Table 1. Overview of studies providing results

	Study reference	Study characteristics (Aim, design, country)	Definition of CUP used	Type of analysis Number of genes	Type of material	Number of CUP patients		Outcomes	Comments
						Total	Number with reportable results (%)		
Next generation sequencing									
1	Review Lombardo (2020) including 11 original studies: 1. Tothill (2013) 2. Gatalica (2014) 3. Ross (2015) 4. Löffler (2016) 5. Kato (2017) 6. Subbiah (2017) 7. Varghese (2017) 8. Clynick (2018) 9. Gatalica (2018) 10. Varghese (2015)	To systematically review the genomic alterations that could be targeted with approved/off-label/in clinical trials drugs in patients with cancer of unknown primary. Systematic review (search performed in June 2019) Italy	No definition provided	Next generation sequencing (NGS) Number of genes ranged between 47 and 701	Not reported in review, apart from one study that used circulating tumor DNA (ctDNA)	3130 (range 16-1806)	Not reported in review	A targetable alteration was identified in a mean of 47.3% of patients with cancer of unknown primary. The most frequent alterations included TP53 (41.9%), KRAS (18.8%), CDKN2A (8.8%) and PIK3CA (9.3%).	The search does not meet the criteria for a comprehensive and systematic search: only one database, and limited number of search terms. Relevant studies may have been missed. No assessment of scientific quality of included studies. <u>Authors' conclusions:</u> Results of this systematic review from nine published studies and two abstracts show 47.3% of patients with CUP present a potentially targetable alteration for which approved/off-label/in

	11. Chandler (2016)								clinical trials drugs are available. The most frequent alterations were found in *TP53, KRAS, CDKN2A, PIK3CA*; interestingly none of the patients had two identical molecular profiles underlying the assumption that CUPs are an individually heterogeneous molecular and clinical entity. NGS represents a chance, although not validated by clinical trials, to improve diagnosis and matched treatment of potential actionable molecular alterations.
2	Yang (2022)	To use targeted gene sequencing to provide more insights into treatment options for patients with cancer of unknown primary Retrospective multicenter study	CUP was defined as follows: 'a histologically proven metastatic malignant tumour whose primary site cannot be identified during pretreatment evaluation. Patient follow-up has also been performed to confirm that the primary tumours remained	Next generation sequencing (NGS) Target gene panel of 416 genes	Peripheral blood, FFPE tumour tissue or fresh tumour tissues	35	Results reported for 35 patients. Two patients were excluded due to the lack of evaluable tumor tissue and plasma or the difference in	*Targetable mutations* Targetable mutations (according to the OncoKB database) were found in 8 out of 35 patients (23%) and included:	<u>Authors' conclusion:</u> The genomic landscape of Chinese CUP patients was similar to the Western population with high frequent alterations in TP53 and KRAS. Interestingly, the incidence of SMAD4

		China	unknown until the time of this study'. Note: It is not clear from this definition which initial diagnostic tests were performed.				the targeted sequencing panel used.	KRAS G12C (n=3; 9%) PIK3CA E545K (n=2; 6%) PIK3CA E542K (n=1; 3%) BRAF V600L (n=1; 3%) EML4-ALK (n=1; 3%) ALK L1196M (n=1; 3%) *Copy number variation (CNVs)* MYC amplification was the most frequently observed CNV (23%), while other frequently observed CNVs included CDKN2A deletion (15%) and ZNF217 amplification (15%). In one patient, a EML4-ALK fusion was identified. *Tumor mutational burden* For the 26 patients with tumor tissue available, the median	alteration (23% in tissue, 14% in plasma) was higher in the Chinese population compared to the Western cohort (6%) (8). The enrichment of TP53, RTK- RAS, and PI3K signaling pathways indicated the possibility of targeted therapies such as RTK and PI3K inhibitors in CUP patients. Further study is warranted to validate our observation and evaluate the efficiency of target therapy and immunotherapy in CUP cohort besides the standard chemotherapy.

								tumor mutational burden (TMB) was 12.5 mutations/Mb. *Microsatellite instability* For all 26 patients, microsatellite instability (MSI) analysis showed that all tissue specimens were microsatellite stable.	
3	Kang (2021)	To examine the clinical and molecular characteristics, treatment patterns, survival outcomes, and efficacy of NGS and targeted therapy in patients with CUP in a real-world setting Retrospective study South Korea	All patients had undergone medical history taking, physical examination, baseline blood and biochemistry analyses, imaging studies including chest and abdomen-pelvis computed tomography (CT), and biopsy. Additionally, 206/218 patients (94%) had undergone (PET)/CT.	Next generation sequencing (NGS) Approximately 300 cancer-related genes	Lymph nodes (59%) Bone (18%) Lung (9%)	218 out of which 22 (10%) underwent NGS	Level 1/2/3/4 alterations level of clinical actionability according to OncoKB 2/22 (9% patients received targeted therapy based on the NGS results		

							(NTRK fusion – entrectinib and AKT2 gain mutation – ipatasertib)		
4	Hayashi (2020)	To assess the clinical use of site-specific treatment, including molecularly targeted therapy based on NGS results, for patients with CUP Phase II multi-centre non-randomized clinical trial Japan	Eligibility criteria included a diagnosis of unfavorable CUP after mandatory examinations, including pathological evaluation by immunohistochemistry, chest-abdomen-pelvis computed tomography scans, and a positron emission tomography scan.	Next generation sequencing (NGS) 257 genes	FFPE tumor tissue samples	111	111 (100%)	Out of 111 patients, 97 received site-specific therapy. Among these 97 patients, the most common genetic alterations were: TP53 (n=45; 46%), KRAS (n=19; 20%), CDKN2A (n=18; 19%) Several genetic driver mutations with implications for treatment were also detected, including those affecting EGFR and KRAS (geen verdere data gepresenteerd)	<u>Authors' conclusion</u> This study's findings suggest that site-specific treatment, including molecularly targeted therapy based on profiling gene expression and gene alterations by NGS, can contribute to treating patients with the unfavorable subset of CUP.
4	Bochtler, 2020	To analyse the prognostic role of mutations and copy number variations	The diagnosis of CUP was revisited in each patient	Targeted next generation	FFPE tumor	252, including 181 (72%)	Not reported?	Most frequent genetic alterations were mutations/deletions	<u>Authors conclusion</u> Besides the identification of drug

		(CNVs) detected in CUP specimens in the context of a comprehensive clinicopathological risk assessment and a meticulous collection of clinical follow-up data. Partly prospective, partly retrospective single center cohort study Germany	according to ESMO guideline	sequencing (NGS) Number of genes? Verschillende panels gebruikt	tissue samples	of patients with unfavorable CUP according to ESMO guidelines		of: TP53 (n=125/252, 50%) CDKN2A (n=48/252, 19%) NOTCH1 (n=33/234, 14%) BAP1 (n=13/165, 8%) STK11 (n=20/252, 8%) SMAD4 (n=16/222, 7%) RB (n=16/252, 6%) PTEN (n=16/252, 6%) TSC2 (n=11/95, 6%) as well as oncogenic activation of: KRAS (n=59/252, 23%) FGFR4 (n=28/188, 15%) PIK3CA (n=27/252, 11%) MYC (n=15/188, 8%) TERT (n=12/177, 7%) AKT1 (n=14/234, 6%) CCND1 (n=11/188, 6%)	targets, panel sequencing in CUP is prognostically relevant, with RAS activation and CDKN2A deletion emerging as novel independent risk factors in a comprehensive assessment with clinicopathological data.

								ERBB2 (n=14/252, 6%)	
5	Bochtler, 2022	To characterize the chromosomal aberration pattern in CUP depending on histological and clinical features and to assess its prognostic impact together with chromothripsis, tumor mutational burden (TMB), microsatellite instabil- ity (MSI), and mutational profiles as potential prognostic biomarkers	Diagnosis of CUP was made according to the ESMO guidelines	Next generation sequencing (NGS) 500 genes?	Not reported	74	For 74 patients TMB values were reported In 59/74 patients, material for CNV was available	*Tumor mutational burden (TMB)* TMB values displayed a wide heterogeneity ranging from 0 to 77.58 mutations/Mb with a median of 4.71 mutations/Mb. Low (<6 mutations/MB): n=45 (61%) Intermediate (≥6 to <12 mutations/Mb): n=18 (24%) High (≥12 mutations/Mb): n=11 (15%) *Copy number variation (CNVs)*	<u>Authors conclusion</u> Overall, CNV, chromothripsis, TMB, and MSI profiles in CUP are reminis- cent of biological characteristics known from other cancer entities without a unifying CUP-specific signature. Markedly, high-level CNV loss is an adverse predictive bio- marker in localized but not disseminated chemotherapy-treated CUP. This implies that chromosomal losses drive CUP progression, but also increase susceptibility to chemotherapy, with both effects apparently leveling out in disseminated CUP.

								The median amount of CNV gains and losses was 168 Mb (range: 0–1217) and 282 Mb (range: 0–1983), respectively.	
6	Posner (2023)	To compare the diagnostic utility of RNA and DNA tests in 215 CUP patients (82% received both tests) in a prospective Australian study Prospective multicenter study Australia	Patients presenting with carcinoma of no confirmed primary site and who had a 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 and DNA tests RNA: microarray [CUPGuide, numer of tumor types unknown] or custom Nanostring 18-class GEP test (18 tumor types) DNA: Comprehensive DNA panel sequencing of 386 cancer-related genes (22 tumor types)	FFPE tumour blocks or unstained sections, blood	215	215 (100%)*	Classification performance in clinicopathology-resolved CUPs: 80% had 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	<u>Authors' conclusion</u> In conclusion, DNA and RNA profiling 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.

								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 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.	
7	Möhrmann (2022)	To describe a cohort of 70 CUP patients characterized by comprehensive molecular profiling within the MASTER	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	RNA sequencing (n=70, 100%) Methylome analysis (n=70, 100%)	Fresh frozen, FFPE, peripheral blood	70	RNA sequencing: 55 (79%)	Transcriptome and methylome analysis provided evidence for the underlying entity in 62/70 (89%) patients. In 48 patients, classification was	<u>Authors' conclusion</u> Our findings indicate that comprehensive molecular analysis of CUP patients can be highly beneficial even

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Richtlijn Primaire tumor onbekend (PTO) 2025

		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. Retrospective multicenter study Germany	necessary initial imaging procedures was lacking (such as CT scans of thorax, abdomen and pelvis).				Methylome analysis: 55 (79%)	possible by both transcriptome and methylome analysis, however in only 20 patients the same entity was predicted by both methods	at late stages or following several rounds of prior treatment. 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 the impact of genomics-based personalized cancer therapy are warranted.
8	Es (2021)	To analyse a large database of CUP samples to identify genomic mutations and copy number alterations to determine potentially druggable targets Retrospective study	Any metastatic epithelial tumour where, following extensive clinical history, physical examination, radiological studies and histopathological investigations, failed to identify the primary site of tumours	Mutation profile analysis: the genomic mutations and copy number alterations of 1709 CUP samples were analyzed, data form a public source.	Part of the GENIE project. Not specified in this paper.	1709		We identifed 52 signifcant mutated genes (SMGs) among CUP samples, in which 13 (25%) of SMGs were potentially targetable with either drugs are approved for the know primary tumour or undergoing clinical trials. The most variants detected were TP53 (43%), KRAS (19.90%), KMT2D (12.60%), and CDKN2A	

		Iran/sponsored by the American Association of Cancer Research		52 significant mutated genes (SMGs) were identified.				(10.30%). Additionally, using pan-cancer analysis, we found similar variants of TERT promoter in CUP and NSCLC samples, suggesting that these mutations may serve as a diagnostic marker for identifying the primary tumour in CUP.	