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Τίτλος Διπλωματικής Εργασίας: "Εκτίμηση της ποιότητας μελετών που δημοσιεύτηκαν μετά το 2005 κι ερευνούν τη διαγνωστική ακρίβεια του Συνδρόμου Lynch, χρησιμοποιώντας τα κριτήρια STARD."

Master of Science Thesis: "Assessment of the reporting quality of studies published after 2005, which investigate the diagnostic accuracy of MMR deficiency in the diagnosis of Lynch Syndrome, using the STARD statement"

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Περίληψη

Εισαγωγή: Η πρώιμη διάγνωση του συνδρόμου Lynch είναι πολύ σημαντική για τον προσυμπτωματικό έλεγχο, την πρόληψη και τη γενετική συμβουλευτική των ασθενών αυτών και των οικογενειών τους. Πολλές κλινικές μελέτες έχουν διεξαχθεί, κυρίως σε πληθυσμούς με κολο-ορθικό καρκίνο και καρκίνο ενδομητρίου, προκειμένου να βρεθεί η καταλληλότερη μέθοδος ανίχνευσης εκείνων των ασθενών που είναι πιο πιθανό να έχουν το σύνδρομο. Κλινικά κριτήρια, μοριακός έλεγχος του όγκου και γενετικός έλεγχος του ασθενή έχουν χρησιμοποιηθεί.

Στόχοι: Να ερευνηθεί η διαγνωστική ακρίβεια των μελετών που προσπαθούν να ανιχνεύσουν την καλύτερη μέθοδο διαλογής εκείνων των ασθενών που πρέπει να υποβληθούν σε γενετικό έλεγχο για τη διάγνωση του συνδρόμου Lynch.

Μέθοδοι: Έγινε συστηματική ανασκόπηση της διεθνούς βιβλιογραφίας για τις μελέτες διαγνωστικής ακρίβειας του συνδρόμου Lynch σε πληθυσμούς κολο-ορθικού καρκίνου και καρκίνου του ενδομητρίου. Απαραίτητη προϋπόθεση ήταν οι μελέτες αυτές να είναι προοπτικές, να έχουν δημοσιευτεί μετά το 2005 και να χρησιμοποιούν μεθόδους ανοσοϊστοχημείας ή /και αλυσιδωτής αντίδρασης πολυμεράσης για την ανίχνευση φαινοτύπου ανεπάρκειας των πρωτεϊνών που συμμετέχουν στο σύστημα επιδιόρθωσης βλαβών στο γενετικό υλικό. Για την αξιολόγηση των μελετών χρησιμοποιήθηκαν τα κριτήρια STARD

Αποτελέσματα: Όσο αφορά τη διάγνωση του συνδρόμου Lynch, οι εργαστηριακές τεχνικές είτε με ανοσοϊστοχημεία, είτε με τη μέθοδο της αλυσιδωτής αντίδρασης πολυμεράσης που ανιχνεύουν την ακεραιότητα των πρωτεϊνών που συμμετέχουν στο μηχανισμό επιδιόρθωσης του γενετικού υλικού ή την ύπαρξη μικροδορυφορικής αστάθειας στον όγκο αντίστοιχα, υπερτερούν ως προς την ευαισθησία, την ειδικότητα και την αρνητική προγνωστική αξία έναντι των κλινικών κριτηρίων, που βασίζονται κυρίως στην ηλικία, το ατομικό και το οικογενειακό ιστορικό, τα κριτήρια Amsterdam και Bethesda. Η εξέταση στον όγκο της ύπαρξης επιγενετικής υπερμεθυλίωσης στο υποκινητή του MLH1 γονιδίου ή μετάλλαξης στο γονίδιο BRAFV600E θα μπορούσε να βελτίωσει περαιτέρω τη διαλογή των ασθενών που πρέπει να παραπεμφθούν για γενετική εξέταση και συμβουλευτική για το σύνδρομο Lynch. Η τήρηση των κριτηρίων STARD φαινεται να τηρειται στις συμπεριληφθείσες μελέτες σε ποσοστό από 47 έως 73%.

Συμπέρασμα: Ο καθολικός έλεγχος στο μηχανισμό επιδιόρθωσης του γενετικού υλικού ή στην ύπαρξη μικροδορυφορικής αστάθειας στον όγκο οδηγεί σε πιο ακριβή κι έγκαιρη αναγνώριση των ασθενών με πιθανό σύνδρομο Lynch, σύμφωνα με τις μελέτες που εξετάστηκαν με τα κριτήρια STARD.

Abstract

Background: Strategies for an early diagnosis of Lynch Syndrome are crucial for the screening, prevention and genetic counseling of susceptible patients and their families. Many studies have been conducted, mainly in colorectal and endometrial cancer

populations, in order to identify the best screening method for Lynch Syndrome, using a combination of clinical criteria, tumor and germline testing approaches.
Objective: To investigate how accurate MMR deficiency recognition is as a screening test before germline diagnosis of Lynch syndrome, using either IHC test for MMR genes or MSI test, and to compare it with the clinical criteria.
Methods: Systematic reviews were conducted of the published literature on diagnostic test accuracy studies of IHC and/or MSI testing for LS, as screening methods for LS. Prospective data after 2005 in CRC and EC populations were included. For the evaluation of the studies the STARD Statement was used.
Results: Overall the compliance of the studies to the standards of the STARD checklist ranges from 47% tom73%. Regarding LS screening strategies, immunochemistry for the identification of MMR gene status and PCR techniques for the identification of MSI status proved to have a higher sensitivity, specificity and negative prognostic value comparing with clinical criteria, such as age, personal and family history, the Amsterdam and the Bethesda criteria, especially for the CRC samples. MLH1 methylation and BRAF V600E testing could even improve the identification of these patients who must refer to a germline test and a genetic counseling.
Conclusion: A universal screening for MMR phenotype in CRC and EC could lead to a more accurate and earlier diagnosis of LS, according on STARD Statement evaluation of the studies used in the present analysis.

2. INTRODUCTION

Lynch syndrome is the most common inherited cause of colorectal cancer (CRC). It is inherited with an autosomal dominant way and instead of CRC, it increases the risk of endometrial cancer as well as of ovary, stomach, small bowel, hepatobiliary system, renal pelvis and ureter, brain (glioma), and sebaceous neoplasms. It is caused by a germline mutation in one of the DNA Mismatch Repair genes, MLH1, MSH6, PMS2 or by an inactivation of the MSH2 gene[1,2]. The most common cause of inactivation of MSH2 is a mutation in the EPCAM gene[1,4]. PMS2 is the most common mutation found in LS, however MLH1 and MSH2 are the riskiest for CRC[2,3]. A mutation in the EPCAM gene is the less common cause and it is responsible for only 1-2% of the cases[4].

The first description of the disease, which was previously known as hereditary nonpolyposis colorectal cancer (HNPCC), was made in the beginning of the 20th century by Aldred Scott Warthin, a "father of cancer genetics"[6]. The diagnosis was initially based only on clinical criteria linked to the family history and the Amsterdam criteria, which were established in 1991 and are still used, slightly modified, to identify the possible carriers of Lynch syndrome[5].

The molecular base of the syndrome was first implied two years later by Peltomäki et al and Lindblom et al[6,7,8]. Then, the first referral to errors in DNA replication as a potent cause of CRC was made and called "Replication error" phenotype, which represent what we today know as Microsatellite instability. This phenotype leads to the creation of certain repetitive DNA motifs, and it was described more often among familiar CRC in comparison with sporadic cases[9,10]. A defect locus in chromosome 2 and 3 was identified and finally, the first two genes MSH2 and MLH1 with a causative relationship of the Lynch syndrome were found out[9,11,12]. The genes PMS2 in chromosome 7 and MSH6 in chromosome 2, were added some years later and the four building proteins which constitute the DNA Mismatch Repair System (MMR) and whose deficit creates the phenotype of LS were established [13,14]. EPCAM gene, which is located in a neighboring position with MSH2, does not belong to MMR system, however, during the first decade of 2000 it was found that EPCAM deletions can silence MSH2 and cause LS. What is important is the fact that in this case the MMR genes are intact. Moreover, as EPCAM deletions are happening epigenetically, patients with this phenotype express a variation in the expression of MSH2 and are more prompt to colon and endometrial cancer[15,16].

The inactivation of the MMR system requires a loss of both alleles of at least one of the before mentioned genes. Patients with LS must have a germline mutation in one of the alleles and the second "hit" comes from a somatic mutation in the other allele or from the epigenetic silencing of the promoter of the gene (hypermethylation)[17]. The created error produces a problematic DNA repair system, which results in regions of repetitive nucleotide sequences in DNA tracts. These regions are called microsatellites.

The diagnosis of LS is based on the identification of the germline mutation in the related genes with Next Generation Sequencing (NGS) techniques. Genetic testing is not a routine

test in patients with CRC. However, there are indirect factors which may indicate the existence of the syndrome, such as the family history, the age of the cancer onset, other extracolonic cancers in the same individual that belong in the range of LS, as well as some characteristics of the colon cancer itself, such as microsatellite instability. Based on clinical information like this, the Amsterdam and the Bethesda criteria have been set up, for the screening of patients and their families for LS[18,19].

As mentioned before, one of the hallmark characteristics of the LS related CRC is the presence of high MSI. Although genetic tests are not a daily practice for CRC, MSI testing it is a routine for the majority of cases with locally advanced and metastatic disease and, sometimes, also for the early disease, because of its prognostic and its predictive value. But MSI can not only be caused by the genetic deficit in at least one the MLH1, MSH2, MSH6 and PMS genes. The result of a deletions in EPCAM gene have been already discussed previously, but there is also epigenetic inactivation of the MLH1 gene through hypermethylation of its promoter may also cause microsatellites in the DNA and consequently the phenotype of an MSI high tumor is arisen, without a background of LS[20]. BRAF testing, another common molecular test in CRC cases, or/and MLH1 promoter methylation could be used to distinguish clearly sporadic from potentially LS in MLH1 negative patients [21,22,23].

Many studies have been conducted with a view to find out the best screening strategy for the identification of these population who should be referred for a germline mutation test and consequently a genetic counselling. Age of cancer diagnosis, a family history, a personal history, Amsterdam criteria, Bethesda guidelines, tumor histology and many other clinical characteristics, as well as, the most objective tests of MMR and MSI status, MLH1 methylation or BRAF V600E mutation or a combination of these information have been examined as possible screening strategies for the diagnose of LS. To assess the utility of each diagnostic test statistical methods, such as sensitivity, specificity, positive and negative predictive value are a prerequisite[24]. But this is not sufficient as diagnostic studies may have many biases in their design, their methods, their statistical analyses, their objectivity. As more and more studies are publishing, additional to statistics standards, worldwide recognized objective tools have been created and established, to facilitate the evaluation of the quality, the credibility and the possible bias of such studies. CASP (Critical Appraisal Skills Programs) diagnostic, CHARMS (CHecklist for critical Appraisal and data extraction for systematic Reviews of prediction Modelling Studies) checklist, the PROBAST (Prediction model Risk of Bias ASsessment Tool) form and the STARD (STAndards for the Reporting of Diagnostic accuracy studies) statement are some of them[25].

The main objective of this analysis is to report the quality of studies which investigate how accurate MMR deficiency recognition is, either with IHC test for MMR genes or with MSI test, as screening test for the diagnosis of Lynch syndrome and to compare it with the clinical criteria.

3. METHODS

3.1 Search and Evaluation Strategy

A systemic electronic research of the published literature was conducted using the PubMed, EMBASE, Cochrane and UPTODATE databases. Studies such as reviews, meta-analysis, case reports were excluded and there was a focus in cohort studies of diagnostic accuracy.

The study selection was at first made by retrieving information from the title and abstracts, based on searching articles which included IHC for detection of MMR proteins or/and PCR for the MSI status in tumor and blood samples of patients with Colorectal or Endometrial Cancer (CRC and EC), older than 18 years old, in order to confirm MMR deficiency. A germline test for at least MLH1, MSH2, MSH6 and PMS2 was also mandatory for confirming the diagnosis of LS. We were focused mainly on colorectal and endometrial cancer, as they are the most common malignancies connected with Lynch Syndrome. The research was restricted in articles published after 2005, which had a reference in the statistical test used for confirming the accuracy of its results. Having identified more than 50 articles the full text was reviewed and we resulted in the then final selection based on specific inclusion and exclusion criteria. We were restricted to include only 5 studies. Both the review of the abstracts and the whole articles was made by one reviewer.

3.2 Inclusion and Exclusion Criteria

The studies included should be prospective cohort studies or case control studies and should be conducted after 2005. The testing was conducted in one of the two most common cancers related to Lynch Syndrome, such as colorectal cancer and endometrial cancer. The use of IHC for the identification of the functionality of MMR was a prerequisite and a parallel test of the Microsatellite instability with PCR techniques was a prerequisite. A genetic test in blood samples based on sequencing techniques was important for the confirmation of the existence of Lynch Syndrome, which should preferably be conducted in all participants or at least in MMR deficient tumors. MLH1 methylation and BRAF V600E were not mandatory tests for this analysis. Clinical criteria such as age o diagnosis of cancer, family history, the Amsterdam criteria or the Bethesda recommendations were also taken under consideration for the choice of papers.

If a study did not mention estimates about its diagnostic accuracy, such as sensitivity, specificity and negative predictive value, it was excluded from the analysis. The diagnostic odds ratio (DOR) was desirable when this analysis started but, because of lack of this information in almost all papers screened for the issue in question, it was decided not to be mentioned as an exclusion criterion. Papers in their full context available were preferable.

3.3 Data analysis

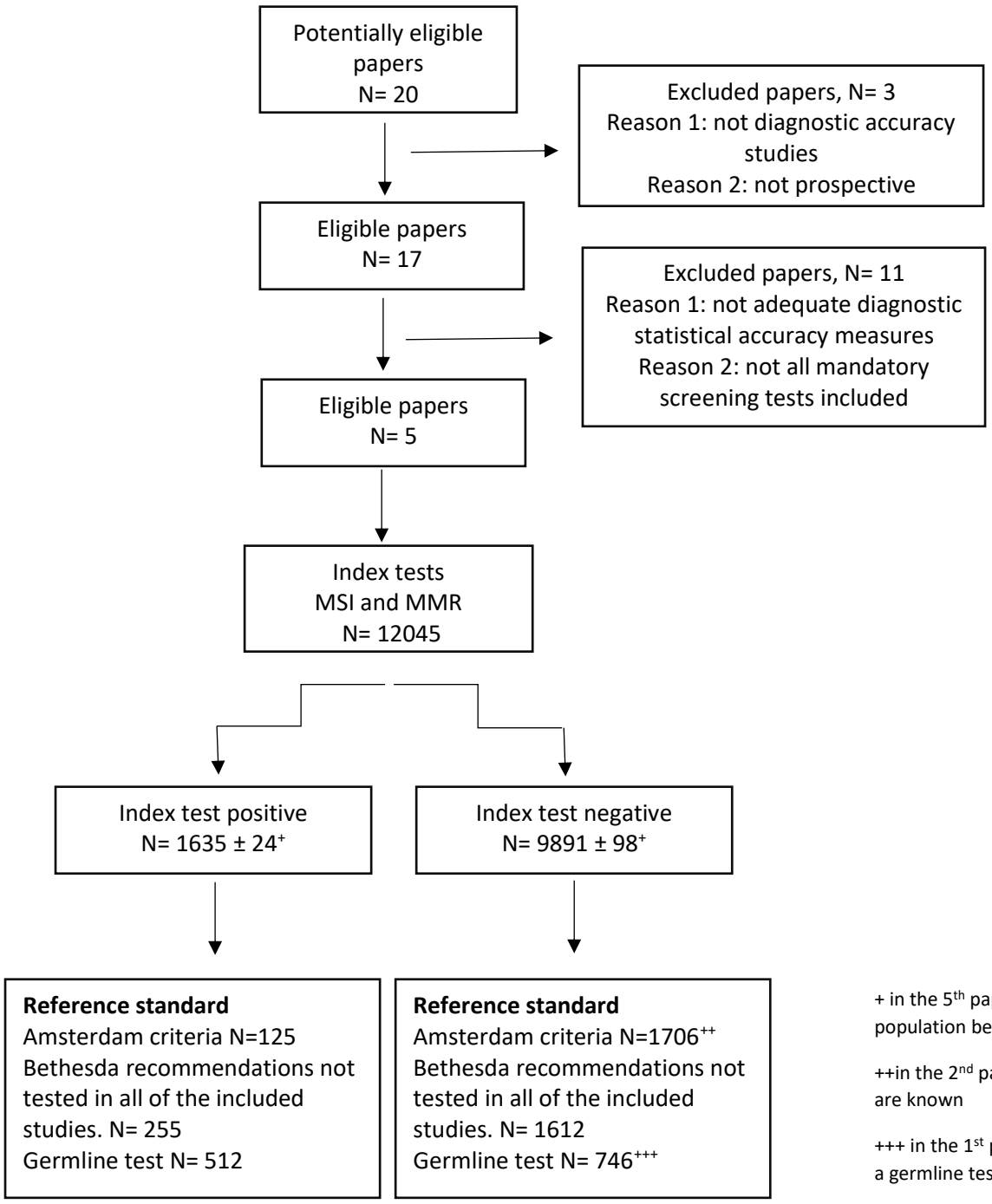
The criteria used to evaluate the quality, the transparency and the potent bias of the studies of interest, are the STARD criteria. We were based on the last update of the STARD statement which was published in 2015 by Cohen et al25,26. Regarding abstracts of the selected papers they were further evaluated using the STARD for abstracts27. Both checklists can be found at https://www.equator-network.org/reporting-guidelines/stard/. Another tool used overall and not in detail or step by step for the validation of the applicability of the results of the trials included in this analysis is the PROBAST tool28.

4. RESULTS

4.1 Search Results

After a detailed search mainly in Pubmed and Cohrane database 20 papers were stood out for further evaluation. Of them, according to abstract data, two were dismissed because they proved not be studies of diagnostic accuracy and one referred to a retrospective study. Of the 17 remained papers, seven did not use the requested statistical measures of diagnostic accuracy, such as sensitivity, specificity and prognostic values and were excluded for further evaluation. Three more studies were considered inappropriate because they did not use both IHC and MSI test and one because it did not analyze the existence of germline mutations in the four MMR genes even in MMR deficient tumors. The Flow STARD diagram of the study participants is presented below in **Diagram 1:**

Diagram 1.: Flow STARD diagram of participants through the study



+ in the 5th paper the overall MMR-d population between is unknown

++in the 2nd paper only the positive results are known

+++ in the 1st paper only MSI-H tumors had a germline test

4.2 Eligible Studies

Finally, the selection of five papers, three with CRC populations and two with EC populations was completed. In **Table 1.** the summary of the basic characteristics of the included studies is described. The data in this table regarding the type of prediction study were based on the PROBAST tool (Prediction model Risk of Bias Assessment Tool), as it was found in http://www.probast.org/.

Table 1.

	Trial Data	Type	Type of prediction study*	Method
1	Hampel H. et al, JCO, 2008, PMID: 18809606[29]	prospective	Dev and Val	IHC MMR, MSI PCR, MLH1 methylation, Amsterdam and Bethesda criteria, genetic mutation analysis
2	Moreira L. et al, Jama, 2012, doi:10.1001/jama.2012.13088[30]	prospective	Dev and Val	Bethesda criteria, Jerusalem Recommendations, multi-variate analyses regarding LS, IHC, MSI testing, germline MMR test
3	Buchanan DD. et al, J Gastr Hepatology,2017 doi:10.1111/jgh.13468[31]	prospective	Dev and Val	IHC, MSI testing, BRAF V600E, MLH1 methylation, germline MMR test, Amsterdam and Bethesda criteria
4	Ferguson S.E. et al, Cancer, 2014 PMID: 25081409[32]	prospective	Dev and Val	IHC, MSI testing, tumor morphology, Family history
5	Chao X.I. et al, Cancer Commun,2019 doi:10.1186/s40880-019-0388-2[33]	prospective	Dev and Val	IHC, MMR, Amsterdam criteria and Bethesda
*According to PROBAST tool Dev and Val: Development and Validation				

4.3 STARD Checklists

The results of the overall evaluation of the abstracts of each study as well as the whole study methodology are presented in **Tables 2 and 3**. respectively. Both Tables are created according to STARD 2015 checklist.

Table 2.: STARD checklist for abstracts

		Included Papers				
		1	2	3	4	5
Backround and Objectives		+	+	+	+	+
Methods	Data collection: whether this was a prospective or retrospective study	-	-	-	+	+
	Eligibility criteria for participants and settings where the data were collected	+	-	-	+	-
	Whether participants formed a consecutive, random or convenience series	-	+	-	-	-
	Description of the index test and reference standard	-	-	+	-	+
Results	Number of participants with and without the target condition included in the analysis	+	+	+	+	+
	Estimates of diagnostic accuracy and their precision (such as 95% confidence intervals)	-	-	-	-	-
Discussion	General interpretation of the results	+	+	-	+	-
	Implications for practice, including the intended use of the index test	-	+	-	+	+
Registration	Registration number and name of registry	-	+	-	-	+

Table 3.: 2015 STARD checklist

			1	2	3	4	5
Title of abstract		1	-	-	-	-	-
Abstract		2	-	-	+	+	+
Introduction	Backround	3	+	+	+	+	+
	Objectives	4	+	+	+	+	+

Methods	Study design	5	+	+	+	+	+
	Partcipants	6	+	+	+	+	+
		7	+	+	+	+	+
		8	+	+	+	+	+
		9	+	+	-	+	+
	Test Methods	10a	+	+	+	+	+
		10b	-	+	+	+	+
		11	-	+	+	+	+
		12a	+	+	+	+	+
		12b	-	+	-	-	+
		13a	-	-	-	-	-
		13b	-	-	-	-	-
	Analysis	14	-	+	+	+	+
		15	+	+	+	+	+
		16	-	+	+	-	-
		17	+	+	-	-	-
		18	-	-	-	-	-
Results	Participants	19	-	+	+	+	+
		20	-	+	+	+	+
		21a	+	+	+	-	+
		21b	+	-	+	-	-
		22	-	-	-	-	-
	Test Results	23	-	-	+	+	+
		24	-	+	-	+	-
		25	+	-	-	-	-
Discussion		26	+	+	-	+	+
		27	+	+	+	+	+
Other Information		28	-	+	-	-	+
		29	-	+	+	+	+
		30	-	+	+	+	+

4.4 Analysis of each study

1st study (Hampel H. et al): The main objective of this study is to answer the question if the IHC for MMR status or the MSI testing are proper methods for screening patients for LS, using a CRC population sample from a metropolitan area in the United States. The reference standard according to this article is testing patients with CRC for the MSI status based on the Amsterdam and the Bethesda criteria and the final diagnosis is validated with the germline test in blood samples for the MMR related genes (MLH1, MSH2, PMS2 and MSH6). The index test under investigation is testing of MMR status to every patient with CRC using both with IHC and MSI testing with PCR. The detailed protocol regarding he whole process methods can be found in a previously published study by the same team[34]. Sample size was 500. All of them had a MSI test and 16.9% were MMR-d, while 483 had an IHC test, which was abnormal in the 14.7%

All MSI positive tumors were tested for a methylation on the proximal region of the promoter of this gene, and this methylation was present in all MSS with MLH1 positive in IHC. Nobody of these who had only this methylation in IHC had LS. Overall, 18 of the 500 patients with CRC had a pathogenic mutation in MMR system and were diagnosed with LS.

All 18 were MSI-H tumors and 17 had also a positive IHC. The majority of patients had a MSH2 mutation and followed the mutation in MLH1, then in MLH6 and last in the PMS2.The most common of them only 7 fulfilled the Amsterdam Criteria.

Sensitivity of MSI and IHC testing was 100% and 94.4% respectively, specificity was 90.5% and 88.4% respectively, while positive predictive value of MSI-high and abnormal IHC was 28.1% and 23.9% respectively and negative predictive value was higher, 100% and 99.8% respectively. At the same time the sensitivity of the Amsterdam and the Bethesda criteria for the diagnosis of LS is very low, 39% and 72% respectively. In every case the 95% CI are not described.

The overall frequency of LS was 2.8% (95%CI: 2.1%- 3.8%). Worth mentioning according to the analysis is also the fact that this prevalence represents the minimum one if more mutations could be tested and proved to be clinically significant (deleterious) or if genetic test could be performed in every participant the number of LS diagnosis could be even higher. Patients with an MSI-H phenotype are as possible as these with abnormal IHC to be diagnosed with LS:20.8% and 21.4% respectively (P-value 0.984). Nobody of the patients with MSS tumor phenotype or/and MLH-1 promoter methylation which tested with a germline test, was diagnosed with LS.

Possible bias of the study are: 1) Germline mutation test was not conducted for every patients regardless of their MMR status, 2) EPCAM was not included in the germline test analysis and 3) not all possible PMS2 gene mutations related to LS were tested. The discordance in the results of IHC and MSI regarding the final MMR status would also be helpful in deciding if only one of the two test is enough for the next step. According the result of this study the sensitivity and specificity of the clinical criteria alone, either Amsterdam or Bethesda recommendations, is not enough to indicate which CRC populations should be screened for LS. IHC and MSI are clearly more accurate and when comparing other factors, such as the cost, the facility and availability of IHC might make this method more suitable as a screening test for LS in combination with clinical characteristics. MLH1 methylation could further increase the accuracy of IHC.

2nd study (Moreira L. et al): Here the participants come from 4 CRC cohorts from 4 different centers. The data were prospectively collected. Test for identifying Germline MMR mutations was performed in patients with MMR deficiency in their tumor confirmed either with PCR or with IHC or with both of them, but also in a sample of 1390 members with MMR proficient tumors (Colon CFR probands) or tumors without a previous test for MMR status, which could affect study's results as the index test should include MMR status. Because of the different source of the data, the analysis of MMR status in each series was performed with different panels according to the practice of each center. However, all tumors were to the end categorized as MSI high or low/stable. 1.8% of the patients was not assessed for MMR status before germline analysis. The germline control for the ascertainment of LS was performed with the same methodology in all series. The overall study population was 10026 samples.

As reference standards were used 4 different combinations: MMR deficient tumors with fulfilling one of the following: 1) the revised Bethesda criteria, 2) Jerusalem recommendations, 3) diagnosis of CRC at age ≤ 70 years and at least one of the Bethesda

criteria (based on a bivariate analysis) and 4) diagnosis of CRC at age ≤ 60 years, at least on first degree relative with CRC diagnosed at age ≤ 50 years or diagnosis of tumors related to LS diagnosed at age ≤ 50 years (multivariate model). The most reliable strategy was considered the 4th as it had the highest sensitivity (94.2 and 88.1% respectively) and negative prognostic value (97% and 97.3% respectively). These standards were compared with the universal screening for MMR status in every CRC patient. The germline mutation MMR test included only three of the involved genes, MLH1, MSH2, MSH6, whereas only the Ohio center performed a gene analysis for the PMS2 gene. The fact that neither EPCAM gene was tested is another limitation. MLH1 promoter methylation or BRAF V600E mutation were not included in the study protocol design.

The median age of diagnosis of CRC related to LS in this study is 48.1 years. The overall frequency of LS is 3.1%, of which only 27.2% fulfilled Amsterdam criteria but 68.6% the Bethesda recommendations. He most common mutation was found in MSH2 gene. It should be underlined that there was a 3.8% of the LS population which was MMR proficient according to the study. But, finally, for these samples it was performed either only IHC, or only MSI test, or both but one them revealed an MMR-proficient and the other a deficient result. However, the discordance between the two tests was only 2.5%.

The results regarding sensitivity, specificity and negative predictive value are presented in **Table 4**.

Table 4.

	MMR testing %	Germline MMR test %	Sensitivity % 95% CI	Specificity % 95%CI	Negative Predictive Value %
Bethesda criteria	31.5	3.5	81.7 (78.9–93.2)	98.3 (96–98)	99.7 (99.5-99.9)
Jerusalem recommendations	57.8	5.1	85.4 (77.1–93.6)	96.7 (96.0–97.2)	99.7 (99.4-99.8)
Bivariate analysis criteria	27	4.4	87.8 (89.8-99)	97.5 (94.7-96.1)	99.7 (99.7-100)
Multivariate model	65.2	6.5	95.1 (72.7-90.6)	95.5 (97.8-98.7)	99.9 (99.3-99.8)
Universal screening	100	9.1	100 (99.3–100)	93 (92.0–93.7)	100 (99.9-100)

In conclusion, relating to clinical models, the multivariate model has better diagnostic values and can better identify these patients who should perform a screening test, however, this model is not more representative than a universal screening of CRC patients for MMR status for the possibility of diagnosing LS, as apart from the samples which were tested with only one of the two index tests, there was no LS diagnosis with MSI stable tumor.

3rd study (Buchanan DD. et al): This is a prospective study and its population comes from the Australian Colorectal Cancer Family Registry (ACCFR) and from the Melbourne Collaborative Cohort Study (MCCS). The main objective of the study was to diagnose LS not only in young but also in older patients investigating the MMR phenotype in their CRC tumor

sample, therefore the population divided in two cohorts: the diagnosis of CRC in the first cohort happened between the age of 18 and 49 years, while in the second between the age of 41 and 80 years.

The clinical criteria, The Amsterdam criteria and the Bethesda recommendations as well as a family history, were available only for the one of the two cohorts. The index tests used for the identification of LS were: MMR deficiency, identified through IHC and MSI status, identified by PCR. BRAF V600E was performed for all samples and MLH1 promoter methylation for those with MLH1 and PMS2 deficiency in IHC. All patients in both cohorts were tested with IHC, while MSI test was performed for 67.8% of the samples in the ACCFR cohort and 96.7% of the samples in the other cohort. The Germline mutation test for LS included all four related genes and also, the EPCAM gene and it was performed, on the one hand, in the Registry cohort, for all MMR deficient tumors, for a part of MMR proficient patients that met Bethesda guidelines, Amsterdam criteria or who had a suspected for LS family history and for a part of MSI-L tumors. For the MCCS population, on the other hand, germline test was performed only for MMR-deficient samples without a MLH1 promoter methylation. A germline test was also available for a small subset of participants with MLH1 methylated CRCs, coming from both cohorts. In this study MMR proficient were characterized the tumors with both MSI-low profile and negative IHC findings, and MMR deficient these with LSI-high profile and/or positive IHC.

Overall, MMR-deficiency was identified in the 11.1% of the ACCFR group and 12.5% of the MCCS cohort and of these 14.4% and 85.2% proved to have a MLH1 promoter methylation. It should be mentioned that MCCS cohort included older patients. The discordance between IHC MMR protein expression and MSI status was 1.1% and 3.9% respectively. The 5.2% and 0.8% in each group had finally a pathogenic germline mutation, which represents the 58.35% of all MMR-deficient samples in the study. The most common mutation was a concurrent loss of both MLH1/PMS2. None of the MSI stable had a germline mutation, but two of the MMR proficient tumors had one. Regarding the clinical criteria in the ACCFR cohort, they were met in 91.5% of the overall germline mutated proportion: 31.2% met the Amsterdam and 55.3% the Bethesda guidelines. About 66% of the MMR deficient tumors with no methylation of the promoter of MLH1, proved negative for germline mutation or had a Variant of unknown significance (VUS) germline mutation status and were characterized as Lynch-like syndromes. The sensitivity, specificity and negative prognostic value for each test is presented in **Table 5.**

Among tumors with MLH1 promoter methylation 61.5% and 77.8% had BRAF V600E mutation in each of the two cohorts, so the diagnosis of LS was excluded. Testing both MLH1 methylation and BRAF V600E could exclude some cases with MMR deficient tumors from a germline test referral.

According to this study 95.7% of patients with LS were diagnosed with CRC before the age of 65 years, all were younger than 70 years old and median age was lower than 50 years. Testing MMR status for screening patients with CRC is a more accurate strategy for identifying patients with LS. When combined with the factor age the specificity grows but the sensitivity and the negative prognostic value falls.

To conclude, by this study it is once again obvious the need for a universal approach of MMR test in CRC patients.

Table 5.

Combined ACCFR and MCCS CRCs	Total	Mutation carriers†	Sensitivity (95%CIs)	Specificity (95%CIs)	Negative Predictive Value (95%CIs)
	262	47			
MMR-proficient CRC	75	2			
MMR-deficient CRC all ages	187	45	95.7 (85.1-99.3)	34.0 (31.6-34.7)	97.3 (90.7-99.5)
MMR-deficient CRC <70 years	129	45	95.7 (85.0-99.3)	60.9 (58.6-61.7)	98.5 (94.7-99.7)
MMR-deficient CRC <60 years	100	43	91.5 (79.8-97.2)	73.5 (70.9-74.7)	97.5 (94.1-99.2)
MMR-deficient CRC <50 years	76	41	87.2 (75.2-94.5)	83.7 (81.1-85.3)	96.8 (93.7-98.6)
MSH2/MSH6 loss	24	10	100 (67.8-100)	94.4 (93.2-94.4)	100 (98.6-100)
MSH6 solitary loss	14	8	66.7 (38.5-87.0)	97.6 (96.2-98.6)	98.4 (97.0-99.4)
PMS2 solitary loss	11	9	100 (69.1-100)	99.2 (98.1-99.2)	100 (98.9-100)
MLH1/PMS2 loss	135	15	93.8 (68.5-99.7)	51.2 (49.6-51.6)	99.2 (96.0-100)
MLH1/PMS2 loss/ *BRAF*^{V600E} wildtype	71	15	93.8 (68.7-99.7)	77.2 (75.6-77.6)	99.5 (97.4-100)
MLH1/PMS2 loss/ *MLH1* methylation negative	50	15	93.8 (69.1-99.7)	85.8 (84.2-86.2)	99.5 (97.7-100)

4th study (Ferguson S.E. et al): This study main objective is to identify the best screening practice for LS in endometrial cancer, which is the most common extra-colonic LS-related tumor. The overall cohort counts 118 women with EC and comes from newly diagnosed EC in a Canadian Center and was selected prospectively. Four different screening tests were used, in order to detect the best screening strategy: IHC for MMR genes in the tumor, MSI test with PCR, a family history questionnaire based on data derived from four different guides and tumor morphology, which was assessed with a blind way by a pathologist. A germline mutation test for all the four related to LS genes (MLH1, MSH2, MSH6, PMS2) as well as for the EPCAM gene was performed in 75% of the patients. The 20% of the patients who denied the germline analysis had a negative result in IHC. About 23% of tumors was MSI high and 28.8% had an MMR deficient result with IHC. Almost all of the IHC deficient tumor were also MSI high, but only 5.9% found finally positive for LS. The most common mutation was detected in MLH1 gene. Hypermethylation of MLH1 in IHC positive samples was not tested. The majority of LS tumors had high risk histology features, which do not seem to play a role in the improvement of the triage in the screening procedure for LS.

The sensitivity, specificity and negative prognostic value from each strategy are presented in **Table 6.** Overall, although there were no statistically significant differences in sensitivity and

specificity among IHC, MSI test, Family questionnaire and tumor morphology, the questionnaire seems to be the less sensitive and tumor morphology the less specific. As all mutations were identified in women <60 years old, the factor age seems to play a very important role in the screening procedure of EC population, as it increases the sensitivity, the positive and the negative predictive value of MSI test . Nevertheless, even this combination does not seem to be superior than IHC, which maintains its high sensitivity and negative predictive value regardless of the age. This is also proved by the fact that two women with LS did not neither the Amsterdam criteria nor had a family history but they were tested because of their positive IHC.

Table 6.

	No.	Sensitivity % (95%CI)	Specificity % (95%CI)	Negative Predictive Value % (95%CI)
IHC	89	100 (59-100)	78.1 (67.5-86.4)	100 (94.4-100)
IHC age <60 y	43	100 (59-100)	86.1 (70.5-95.3)	100 (88.8-100)
MSI test[1]	87	100 (54.1-100)	81.5 (71.3-89.3)	100 (94.6-100)
MSI test[2]	89	85.7 (42.1-99.6)	81.7 (71.6-89.4)	98.5 (92.1-100)
MSI test age <60 y	41	100 (54.1-100)	88.6 (73.3-96.9)	100 (88.8-100)
MSI test age >60 y	43	85.7 (42.1-99.6)	88.9 (73.9-96.9)	97 (84.2-99.9)
Family Questionnaire	82	71.4 (29.6-96.3)	86.7 (76.8-93.4)	97 (89.6-99.6)
Tumor /histology	83	71.4 (29-96.3)	42.1 (30.9-54)	94.1 (80.3-99.3)
1: Excludes MSI equivocal results (small number of samples included) 2: Includes the MSI equivocal results and categorizes them as negative.				

5th study (Chao X.I. et al): This prospective study aimed to identify which is the best screening or combination of tests, among clinical criteria, which were considered as a reference standard (Amsterdam criteria and Bethesda guidelines), IHC for MMR proteins and MSI test, which were considered as the index tests, for the diagnosis of LS. The whole sample (N=111) comes from one center in China and consists of newly diagnosed with EC with surgical staging of their disease. A germline mutation test was performed for all the women included in the study and for all genes related with LS (MMR genes and EPCAM gene) and, finally, 5.6 % of them was diagnosed with EC related to LS. MSH6 was the most common germline mutation identified. Although age was not one of the criteria for the selection of the study population, none of the women with EC and LS was above 70 years old at the time of diagnosis. All of participants were evaluated for the clinical criteria, while the 91.9% was assessed with IHC and 74.8% with MSI test. In cases with MLH1 deficient IHC the study investigated also the possibility of a methylation of MLH1.

In **Table 7.** table the sensitivity, specificity and negative prognostic value for each screening test are presented. Noteworthy key points of the study are the fact that taking into consideration only the Amsterdam criteria and the Bethesda recommendations the 85.7% cases will be lost, the high agreement between the results of IHC and MSI test and the important role of MLH1 promoter hypermethylation test, when a loss of this protein in IHC is detected. MSI test seem to be the most accurate regarding the diagnosis of LS and its sensitivity, specificity and negative prognostic values increase more when combined with IHC test. Although the basic statistical estimates for a diagnostic accuracy trial, sensitivity, specificity and negative predictive value, are used to identify the best screening strategy, the 95% CI, is not written on this study.

Table 7.

	NGS (cases)		Sensitivity %	Specificity %	Negative Predictive Value %
	LS-EC	Non LS-EC			
Clinical criteria	6	105	33.3	88.6	95.9
IHC	6	96	66.7	75	100
MSI test	4	79	100	89.9	100
IHC plus MSI	6	87	100	72.4	100
Clinical Criteria: Amsterdam criteria II and Bethesda Recommendations					

4.5 STARD results

Regarding the evaluation of all studies according to STARD statement it is obvious from Table 3. that in the main body of the included papers, the majority of the requested data in each part of a diagnostic accuracy trial is fulfilled. In abstracts, in contrast, the results are more disappointing. Although the objectives are well mentioned in every abstract, in part "Methods" neither the eligibility criteria, nor the type of the study is described. Most of the times there is a simple reference in the index test under investigation while only one paper gives information about the sampling process (consecutive, random or convenience). The part "Results", apart from a more detailed description of the statistical estimates of diagnostic accuracy, met the relative STARD criteria. The way the part "Discussion" is presented is also adequately presented.

As far as the main body of the paper is concerned, the presentation and the structure represent a more consistent and comprehensive reflection of the STARD statement. Except from the title (number 1 in the list), the blindness of the performers, readers and assessors of the index test and the reference standard (number 13a and b), the way the sample size was predetermined (number 18) and information about the registration details of each study (number 28), all the other features are more or less well reported. Analyses of variability is not important in this study as both MSI and MMR test have specific and

concrete ways of validation and there is not a way to show variability among different samples of CRC or EC. Moreover, it is not clear if the investigating index tests were prespecified before data were collected, but it is doubtful if this fact affects the diagnostic accuracy of the MSI and MMR test (number 17 in the list). Another feature that is not described in any of the studies is the number 22 of the list, which refers to time interval and the clinical interventions between the index test and the reference standard. As the aim is the screening and diagnosis of a germline mutation, and the index tests are performed in tumor tissue and blood samples, neither time nor any intervention could affect the result. Finally, as the studies include only a blood sampling as an invasive procedure there are no adverse events worth mentioning (number 25 in the list).

Overall, we conclude that the most noteworthy omissions of the studies included, are the titles and the abstracts. Although both of these parts could be designated as the mirror of a trial, they do not clarify from the beginning that the study that follows is a diagnostic accuracy study.

5. DISCUSSION

The present analysis reports the quality of five studies investigating the diagnostic accuracy of MMR deficiency as a screening test for the further diagnosis of LS. The index tests in all studies were either IHC for the four MMR proteins or/and PCR test for MSI status. The reference standards were the clinical characteristics, which means the Amsterdam and the Bethesda Criteria and most of the times age and the family history, too.

All studies agree on the diagnostic value of the index tests, which in all studies share high levels of concordance. Sensitivity, specificity and negative predictive value exceeds 90% and regarding sensitivity and NPV touches 100% many cases. The diagnostic accuracy of the tests increases when a patient performs both of the index tests, while the combination with the factor age seems to play an important role in women with endometrial cancer. From the studies with this population, it is concluded that almost no woman above 70 years old is diagnosed with Lynch syndrome and the sensitivity, specificity and negative prognostic value of the IHC and especially for MSI test can be increased about 3-5%, if the factor age is added.

At the same time the diagnostic accuracy of clinical approaches is significantly lower and has great variability among the different studies. The sensitivity and specificity range from 33%-95% and 72%- 97.5% respectively, depending on the study population and the use of only a family history or the use of a combination of Amsterdam criteria II, Bethesda recommendations and other clinical data.

Although the use of MLH1 promoter methylation and BRAF V600E mutation were not inclusion criteria for the selected studies, in cases they were tested they added benefit in diagnostic process. In tumor samples with MLH1 loss and/or PMS2 staining, testing of a BRAF V600E mutation and/or MLH1 promoter methylation can exclude Lynch syndrome if the test proves positive[37,38].

Examining all studies as a whole some limitations are identified. First, the MSI-low tumors are categorized in different ways. In some studies, they are counted in MSI high tumors and in others in MSI low. Nevertheless, when mentioned, as, for instance, in the first study, MSI-low tumors are negative for germline mutation, but this needs further evaluation. Second, in studies with cohorts of many different sources the panels used for the test of MMR status

may differ with each other, a fact that could create bias. Third, in no study germline test was performed for the whole population. Sometimes this was prespecified but other times this happened due to participants personal choice. A universal germiline test for every patients could affect the diagnostic accuracy of index test, not necessarily in an unfavorable way. Last, in the majority of the studies there were germline test results with Variant of unknown significance (VUS). In some cases, they were excluded from the analysis of diagnostic accuracy but in others it is unknown if they participated in the analysis. This is probably another source of bias.

The evaluation of Lynch syndrome in everyday clinical life, according to National Comprehensive Cancer Network (NCCN) guidelines, is based on clinical characteristics at first, and on MMR status consequently. Family history and personal history, including Amsterdam criteria II and Bethesda recommendations, are the first criteria, which may lead to a test of MMR status with either IHC or PCR or both, depending on the available methods in the institution where the patients is monitored. NCCN recommends also the use of predictive models such as $PREMM_5$, MMpro and MMRpredict, which are all clinical models, for the referral to a germline test and genetic counceling[35]. Among them $PREMM_5$ includes all related to LS genes and is based on personal characteristics, on family history (first and second degree relatives) and is available online: https://premm.dfci.harvard.edu/. Although NCCN cites these guidelines, the panel of NCCN finally recommends all colorectal and endometrial tumors for MMR deficiency screening. The European Society for Medical Oncology on the other hand provides recommendation only for CRC, suggesting also a universal screening either with IHC or with MSI test regardless of clinical characteristics[36]. The truth is, that nowadays, especially for Colorectal cancer, searching for MSI or MMR status is a daily practice. This is not a priority because of the need of screening for Lynch Syndrome, but it is happening because the result has also, a prognostic and a predictive value for patients.

Regardless of the screening test chosen, the diagnosis of Lynch syndrome in early life affects the prognosis of these patients. An early diagnosis changes the follow-up of these patients and also may affect the life of their family. Additionally, it helps them adopt life saving strategies for cancer prevention or early diagnosis of cancer which has an impact also in the treatment of cancer[36, 37].

Finally, every trial of diagnostic accuracy should follow one of the many recognized and online published guidance for the rational reporting its results. The checklists could be used even for the study design. These tools have been proved useful not only for investigators but also for the authors, the editors, the reviewers of study of diagnostic accuracy and the decision makers. STARD Checklist is comprehensible, easy to use and can prevent many mistakes which could lead to a failure.

6.CONCLUSION

To conclude, MMR status either with IHC for the MMR proteins or with test for MSI status are of greater sensitivity, specificity and negative prognostic value as screening tests for Lynch Syndrome, when compared to clinical criteria. A strategy that combines both diagnostic procedures could be ideal for the detection of the suspected population and the

prompt diagnosis. Every study of diagnostic accuracy should meet the checklist points of diagnostic accuracy tool, such as STARD Statement Tool.

7.ABBREVIATIONS

CRC: Colorectal cancer
EC: Endometrial cancer
EPCAM: Epithelial Cell Adhesion Molecule
LS: Lynch syndrome
MMR: Mismatch Repair
MLH1: Mutator L homolog 1
MSH2: Mutator S homolog 2
MSH6: Mutator S homolog 6

MSI: Microsatellite instability
NGS: Next Generation Sequencing
PMS2: Postmeiotic segregation increased 2
PCR: Polymerase chain reaction

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