## Table 2. Overview of studies providing results on indication of origin for various comprehensive molecular analyses

| | Study<br>reference | Study characteristics (Aim, design, country) | Definition of CUP used | Type of analysis<br>Number of tumor types in classification system | Type of material | Number of CUP patients | | Outcomes | Comments |
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| | | | | | | Total | Number with reportable results (%) | | |
| Gene-expression based assays | | | | | | | | | |
| 1 | Qi, 2022 | To describe the clinicopathological, molecular, and prognostic characteristics of Chinese CUP patients<br>Retrospective single center study<br>China | Definition according to ESMO<br>Patients clinically diagnosed with CUP with histopathologically confirmed metastatic tumors without a detectable tumor tissue of origin after standard evaluation (medical history, physical examination, blood counts, chest-abdomen-pelvis computed tomography scans, and directed assessment of all symptomatic areas | 90-gene expression assay<br>21 tumor types | FFPE | 58 | 53 (91%) | In 53 out of 58 patients (and 100% of patients with reportable results), a primary site was identified.<br>The most common diagnoses were:<br>breast (N = 9, 17%) gastroesophageal (N = 7, 13%) ovary (N = 6, 11%)<br>colorectum (N = 6, 11%)<br>lung (N = 6, 11%) | Authors' conclusion<br>Cancer of unknown primary remains a difficult cancer to diagnose and manage. Our findings improve our understanding of Chinese CUP patient characteristics, leading to improved care and outcomes for CUP patients. |
| 2 | Vibert, 2021 | To develop and evaluate a classifier tool based on the training of a variational autoencoder | Diagnostic workup included standard biological and radiological procedures | RNA sequencing (TransCUPtomics) | Fresh frozen | 48 | 48 (100%)* | The tissue of origin (TOO) could be identified in 38 (79%) of 48 CUP patients. | Authors' conclusion<br>TransCUPtomics confidently |

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| | | to predict tissue of origin based on RNA-sequencing data.<br>Retrospective single center study<br>France | to search for the primary tumor, as well as appropriate extensive pathologic examination and immunohistochemistry (IHC) testing. | 39 tumor types | | | | | predicted TOO for CUP and enabled tailored treatments<br>leading to significant clinical responses. |
| 3 | Ye, 2020 | To develop a gene expression assay for tumor molecular classification and integrate it with clinicopathologic evaluations to identify the tissue origin for cancer of uncertain primary<br>Retrospective multicenter study<br>China | Population not clearly described. Physical examination, imaging, light microscopy, and immunohistochemical (IHC) staining were done at each participating center. | 90-gene expression assay<br>21 tumor types | FFPE | 157 | 141 (90%) | In a real-life cohort of 141 CUP patients, predictions by the 90-gene expression signature were consistent or compatible with the clinicopathologic features in 71.6% of patients (101/141). The most common diagnoses were: breast cancer (26; 18%), lung cancer (14; 10%), pancreatic cancer (11; 8%), hepatocellular carcinoma or cholangiocarcinoma (10; 7%), head and neck carcinoma (9; 6%), colorectal carcinoma (9; 6%), and gastroesophageal carcinoma (8; 6%). | Authors’ conclusion<br>Findings suggest that this novel gene expression assay could efficiently predict the primary origin for a broad spectrum of tumor types and support its diagnostic utility of molecular classification in difficult-to-diagnose metastatic cancer. Additional studies are ongoing to further evaluate the clinical utility of this novel gene expression assay in predicting primary site and directing |

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| | | | | | | | | | therapy for CUP patients. |
| 4 | Thomas, 2018 | To assess the clinical impact of the 92-gene assay on diagnostic and treatment decisions for patients with unknown or uncertain diagnoses.<br>Prospective multicenter study<br>USA | Factors that contributed to an oncologist's decision to order the 92-gene assay were multidisciplinary and included the following: no primary site of origin after clinical review and imaging (42%), a pathology report that indicated a differential diagnosis (21%) or that indicated an unknown primary site (20%), and distinguishing between new cancer versus recurrence (16%). Data that were collected to better characterize the sequence of diagnostic testing demonstrated that 72% of physicians responded that patients had pathology and IHC studies performed before the 92-gene assay, 14% of samples were submitted for pathology and IHC evaluation and the 92-gene assay concurrently, and approximately 14% of samples were submitted without an indication of | 92-gene expression assay (CancerTYPE ID)<br>50 tumor types | Not reported? | 444 | 397 (89%) | Of the 397 patients with sufficient tissue and RNA for a reportable result, the 92-gene assay provided a molecular-based tumor type and histologic subtype diagnosis in 379 patients (95.5%), whereas 4.5% had an indeterminate molecular diagnosis.<br>Across all submitted cases, the assay predicted 22 different tumor types. The most common diagnoses were pancreaticobiliary (21.9%), squamous cell carcinoma (10.1%), lung adenocarcinoma (9.3%), and intestinal (8.6%) type tumors. | Authors' conclusion<br>This study demonstrated that the 92-gene assay affected diagnosis and treatment selection in a significant proportion of patients, which supports the clinical utility of the assay as a standardized molecular approach to help streamline additional diagnostic testing in patients with metastatic cancer with unknown or uncertain diagnoses. |

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| | | | the diagnostic sequence. The most common imaging tests were computed tomography scans (86%), fusion positron emission tomography/computed tomography scans (57%), magnetic resonance imaging (29%), ultrasound (28%), regular film radiographs (11%), or mammogram (11%; data not shown).<br>For pathologists, inconclusive IHC (50%) was the most common reason for ordering the 92-gene assay. In these cases, 90% were submitted for 92-gene assay testing after the first set of IHC stains were performed. The mean number of IHC stains performed before the molecular assay was ordered was 10 (median, nine; range, zero to 23; data not shown). | | | | | | |
| 5 | Greco, 2015 | To identify CUP patients with undifferentiated neoplasms seen at our referral center, and perform gene expression | Twenty-eight of 30 patients presented with advanced cancers (two patients had single-site lesions) and had no anatomical primary site | 92-gene expression assay (CancerTYPE ID) | FFPE | 30 | 29 (97%) | Lineage diagnoses were made by MCCA in 25 of 30 (83 %) patients, including ten carcinomas (three germ cell, two | Authors' conclusion<br>The MCCA provided a specific lineage diagnosis |

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| | | profiling on archived biopsy tissue in an attempt to accurately identify the tumor lineage.<br>Retrospective multicenter study<br>USA | detected after a standard work-up for CUP. | Number of tumor types not described | | | | neuroendocrine, five others), eight sarcomas [three peritoneal mesotheliomas, one primitive neuroectodermal tumor (PNET), four others], five melanomas, and two lymphomas. Additional IHC and genetic testing [BRAF, i(12)p] supported the MCCA diagnoses in 11 of 16 tumors. | and tissue of origin in most patients with PDNs unclassifiable by standard pathologic evaluation. Earlier use of MCCA will expedite diagnosis and direct appropriate first-line therapy, which is potentially curative for several of these tumor types. |
| 6 | Greco, 2013 | To better define the accuracy of the molecular tumor profiling assay and its role in the diagnostic evaluation of cancer of unknown primary patients<br>Prospective (n=151) and retrospective (n=20) single center study<br>USA | Patients where no anatomical primary site was detected after an evaluation consisting of complete history; physical examination; complete blood count; chemistry profile; prostate specific antigen (PSA) in men; urinalysis; computed tomography scans of chest, abdomen, and pelvis; mammography in women; and appropriate additional targeted evaluation of any specific signs or symptoms. | 92-gene expression assay (CancerTYPE ID)<br>26 tumor types | FFPE | 171 | 149 (87%) | In five patients, the assay was successful but was not diagnostic of a single tissue of origin (unclassifiable). In 144 of 149 patients with adequate tumor specimens, a single diagnosis was rendered (96%). Twenty-three tumor types were predicted.<br>Eighteen of 24 patients with latent primaries discovered months to years later had correct diagnoses by MTP (75%), and these diagnoses compared favorably with IHC. Single IHC diagnoses | <u>Authors’ conclusion</u><br>The diagnostic accuracy of this MTP assay was supported by a high level of agreement with identified latent primaries (75%), single IHC diagnoses (77%), and additional directed IHC and/or clinical/histologic findings (74%) prompted by the MTP diagnoses. MTP complements standard pathologic |

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| | | | | | | | | matched MTP diagnoses in 40 of 52 patients (77%). IHC predictions of 2 or more possible primaries compared poorly with MTP diagnoses. However, additional targeted IHC and clinical/histologic evaluation supported the MTP diagnosis in 26 of 35 patients (74%). Clinical features were usually consistent with MTP diagnoses (70%). | evaluation in determining the tissue of origin in patients with CUP, particularly when IHC is inconclusive. |
| 7 | Hainsworth, 2013 | To evaluate the clinical value of molecular tumor profiling to determine the tissue of origin in patients with carcinoma of unknown primary site<br>Prospective trial at 14 sites in the Sarah Cannon Oncology Research Consortium<br>USA | Eligible patients had a diagnosis of CUP after a standard evaluation (medical history, physical examination, blood counts, chemistry profile, chest/ abdomen computed tomography scans, positron emission tomography scan, and directed evaluation of all symptomatic areas). | 92-gene expression assay (CancerTYPE ID)<br>Type I of the assay: 26 tumor types (n=61)<br>Type II of the assay: 50 tumor types (n=228) | FFPE | 289 | 252 (89%) | The molecular profiling assay predicted a tissue of origin in 247 (98%) of 252 patients. Twenty-six different tissues of origin were predicted. The four most commonly predicted tissues of origin were biliary tract, urothelium, colorectal, and non– small-cell lung cancer (NSCLC); these sites accounted for 55% of all patients. One hundred nineteen assay predictions (48%) were made with 80% probability, whereas 128 (52%) were made with less than 80% probability. | <u>Authors' conclusion</u><br>In this large prospective trial, molecular tumor profiling predicted a tissue of origin in most patients with CUP. The median survival time of 12.5 months for patients who received assay-directed site-specific therapy compares favorably with previous results using empiric CUP regimens. Patients with CUP predicted to have more responsive tumor types had longer |

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| | | | | | | | | | survival compared with patients with less responsive tumor types. Molecular tumor profiling contributes to the management of patients with CUP and should be a part of their standard evaluation. |
| 8 | Greco, 2010 | To evaluate the accuracy of tissue-of-origin prediction by molecular profiling in patients with carcinoma of unknown primary site<br>Retrospective multicenter study<br>USA | All patients fulfilled our definition of CUP and had no detectable primary site after a diagnostic evaluation consisting of: a complete history, physical examination, CBC, chemistry profile, and computed tomography (CT) scans of the chest, abdomen, and pelvis; mammography in women; and evaluation of serum prostate-specific antigen (PSA) in men. | 92-gene expression assay (Cancer Type ID)<br>39 tumor types | FFPE | 28 | 20 (71%) | In 18 out of 20 patients (90%) a prediction of the site of origin was available.<br>Fifteen of the 20 assay predictions (75%) were correct (95% confidence interval, 60%–85%), corresponding to the actual latent primary sites identified after the initial diagnosis of CUP. Primary sites correctly identified included breast (four patients), ovary/primary peritoneal (four patients), non-small cell lung (three patients), colorectal (two patients), gastric (one patient), and melanoma (one patient). Three predictions were incorrect (intestinal, | <u>Authors' conclusion</u><br>These data validate the reliability of this assay in predicting the primary site in CUP patients and may form the basis for more successful site-directed therapy, when used in concert with clinicopathologic data. |

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|  |  |  |  |  |  |  |  | testicular, sarcoma) in patients with gastro-esophageal, pancreatic, and non-small cell lung cancer, respectively, and two were unclassifiable in patients with non-small cell lung cancer. |  |
| 9 | Monzon, 2010 | To evaluate the clinical utility of the TOO test (a microarray-based gene expression test) in identifying the primary site in specimens from patients diagnosed with CUP<br>Retrospective study using specimens from several tissue banks<br>USA | Most patients whose specimens were included had received a complete history, physical, and full clinical, laboratory, imaging, and pathologic workups, including standard histologic and IHC examination, prior to their designation as CUP. | Pathwork® TOO Test (gene expression test of 1550 genes)<br>15 tumor types | Fresh-frozen | 21 | 21 (100%) | The TOO Test yielded a clear single positive call for the primary site in 16 of 21 (76%) specimens and was indeterminate in 5 (24%). The positive results were consistent with clinicopathologic suggestions in 10 of the 16 cases (62%). In the remaining six cases the positive results were considered plausible based on clinical information. Positive calls included colorectal (5), breast (4), ovarian (3), lung (2), and pancreas (2). The TOO Test ruled out an average of 11 primary tissues in each CUP specimen. | Authors' conclusion<br>The Pathwork TOO Test reduced diagnostic uncertainty in all CUP cases and could be a valuable addition or alternative to current diagnostic methods for classifying uncertain primary cancers. |
| 10 | Morawietz, 2010 | To compare results and feasibility of GEP with results from an experienced histopathological institute utilizing a broad | Complete history, physical examination, chest X-ray, CT scan of chest and abdomen, upper intestinoscopy, colonoscopy, | Gene expression profiling (CupPrint array, 495 genes) | FFPE | 42 | 24 (57%) | In 13 out of the remaining 24 cases (54%), the same primary was proposed by IHC and GEP and was supported by the clinical findings, | Authors' conclusion<br>Overlap between IHC and GEP results and the clinical presentation was |

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| | | spectrum of immunohistochemical markers on patients with true CUP syndrome.<br>Prospective multicenter study (analysis of samples from patients who participated in a randomized phase II trial)<br>Germany | mammography (in women), PSA, AFP, and hCG (in men), and directed workup of symptomatic areas were mandatory before inclusion in the RCT. | 49 tumor types | | | | furthermore leading to the doubtless identification of the primary in four out of these. In seven cases, there was discordance between IHC and GEP, with the clinical picture being more in line with IHC in three and with GEP in four cases. Four cases had to remain undecided because the primary tumors suggested by IHC and GEP were not supported. | noted in the majority of those true CUP cases that were evaluable with both techniques. Therefore, GEP can be a complementary diagnostic technique assisting immunohistochemical profiling of cancer biopsies with unknown primary. |
| Micro-RNA based assays | | | | | | | | | |
| 11 | Pentheroudakis, 2013 | To test the performance of a microRNA-based assay in formalin-fixed paraffin-embedded samples from 84 CUP patients<br>Retrospective multicenter study<br>Greece | Patients diagnosed with CUP according to a standardized clinico-pathologic diagnostic algorithm | 64 microRNA assay<br>42 tumor types | FFPE | 92 | 84 (91%) | The microRNA based assay agreed with the clinical diagnosis at presentation in 70% of patients; it agreed with the clinical diagnosis obtained after patient management, taking into account response and outcome data, in 89% of patients; it agreed with the final clinical diagnosis reached with supplemental immunohistochemical stains in 92% of patients, indicating a 22% | <u>Authors’ conclusion</u><br>This novel microRNA-based assay shows high accuracy in identifying the final clinical diagnosis in a real-life CUP patient cohort and could be a useful tool to facilitate administration of optimal therapy. |

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|  |  |  |  |  |  |  |  | improvement in agreement from diagnosis at presentation to the final clinical diagnosis. In 18 patients the assay disagreed with the presentation diagnosis and was in agreement with the final clinical diagnosis, which may have resulted in the administration of more effective chemotherapy. In three out of four discordant cases in which supplemental IHC was performed, the IHC results validated the assay's molecular diagnosis. |  |
| 12 | Varadhachary, 2011 | To prospectively evaluate the clinical utility of ToO predictions generated by this micro-RNA-based assay for metastases in CUP patients in the context of currently available immunohistochemistry (IHC) and clinicopathologic "working diagnoses"<br>Prospective single center study | All patients were diagnosed with CUP at presentation, in that a primary cancer was not detected after a complete history and physical examination, detailed laboratory studies, imaging, and when indicated, invasive studies including endoscopy and colonoscopy as directed by symptoms, signs, and pathology. | microRNA-based assay (48 microRNAs)<br>25 tumor types | FFPE | 87 | 74 (85%) | The assay result was consistent or compatible with the clinicopathologic features in 84% of cases processed successfully (71% of all samples attempted). In 65 patients, pathology and immunohistochemistry (IHC) suggested a diagnosis or (more often) a differential diagnosis. Out of those, the assay was consistent or compatible with the | <u>Authors' conclusion</u><br>In this prospective study, the microRNA diagnosis was compatible with the clinicopathologic picture in the majority of cases. Comparative effectiveness research trials evaluating the added benefit of molecular profiling |

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| | | USA | | | | | | clinicopathologic presentation in 55 (85%) cases. Of the 9 patients with noncontributory IHC, the assay provided a ToO prediction that was compatible with the clinical presentation in 7 cases. | in appropriate CUP subsets are warranted. MicroRNA profiling may be particularly helpful in patients in whom the IHC profile of the metastasis is nondiagnostic or leaves a large differential diagnosis. |
| DNA sequencing assays | | | | | | | | | |
| 13 | Schipper, 2022 | To investigate the clinical value of whole genome sequencing (WGS) in terms of primary tumor identification and detection of actionable events, in the routine diagnostic work-up of CUP patients.<br>Retrospective single center study<br>The Netherlands | Patients with a clinical diagnosis of CUP, for whom extensive pathological, radiological, and endoscopic modalities failed to identify a primary tumor type | WGS (CUPPA algorithm)<br>29 tumor types | Fresh frozen | 72 | 72 (100%)* | When integrated in the diagnostic work-up of CUP patients, CUPPA could identify a primary tumor type for 49/72 patients (68%). | Authors' conclusion<br>Genome-based tumor type prediction can predict cancer diagnoses with high accuracy when integrated in the routine diagnostic work-up of patients with metastatic cancer. With identification of the primary tumor type in the majority of patients and detection of actionable events, WGS is a valuable |

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| | | | | | | | | | diagnostic tool for patients with CUP. |
| DNA methylation assays | | | | | | | | | |
| 14 | Moran, 2016 | To examine the feasibility of using DNA methylation profiles to determine the occult original cancer in cases of cancer of unknown primary.<br>Retrospective and prospective multicenter study<br>USA, Spain, Germany, Italy, Australia | Cancers of unknown primary was defined following the European Society of Medical Oncology guidelines as metastatic tumours for which the standardised diagnostic work-up failed to identify the site of origin at the time of diagnosis. Each health center had their own cancer of unknown primary institutional diagnostic work-up.<br>Molecular screening of alterations in the main oncodrivers, and immunohistochemical stainings routinely analysed in clinical care were done at each participating centre. | DNA methylation based assay<br>38 tumor types | FFPE or fresh frozen | 216 | 216 (100%)* | DNA methylation profiling predicted a primary cancer of origin in 188 (87%) of 216 patients with cancer with unknown primary.<br>The six most commonly predicted tissues of origin were non-small-cell lung carcinoma (NSCLC; 39 [21%] of 188), head and neck squamous cell carcinoma (18 [10%]), breast carcinoma (17 [9%]), colon carcinoma (16 [9%]), hepatocellular carcinoma (14 [7%]), and pancreatic carcinoma (14 [7%]). | Authors' conclusion<br>We show that the development of a DNA methylation based assay can significantly improve diagnoses of cancer of unknown primary and guide more precise therapies associated with better outcomes. Epigenetic profiling could be a useful approach to unmask the original primary tumour site of cancer of unknown primary cases and a step towards the improvement of the clinical management of these patients. |
| Combination or comparison of different assays | | | | | | | | | |
| 15 | Posner, 2023 | To compare the diagnostic utility of RNA and DNA tests in 215 CUP patients (82% | Patients presenting with carcinoma of no confirmed primary site and who had a | RNA and DNA tests | FFPE tumour blocks or unstained | 215 | 215 (100%)* | Classification performance in clinicopathology-resolved CUPs: 80% had | Authors' conclusion<br>In conclusion, DNA and RNA profiling |

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|  |  | received both tests) in a prospective Australian study<br>Prospective multicenter study<br>Australia | preliminary diagnostic workup including, but not limited to, a detailed clinical assessment; a CT scan of the chest, abdomen, and pelvis; pathological review of tumour tissue; and other gender-appropriate diagnostic tests; | RNA: microarray [CUPGuide, numer of tumor types unknown] or custom Nanostring 18-class GEP test (18 tumor types)<br>DNA: Comprehensive DNA panel sequencing of 386 cancer-related<br>genes<br>(22 tumor types) | sections, blood |  |  | high–medium predictions and 94% were concordant with pathology. Notably, only 56% of the clinicopathology-unresolved CUPs had high–medium confidence GEP predictions. Among the clinicopathology-unresolved CUPs, mutations and mutational signatures provided additional diagnostic evidence in 31% of cases. GEP classification was useful in only 13% of cases and oncoviral detection in 4%. Among CUPs where genomics informed TOO, lung and biliary cancers were the most frequently identified types, while kidney tumours were another identifiable subset. We showed that DNA and RNA tests help to resolve a third of CUP cases where clinicopathological data alone were insufficient to designate a likely TOO diagnosis. Importantly, despite GEP being the most commonly explored molecular diagnostic test | supported an unconfirmed TOO diagnosis in one-third of CUPs otherwise unresolved by clinicopathology assessment alone. DNA mutation profiling was the more diagnostically informative assay. |

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| | | | | | | | | for CUP to date, we found that DNA sequencing may be of greater diagnostic value, as many CUP tumours appear to have an atypical transcriptional profile yet retain identifiable and compelling diagnostic mutational features. | |
| 16 | Möhrmann, 2022 | To describe a cohort of 70 CUP patients characterized by comprehensive molecular profiling within the MASTER program of the National Center for Tumor Diseases and the German Cancer Consortium (NCT/DKTK) combining whole-exome/ genome sequencing, transcriptome and methylome analysis in a clinical workflow to identify therapeutic targets.<br>Retrospective multicenter study | Seventy CUP patients were included of whom 61 met the criteria defined by the ESMO clinical practice guideline. In the remaining nine cases documentation of necessary initial imaging procedures was lacking (such as CT scans of thorax, abdomen and pelvis). | RNA sequencing (n=70, 100%)<br>Methylome analysis (n=70, 100%) | Fresh frozen, FFPE, peripheral blood | 70 | RNA sequencing: 55 (79%)<br>Methylome analysis: 55 (79%) | Transcriptome and methylome analysis provided evidence for the underlying entity in 62/70 (89%) patients. In 48 patients, classification was possible by both transcriptome and methylome analysis, however in only 20 patients the same entity was predicted by both methods | <u>Authors' conclusion</u><br>Our findings indicate that comprehensive molecular analysis of CUP patients can be highly beneficial even at late stages or following several rounds of prior treatment.<br>We provide valuable insight into the heterogenic genomic, transcriptomic and epigenetic landscape of CUP and show potentially actionable alterations in a large proportion of patients. Further prospective clinical studies to assess |

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| | | Germany | | | | | | | the impact of genomics-based personalized cancer therapy are warranted. |

**** No failed analyses are reported in the paper, however it is not clear whether only successfully analyzed patients are included in the study***

***CUP: Cancer of unknown primary***

***FFPE: Formalin-Fixed Paraffin-Embedded***

***TOO: Tissue of Origin***