The method of claim 23, wherein the gene expression levels are measured using RT-PCR.

25. A method for assessing pre-test probability of a positive sentinel lymph node biopsy (SLNB) for a subject with cutaneous melanoma, comprising:

determining whether a subject has taken a statin, and

classifying the subject as:

having a relatively lower risk of a positive SLNB if the subject has taken a statin; and

having a relatively higher risk of positive SLNB if the subject has not taken a statin.

26. The method of claim 25, further comprising considering at least one additional measure or marker of melanoma disease state, disease severity, or disease progression in classifying the subject.

27. The method of claim 25, further comprising factoring in Breslow depth and ulceration status of the primary melanoma tumor of the subject in classifying the subject.

28. The method of claim 25, further comprising analyzing a genetic expression profile (GEP) of the primary melanoma in classifying the subject.

29. The method of claim 28, wherein the GEP comprises measurement of the expression level of one or more genes selected from: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4,

PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

30. The method of claim 28, wherein the GEP comprises measurement of the expression level of:

at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least 10, at least 15, at least 20, at least 25, or at least 30 genes selected from GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

31. The method of claim 28, wherein the GEP comprises measurement of the expression level of all of the following genes: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

32. The method of claim 28, wherein the GEP comprises measurement of the expression level of any one or more of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI.

33. The method of claim 32, wherein the GEP comprises measurement of the expression level of each of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI.

34. The method of claim 28, wherein the GEP comprises measurement of the expression level of any or more of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

35. The method of claim 34, wherein the GEP comprises measurement of the expression level of each of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1,

SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

36. The method of claim 25, further comprising factoring in Breslow depth, ulceration status of the primary melanoma tumor, and a genetic expression profile (GEP) of the primary melanoma tumor in classifying the subject.

37. The method of claim 36, wherein the GEP comprises measurement of the expression level of one or more genes selected from: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

38. The method of claim 37, wherein the GEP comprises measurement of the expression level of:

at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least 10, at least 15, at least 20, at least 25, or at least 30 genes selected from GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

39. The method of claim 37, wherein the GEP comprises measurement of the expression level of all of the following genes: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

40. The method of claim 37, wherein the GEP comprises measurement of the expression level of any one or more of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI.

41. The method of claim 41, wherein the GEP comprises measurement of the expression level of each of: GDF15, MLANA, PLAT, IL8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI and TGFBRI.

42. The method of claim 41, wherein the GEP comprises measurement of the expression level of any or more of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

43. The method of claim 42, wherein the GEP comprises measurement of the expression level of each of: BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6.

44. The method of claim 25, wherein classifying the subject is used to determining a treatment and/or diagnostic work-up schedule for the individual.

45. The method of claim 25, wherein the statin comprises at least one of fluvastatin, pitavastatin, atorvastatin, simvastatin, lovastatin, rosuvastatin, or pravastatin.

46. The method of claim 25, further comprising considering the age of the subject in classifying the subject.

47. The method of any one of claims 25-35 or claim 37-43, wherein the gene expression levels are measured using one or more of polymerase chain reaction (PCR), real-rime polymerase chain reaction (RT-PCR), direct DNA expression in microarray, Sanger sequencing analysis, Northern blot, direct RNA expression detection, serial analysis of gene expression (SAGE), or next-generation RNA-sequencing.

48. The method of claim 47, wherein the gene expression levels are measured using RT-PCR.

49. An improved method of classifying a subject with melanoma, the improvement comprising taking into account in the classifying whether the subject has a history of taking a statin, wherein:

a history of taking a statin classifies the subject as having one or more of: a relatively lower risk of a positive sentinel lymph node biopsy, a relatively low risk of sentinel lymph node metastasis, and a relatively good prognosis; and

no history of taking a statin classifies the subject as having one or more of: a relatively higher risk of positive sentinel lymph node biopsy, a relatively high risk of sentinel lymph node metastasis, and a relatively poor prognosis.

FIG. 1

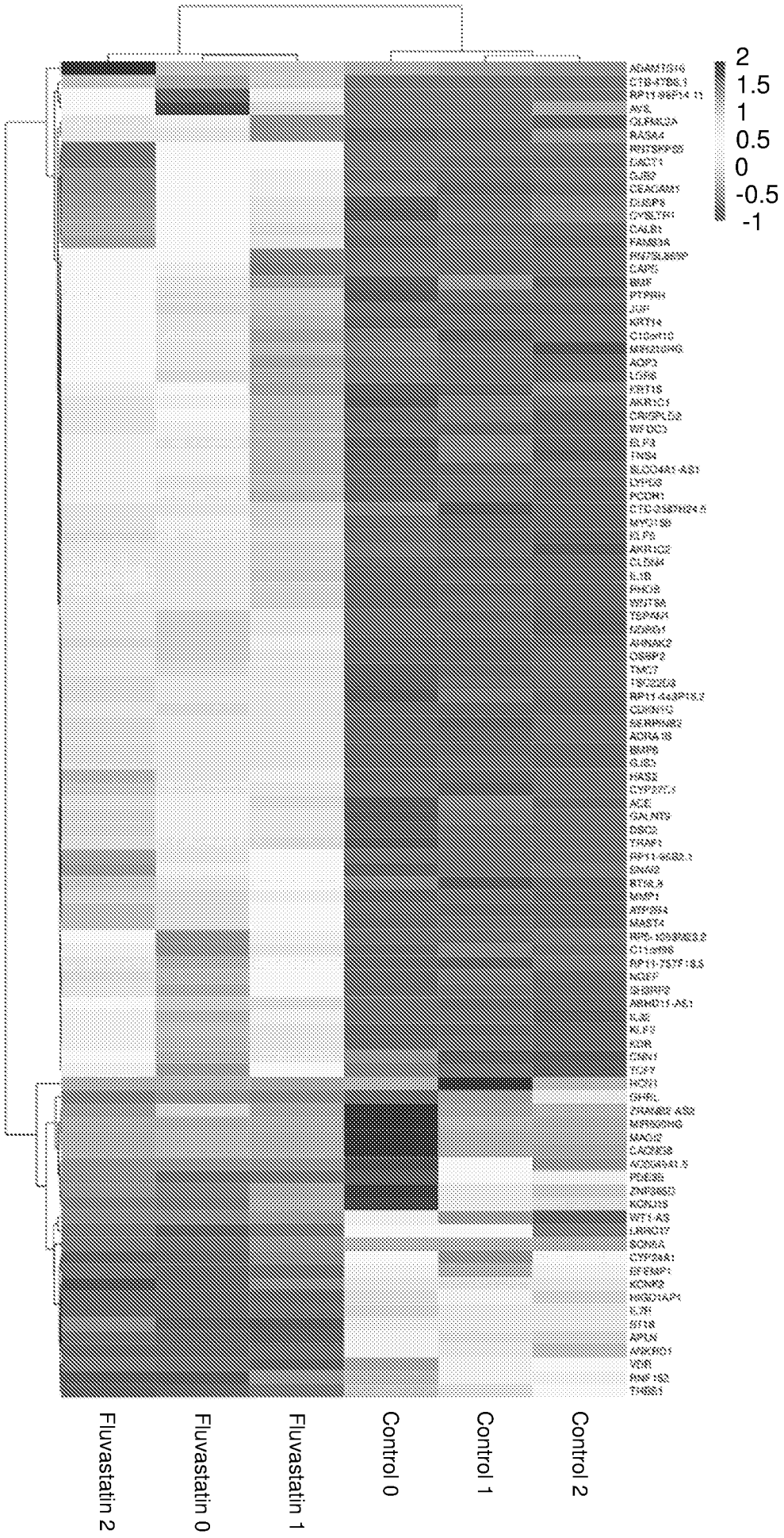
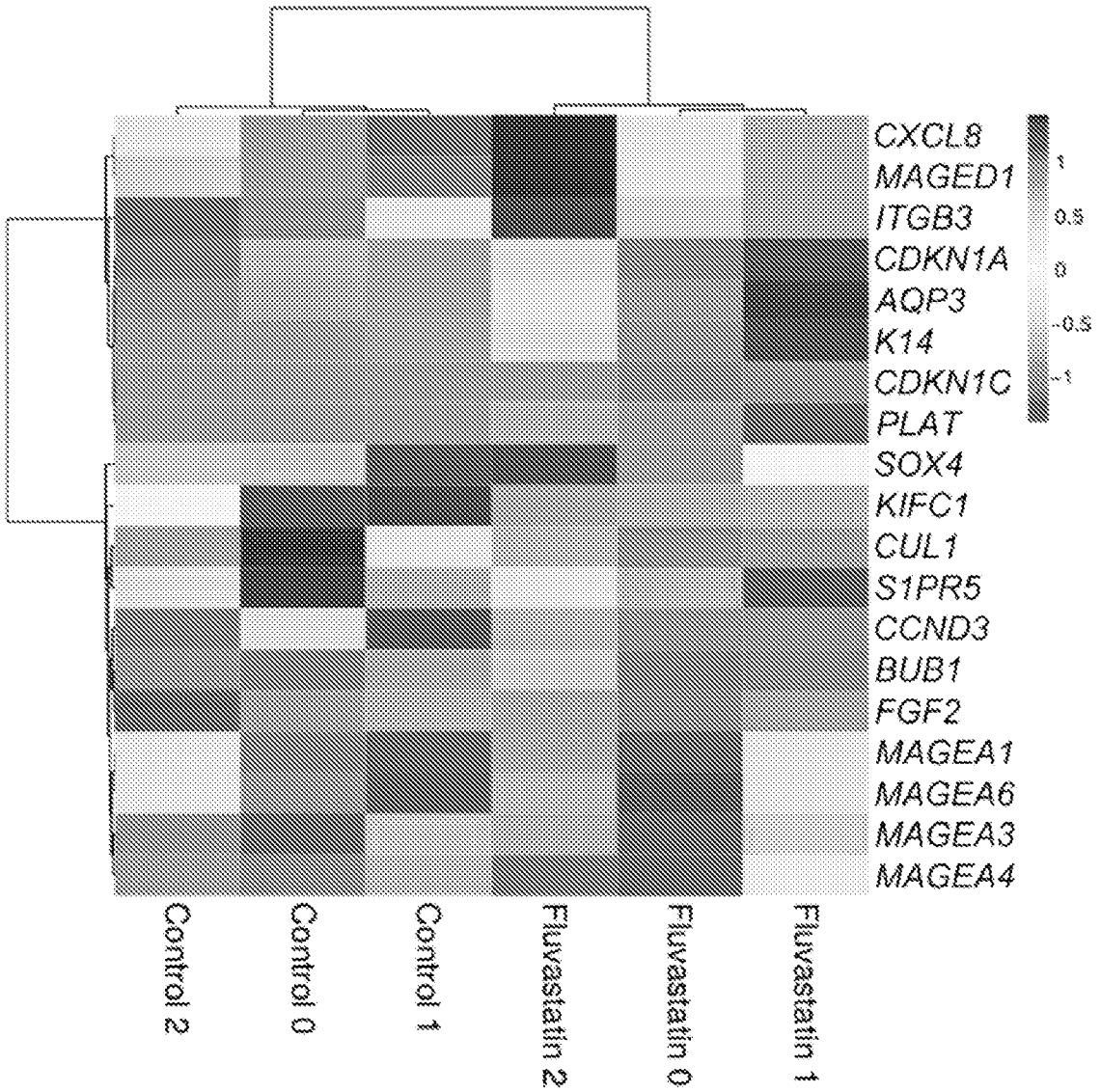

2
1.5
1
0.5
0
-0.5
-1
Fluvastatin 2
Fluvastatin 0
Fluvastatin 1
Control 0
Control 1
Control 2

FIG. 2


CXCL8
MAGED1
ITGB3
CDKN1A
AQP3
K14
CDKN1C
PLAT
SOX4
KIFC1
CUL1
S1PR5
CCND3
BUB1
FGF2
MAGEA1
MAGEA6
MAGEA3
MAGEA4
1
0.5
0
-0.5
-1
Control 2
Control 0
Control 1
Fluvastatin 2
Fluvastatin 0
Fluvastatin 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2022/011266

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/404; A61K 31/405; A61P 35/00; C12Q 1/6886; G01N 33/574 (2022.01)
CPC - A61K 31/404; A61K 31/405; A61P 35/00; C12Q 2600/106; C12Q 2600/158; G01N 33/5743 (2022.05)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
see Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
see Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
see Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2020/0318200 A1 (THE BRIGHAM AND WOMEN'S HOSPITAL INC.) 08 October 2020 (08.10.2020) entire document	1-4, 7, 11, 12, 15, 19-29, 32, 36, 37, 40, 44-49
Y	US 2014/0162888 A1 (KUSLICH et al) 12 June 2014 (12.06.2014) entire document	1-4, 7, 11, 12, 15, 19-29, 32, 36, 37, 40, 44-49
A	US 2016/0271106 A1 (THE BRIGHAM AND WOMEN'S HOSPITAL INC.) 22 September 2016 (22.09.2016) entire document	1-4, 7, 11, 12, 15, 19-29, 32, 36, 37, 40, 44-49
A	US 2017/0283885 A1 (DAIICHI SANKYO COMPANY LIMITED) 05 October 2017 (05.10.2017) entire document	1-4, 7, 11, 12, 15, 19-29, 32, 36, 37, 40, 44-49
A	US 2021/0147948 A1 (GENECENTRIC THERAPEUTICS INC. et al) 20 May 2021 (20.05.2021) entire document	1-4, 7, 11, 12, 15, 19-29, 32, 36, 37, 40, 44-49
A	US 2019/0358194 A1 (UNIWERSYTET JAGIELLONSKI) 28 November 2019 (28.11.2019) entire document	1-4, 7, 11, 12, 15, 19-29, 32, 36, 37, 40, 44-49
A	MULDER et al., "Validation of a clinicopathological and gene expression profile model for sentinel lymph node metastasis in primary cutaneous melanoma," The British Journal of Dermatology, 02 November 2020 (02.11.2020), Vol. 184, No. 5, Pgs. 944-951. entire document	1-4, 7, 11, 12, 15, 19-29, 32, 36, 37, 40, 44-49

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 May 2022	MAY 23 2022
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Harry Kim Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2022/011266

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See extra sheet(s).

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-4, 7, 11, 12, 15, 19-29, 32, 36, 37, 40, 44-49

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2022/011266

Continued from Box No. III Observations where unity of invention is lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I+: claims 1-49 are drawn to methods for assessing risks of future melanoma metastasis, lymph node status, prognosis, and/or disease progression.

The first invention of Group I+ is restricted to a gene selected to be GDF15, and a statin selected to be fluvastatin, and methods comprising the same. It is believed that claims 1-4, 7, 11, 12, 15, 19-29, 32, 36, 37, 40, and 44-49 read on this first named invention and thus these claims will be searched without fee to the extent that they read on GDF15 and fluvastatin.

Applicant is invited to elect additional genes and therapeutics to be searched in a specific combination by paying additional fee for each set of election. An exemplary election would be a gene selected to be MLANA, and a statin selected to be pitavastatin, and methods comprising the same. Additional genes and therapeutics will be searched upon the payment of additional fees. Applicants must specify the claims that read on any additional elected inventions. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined.

The inventions listed in Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The Groups I+ formulas do not share a significant structural element responsible for assessing risks of future melanoma metastasis, lymph node status, prognosis, and/or disease progression, requiring the selection of alternative genes and therapeutics where "the GEP comprises measurement of the expression level of one or more genes selected from: GDF15, MLANA, PLAT, 1L8, ITGB3, LOXL4, PRKCB, SERPINE2, ADAM12, LGALSI, TGFBRI, BAP1_varA, BAP1_varB, MGP, SPP1, CXCL14, CLCA2, S100A8, BTG1, SAP130, ARG1, KRT6B, GJA, ID2, EIF1B, S100A9, CRABP2, KRT14, ROBO1, RBM23, TACSTD2, DSC1, SPRR1B, TRIM29, AQP3, TYRP1, PPL, LTA4H, and CST6," and "wherein the statin comprises at least one of fluvastatin, pitavastatin, atorvastatin, simvastatin, lovastatin, rosuvastatin, or pravastatin."

Additionally, even if Groups I+ were considered to share the technical features of a method, comprising: determining whether a subject with a primary melanoma has taken inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (a statin), and classifying the subject as: having a relatively lower risk of sentinel lymph node metastasis and/or a relatively positive prognosis if the subject has taken a statin; and having a relatively higher risk of sentinel lymph node metastasis and/or a relatively negative prognosis if the subject has not taken a statin; a method for assessing pre-test probability of a positive sentinel lymph node biopsy (SLNB) for a subject with cutaneous melanoma, comprising: determining whether a subject has taken a statin, and classifying the subject as: having a relatively lower risk of a positive SLNB if the subject has taken a statin; and having a relatively higher risk of positive SLNB if the subject has not taken a statin; an improved method of classifying a subject with melanoma, the improvement comprising taking into account in the classifying whether the subject has a history of taking a statin, wherein: a history of taking a statin classifies the subject as having one or more of: a relatively lower risk of a positive sentinel lymph node biopsy, a relatively low risk of sentinel lymph node metastasis, and a relatively good prognosis; and no history of taking a statin classifies the subject as having one or more of: a relatively higher risk of positive sentinel lymph node biopsy, a relatively high risk of sentinel lymph node metastasis, and a relatively poor prognosis. However, these shared technical features do not represent a contribution over the prior art.

Specifically, US 2020/0318200 A1 to The Brigham And Women's Hospital Inc. discloses a method, comprising: determining whether a subject with a primary melanoma has taken inhibitor (methods for the treatment of subjects identified as at risk of developing metastatic melanoma. In some embodiments, the subject has primary melanoma, Para. [0092]; reduce risk of developing metastatic melanoma, Para. [0093]; among patients undergoing or previously having undergone treatment for melanoma, thereby greatly improving prediction of melanoma recurrence and guiding therapeutic treatment among patients identified as being at risk for melanoma progression, Para. [0006]); and classifying the subject as: having a relatively lower risk of sentinel lymph node metastasis and/or a relatively positive prognosis if the subject has taken a drug; and having a relatively higher risk of sentinel lymph node metastasis and/or a relatively negative prognosis if the subject has not taken a drug (demonstrated the association of lymphocyte infiltration of melanomas with longer progression-free and overall survival8,9,10-15 and other groups have studied "tumor infiltrating lymphocytes," reporting an inverse correlation between TIL grade and presence of sentinel lymph node metastase, Para. [0057]; Patients with low TCFr and higher risk for recurrence may have some benefit, Para. [0065]; [c]lassification or staging of melanomas can be done using, e.g., Breslow classification; TNM (tumor, node, metastasis) system for clinical staging as designated by the American Joint Committee on Cancer (AJCC) staging system, Para. [0067]); a method for assessing pre-test probability of a positive sentinel lymph node biopsy (SLNB) for a subject with cutaneous melanoma (methods for the treatment of subjects identified as at risk of developing metastatic melanoma. In some embodiments, the subject has primary melanoma, Para. [0092]; reduce risk of developing metastatic melanoma, Para. [0093]; among patients undergoing or previously having undergone treatment for melanoma, thereby greatly improving prediction of melanoma recurrence and guiding therapeutic treatment among patients identified as being at risk for melanoma progression, Para. [0006]; TIL grade and presence of sentinel lymph node metastases, Para. [0057]), comprising: determining whether a subject has taken a drug, and classifying the subject as: having a relatively lower risk of a positive SLNB if the subject has taken a drug; and having a relatively higher risk of positive SLNB if the subject has not taken a drug (demonstrated the association of lymphocyte infiltration of melanomas with longer progression-free and overall survival8,9,10-15 and other groups have studied "tumor infiltrating lymphocytes," reporting an inverse correlation between TIL grade and presence of sentinel lymph node metastase, Para. [0057]; Patients with low TCFr and higher risk for recurrence may have some benefit, Para. [0065]; [c]lassification or staging of melanomas can be done using, e.g., Breslow classification; TNM (tumor, node, metastasis) system for clinical staging as designated by the American Joint Committee on Cancer (AJCC) staging system, Para. [0067]); an improved method of classifying a subject with melanoma, the improvement comprising taking into account in the classifying whether the subject has a history of taking a drug, wherein: a history of taking a statin classifies the subject as having one or more of: a relatively lower risk of a positive sentinel lymph node biopsy, a relatively low risk of sentinel lymph node metastasis, and a relatively good prognosis; and no history of taking a statin classifies the subject (methods for the treatment of subjects identified as at risk of developing metastatic melanoma. In some embodiments, the subject has primary melanoma, Para. [0092]; reduce risk of developing metastatic melanoma, Para. [0093]; among patients undergoing or previously having undergone treatment for melanoma, thereby greatly improving prediction of melanoma recurrence and guiding therapeutic treatment among patients identified as

being at risk for melanoma progression, Para. [0006]; TIL grade and presence of sentinel lymph node metastases, Para. [0057])as having one or more of: a relatively higher risk of positive sentinel lymph node biopsy, a relatively high risk of sentinel lymph node metastasis, and a relatively poor prognosis (methods for the treatment of subjects identified as at risk of developing metastatic melanoma. In some embodiments, the subject has primary melanoma, Para. [0092]; reduce risk of developing metastatic melanoma, Para. [0093]; among patients undergoing or previously having undergone treatment for melanoma, thereby greatly improving prediction of melanoma recurrence and guiding therapeutic treatment among patients identified as being at risk for melanoma progression, Para. [0006]; TIL grade and presence of sentinel lymph node metastases, Para. [0057]).

Further, US 2016/0271106 A1 to The Brigham And Women's Hospital Inc. teaches an inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (a statin) (a method for treating cancer in a subject comprising administering to a subject in need thereof a therapeutically effective amount of a statin, Para. [0023]; a composition comprising a statin, Para. [0024]).

The inventions listed in Groups I+ therefore lack unity under Rule 13 because they do not share a same or corresponding special technical features.