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Review article

Biomarker identification for endometriosis as a target for real-time intraoperative fluorescent imaging: A new approach using transcriptomic analysis to broaden the search for potential biomarkers

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ABSTRACT

Intra-operative fluorescent imaging of endometriosis could help to optimize surgical treatment. Potential biomarkers to use as target for endometriosis-binding fluorescent probes were identified using a new five-phase transcriptomics-based approach to broaden the search for biomarkers. Using publicly available datasets, a differentially expressed gene (DEG) analysis was performed for endometriosis versus surgically relevant surrounding tissue (peritoneum, bladder, sigmoid, rectum, transverse colon, small intestine, vagina, and fallopian tubes) for which data was available. The remaining relevant surrounding tissues were analyzed for low expression levels. DEGs with a predicted membranous or extracellular location and with low expression levels in surrounding tissue were identified as candidate targets. Modified Target Selection Criteria were used to rank candidate targets based on the highest potential for use in fluorescent imaging. 29 potential biomarkers were ranked, resulting in Folate receptor 1 as the most potential biomarker. This is a first step towards finding a fluorescent tracer for intra-operative visualization of endometriosis. Additionally, this approach, using transcriptomics analysis to identifying candidate targets for a specific type of tissue for use in fluorescence-guided surgery could be translated to other surgical fields.

Tweetable abstract: A new approach using transcriptomics analysis is shown to identify candidate targets for intra-operative fluorescent imaging for endometriosis, resulting in 29 potential candidates.

Introduction

Endometriosis is a complex disease, characterized by endometrial-like tissue outside the uterus. Endometriosis affects approximately 10% of all reproductive women [1] and seriously impacts quality of life [2]. Surgery is an important treatment modality that aims to remove visible lesions. However, postoperative recurrence is a frequent problem with rates of 2–43% [3], depending on definition and length of follow up, with incomplete resection being a risk factor [3,4]. Therefore, complete surgical resection is desirable, which could be complicated by difficult intra-operative recognition of deep (DE) and superficial endometriosis (SE) [5]. Optimized intra-operative visualization of endometriosis could help to achieve complete resection.

Fluorescence-guided surgery (FGS) is an innovative imaging technique for intra-operative visualization by creating a contrast between the tissue of interest and its surrounding tissue. Although FGS is mainly used in oncological surgery, it has the potential to be translated to other surgical fields, such as endometriosis surgery. The main advantage is that this technique does not change the surgical field, as the used near-infrared light is not visible to the human eye and can be made visible with a specialized camera system. Activating this camera, results in the targeted tissue highlighted in fluorescent, by software translated to, green light, in comparison to the surrounding non fluorescent background. A fluorescent tracer that highlights the target tissue is essential [6–10]. These tracers are mostly administered intravenously or topically, depending on the location of the target tissue [11]. The time

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between administration and visualization differs between tracers and target tissue. Fluorescent tracers can be divided into two types: non-targeted and targeted tracers. A non-targeted tracer does not bind specifically to a structure, but accumulates in or around the tissue using various tissue characteristics [12–15]. A targeted tracer is a dye conjugated to a structure, which specifically binds to a tissue [16]. Within surgical endometriosis resection, Indocyanine green (ICG), a non-targeted tracer, has been studied with conflicting clinical results, showing no clear benefit to visualize endometriosis itself [17–23]. Therefore, targeted fluorescent tracers are needed for optimized intraoperative visualization of endometriosis, which are not yet researched in endometriosis.

To develop a targeted fluorescent tracer, a biomarker to use as a target needs to be identified. The biomarker should be upregulated in endometriosis compared to the healthy surrounding tissue. In endometriosis, one of the most important affected surrounding tissues is the peritoneum as it is primarily a peritoneal disease, with deeper infiltration in deep endometriosis (DE). Other relevant surrounding tissues include the rectosigmoid, colon, intestine, bladder, vagina, and fallopian tubes. Additionally, the biomarker must be expressed at a membranous or extracellular location to enable the fluorescent tracer to reach the target upon intravenous or intraperitoneal administration.

To identify candidate targets, the most commonly used method is a systematic review of protein expression experiments of specific biomarkers in the tissue of interest [24–31]. However, this approach is biased due to the dependency on selected biomarkers chosen by other researchers, often for different goals than fluorescent-guided surgery. For example, van den Berg et al. is, to our knowledge, the only study that performed an immunohistochemical analysis of potential biomarkers for targeted fluorescence imaging in endometriosis [32]. However, the choice of biomarkers was mostly based on practical considerations instead of extensive analysis to identify markers specific for endometriosis [32]. To avoid these disadvantages and optimize the chance of finding candidate targets, one could opt for a transcriptomics approach instead of protein expression approach. In such an approach, no selection of targets is made beforehand, as total RNA in a specific tissue or cell type is studied. Transcriptomic analyses are often used for the pathophysiological understanding of diseases but, in our opinion, could also be used for biomarker identification for fluorescent imaging by analysis of expression levels between tissues.

The current evidence of targeted fluorescence-guided surgery is scarce. This study aims to identify biomarkers as candidate targets for the use in fluorescent imaging in endometriosis by using a transcriptomic analysis, combined with target selection criteria.

Materials and methods

The search for candidate biomarkers was divided into five phases, according to predetermined criteria for a potential target: upregulation of the biomarker in endometriosis compared to surrounding tissue and a membranous or extracellular location. Phases 1–4 result in genes that meet all our pre-set criteria. Phases 1 and 2 show the differentially expressed genes (DEG) analysis comparing endometriosis to relevant surrounding tissue. Phase 3 filters biomarkers based on the subcellular location. Phase 4 selects biomarkers that additionally have a low expression in the remaining surrounding tissues, which could not be included in the DEG analysis. In phase 5, all potential biomarkers are ranked according to the potential for use as a target in fluorescent imaging. Institutional Review Board was not applicable as only publicly available data was used.

Phase 1: Identification of a publicly available dataset

Gene Expression Omnibus (GEO) is a publicly available genomics database that contains data on gene expression, chips, and microarrays [33]. GEO consists of Databases, Sample Records, and Platform Records. A Platform Record is composed of a summary description of the array or sequencer and, for array-based Platforms, a data table defining the array template. A GEO Series record is an original submitter-supplied record that summarizes an experiment. These data are reassembled by GEO staff into GEO Dataset records [33]. GEO was searched with the keyword "endometriosis". Available sets were screened for data from patients in which both endometriosis and relevant surrounding tissue was available. As relevant affected surrounding tissues, the following tissues were identified: bladder, sigmoid, rectum, transverse colon, small intestine, vagina, and fallopian tubes.

Phase 2: Identification of differentially expressed genes

With the extracted data from the detected dataset during phase 1, we used RStudio (version 1.4.1106) to screen for differentially expressed genes (DEG) between endometriotic lesions and peritoneum, the relevant surrounding tissue included in the data. Specifically; the Readr [34], Biopeak [35], and limma [36] software packages were used. To identify DEGs, the fold change (log2 (fold change)), hereafter called logFC, was determined, together with p-values and adjusted p-values for multiple testing. The complete R script is available in Supplemental Materials A. To select the most potential DEGs to include in phase 3, a cut-off was determined, as discussed in the results section.

Phase 3: Predicted location of the biomarker

The most commonly used cellular sublocation for biomarkers to act as a target for fluorescent tumor imaging is membrane-bound or in close proximity to the cell [37]. As endometriosis consists of different cellular components, namely epithelial glands, stroma, and fibrosis, a membranous or extracellular location of the target was considered suitable. The predicted location was extracted from UniProt [38]. Only differentially expressed genes located at one of these locations were selected to enter phase 4.

Phase 4: Low expression level of selected biomarkers in relevant surrounding tissues

As the final criterium for the biomarkers to be potentially used as a target for fluorescent imaging, the expression levels should be low on surrounding tissues. To determine the expression levels of the remaining surrounding tissues, Euretos platform was used [39]. Euretos platform is an artificial intelligence platform that uses findability, accessibility, interoperability, and reuse of digital assets (FAIR) data. The platform interlinks multi-omics data to the scientific literature by integrating over 250 public life sciences databases and textual sources. For RNA expression values, the information is extracted from The Genotype-Tissue Expression (GTEx) project [40] or The Cancer Genome Atlas (TCGA) for healthy rectum [41]. All identified relevant surrounding tissues were included in the used databases (bladder, sigmoid, colon transverse, fallopian tube, small intestine, vagina, and rectum).

To select the biomarkers, a cutoff value for expression level is needed. In literature, a tumor to background protein expression ratio of 10 is suggested to compare the targeted tissue with healthy surrounding tissue [37]. As our analysis used transcriptomics data instead of protein expression data, this ratio was not suitable for our approach. Therefore, an approach including RNA expression data was included. RNA expression from surrounding tissues in GTEx and TCGA is expressed in Transcripts per Million (TPM) [42], a normalized method to compare expression levels. To identify potential biomarkers with low expression levels in relevant surrounding tissues, a cutoff for TPM values for the surrounding tissues is determined and discussed in the Results section.

Phase 5: Modified target selection criteria (TASC)

The biomarkers that enter phase 5 are candidate biomarkers to use as

a target based on the preset criteria. However, multiple characteristics may influence the potential for using such biomarkers as a target for clinical fluorescent tracers. Van Oosten et al. developed the TASC to select the best biomarkers for imaging purposes [37]. It highlights the seven most favorable characteristics for potential targets and creates a ranking based on the potential for use as a target for fluorescent imaging. However, these TASC are based on protein expression, and therefore not fully applicable to our dataset. Therefore, the TASC was modified to score the biomarkers based on RNA expression and, if available, protein expression, among other criteria. Table 1 shows an overview of the criteria and scoring of the modified TASC.

Supplemental materials B shows a detailed description of the criteria of the modified TASC. In summary, the modified TASC is divided into two parts; "biological characteristics" and "feasibility for clinical translation". The first section incorporates information from the DEG analysis (magnitude of fold change and presence of target in deep and/or superficial endometriosis analysis), literature association with endometriosis and fibrosis, level of RNA expression in the surrounding tissue, and proof of protein expression in endometriosis. Additionally, it contains scoring for the level of protein expression of endometrium, being the best available surrogate tissue of endometriosis, and the relevant surrounding tissues. For these last categories, the availability of evidence was taken into account. The availability of evidence was determined based on Pubmed search strategies or Human Protein atlas, as mentioned in Supplemental materials B and C. If no evidence was available, the score 'Not Applicable' (NA) was assigned. If NA was assigned to the scoring, there was no opportunity to award points for that category and therefore simultaneously no points were added to the maximum possible score. By creating this scoring option, beneficial evidence was rewarded, adverse evidence was penalized and concurrently the absence of protein expression data was not penalized. This results in a different maximum possible score per biomarker.

The 'feasibility for clinical translation' part consists of information on previous use of the biomarker for imaging purposes, the availability of a clinical tracer, and if the biomarker encodes for a receptor, as this makes it relatively easier to develop a fluorescent tracer as a ligand may be available.

The final score is the percentage of the awarded score compared to the maximum possible score for that biomarker.

Results

The selection of potential biomarkers to act as a target for fluorescent imaging of endometriosis was divided into five phases, of which the results are described below. Fig. 1 shows the selection flowchart of all biomarkers.

Phase 1: Identification of a publicly available dataset

To find a relevant publicly available dataset, GEO was searched for datasets and series records with the terms given in the methods section. Nine relevant DataSets and 122 Series records were identified in GEO. Only one series record, GSE141549, contained expression profiles from endometriosis and a relevant surrounding tissue, i.e. peritoneum, of the same patient [43,88]. The data was generated on the Sentrix Illumina Human WG-6 v2 Expression BeadChips (Illumina, USA) and Illumina HumanHT-12 v4.0 Expression BeadChips (Illumina, USA) microarray platforms [43]. Normalized datasets were downloaded from GEO. GSE141549 contained gene expression profiles of 392 samples (115 patients, 53 women without endometriosis (controls)). The samples included deep endometriotic (DE) tissue (intestinal, bladder, rectovaginal, and sacro-uterine ligament tissue), ovarian endometriotic tissue, and superficial endometriotic (SE) tissue (black, red, and white endometriosis spots), patient peritoneum tissue, patient endometrium tissue, control endometrium tissue, and control peritoneum tissue. This was a diverse group of patients, with a mean age of 32 years old and median BMI of 23. 13% was revised American Fertility Society (rAFS) stage 1, 13% rAFS stage 2, 23% stage 3 and 49% stage 4, for 2% the rAFS stage was missing. All stages of menstrual cycle were included, including 30.4% of the patients that used hormonal medication to suppress endometrial activity [43]. The relevant samples for our research question (deep and superficial endometriosis and patient peritoneum) were extracted and used, resulting in 202 patient samples being used for the identification of DEGs.

Phase 2: Identification of differentially expressed genes

GSE 141549 contained gene expression data regarding 28247 genes. Low expressed genes were identified and the bottom 30% were excluded from the analysis. For the DEG analysis, two different comparisons were created: DE versus peritoneum and SE versus peritoneum. Theoretically, genes are considered differentially expressed if the observed difference between the two tissues is statistically significant [44]. However, a widely accepted cutoff for clinical value is logFC > 1 combined with an adjusted p-value of $p < 0.05$ [45]. To ensure no potentially interesting biomarkers would be missed for the research question, our cutoff was set at a logFC ≥ 0.9 combined with an adjusted p-value of $p < 0.05$. DEG analysis resulted in 3597 upregulated genes in the DE versus peritoneum comparison and 3467 upregulated genes in the SE versus peritoneum comparison. Fig. 2 show volcano plots of respectively DE versus peritoneum and SE versus peritoneum. LogFC values ranged from 0 to 3.68. The volcano plots clearly show multiple interesting biomarkers for further analysis (green dots show biomarkers with logFC ≥ 0.9 and adjusted $p < 0.05$). 249 unique genes within the two comparisons met our preset requirements of differentially expressed genes. The modified TASC in phase 5 took into account if the biomarker was identified as DEG in one or both of the comparisons. To visualize the difference between sample groups, Supplemental Figs. 1 and 2 show boxplots of the highest ranked biomarker for respectively DE (FOSB) and SE versus peritoneum (MMP7).

Phase 3: Predicted location of the biomarker

The predicted location of all 249 biomarkers was extracted from UniProt. Biomarkers with notations of membranous, extracellular, or secreted subcellular locations were included. 118 biomarkers with other predicted locations were excluded, resulting in 131 genes entering phase 4.

Phase 4: Low expression level of selected biomarkers in relevant surrounding tissues

A cutoff value for TPM levels was determined to result in biomarkers enabling to distinguish endometriosis from relevant surrounding tissues. The goal for the cutoff value was to ensure a relatively low expression level in tissues surrounding endometriosis, but concurrently not exclude potential targets by choosing a strict TPM level as RNA expression may not be directly related to protein expression levels. Therefore, the maximum level for normal tissue was set at 10 TPM. 102 biomarkers were excluded due to a TPM value above 10 in one or multiple of the predetermined relevant surrounding tissues. This resulted in 29 biomarkers (Table 2) that were included for further analysis in the modified TASC ranking process.

Phase 5: Modified target selection criteria (TASC)

In Table 2, all 29 potential biomarkers were scored according to the modified TASC, of which the criteria were elaborated in the methods section. For only 13 biomarkers, published experiments were available showing protein expression. For 10 biomarkers, no information was available on protein expression in the endometrium and surrounding tissue. Some biomarkers were already often mentioned in literature

Table 1
Overview of criteria and scoring in modified Target Criteria Score (TASC).

Scoring	Biological characteristics										Feasibility for clinical translation		
Points	Location[1]	LogFC[2]	Endometriosis subtype[3]	Endometriosis association[4] (nr. of references)	Fibrosis association[5] (nr. of references)	Transcripts per Millions (TPM) surrounding tissue[6]	Proof of protein expression in endometriosis[7]	Protein expression in endometriosis among patients (%)[8]*	Level of protein expression endometrium[9] *	Level of protein expression surrounding tissue[10]*	Previously imaged in vivo[11]	Clinically available tracer[12]	Gene encodes for receptor[13]
0				0	0		No	<10	Not detected	High	No	No	No
1		<1	Superficial or deep	<10	<10	TPM ≤ 10		10–50	Low	Medium			
2		1.0–1.99	Superficial and deep	10–99	10–99	TPM ≤ 5			Medium	Low	Yes	Yes	Yes
3	Cell secretion	2.0–2.99		100–499	100–499	TPM ≤ 3	Yes	51–70	High	Not detected			
4		≥3.0		≥500	≥500								
5	Extracellular or membranous							71–90					
6								>90					

[1] Location. *Information extracted from UniProt.*
[2] logFC. *Results from the DEG analysis.*
[3] Endometriosis subtype. *Based on the DEG analysis. The target could be present in one or both of the contrasts with both logFC ≥ 0.9 and significant adjusted p-value.*
[4] Endometriosis association. *Based on the references combining the target and 'endometriosis', found via Euretos, which shows results from database annotations and publications.*
[5] Fibrosis association. *Based on the references combining the target and 'endometriosis', found via Euretos, which shows results from database annotations and publications.*
[6] TPM Surrounding tissue. *Extracted from GTEx or TCGA. The TPM score of all tissues should be within the mentioned group.*
[7] Proof of endometriosis protein expression in endometriosis. *Awarded as 'yes' if any available reference showed proof of protein expression.*
[8] Protein expression in endometriosis among patients (%). *Available immunohistochemical experiments were studied to extract this information.*
[9] Protein expression endometrium. *As best surrogate for endometriosis, level of protein expression of endometrium was included in the score. Information was extracted from the Human Protein Atlas.*
[10] Protein expression surrounding tissue. *Level of protein expression of relevant healthy surrounding tissues. Information was extracted from the Human Protein Atlas. Score was based on the surrounding tissue with the highest expression. Tissues included: Urinary Bladder, Colon, Rectum, Small Intestine, Vagina, Fallopian tube.*
[11] Previously imaged in vivo. *A Pubmed search was performed to discover if this target was previously imaged* in vivo. *Animal experiments were allowed, and imaging could be by multiple ways (not necessarily fluorescent).*
[12] Clinically available tracer. *A Pubmeb search was performed to discover if a clinically available tracer was available. This could be for imaging but also for treatment.*
[13] Gene encodes for receptor. *This criterium was based on the fact if the target was a receptor, based on information from Uniprot. For a receptor it is relatively easy to modify a ligand to a fluorescent tracer.*
* If evidence for this criterium was not available for a target, the score 'Not Applicable' (NA) was awarded and no points were added to 'Maximum possible score'.

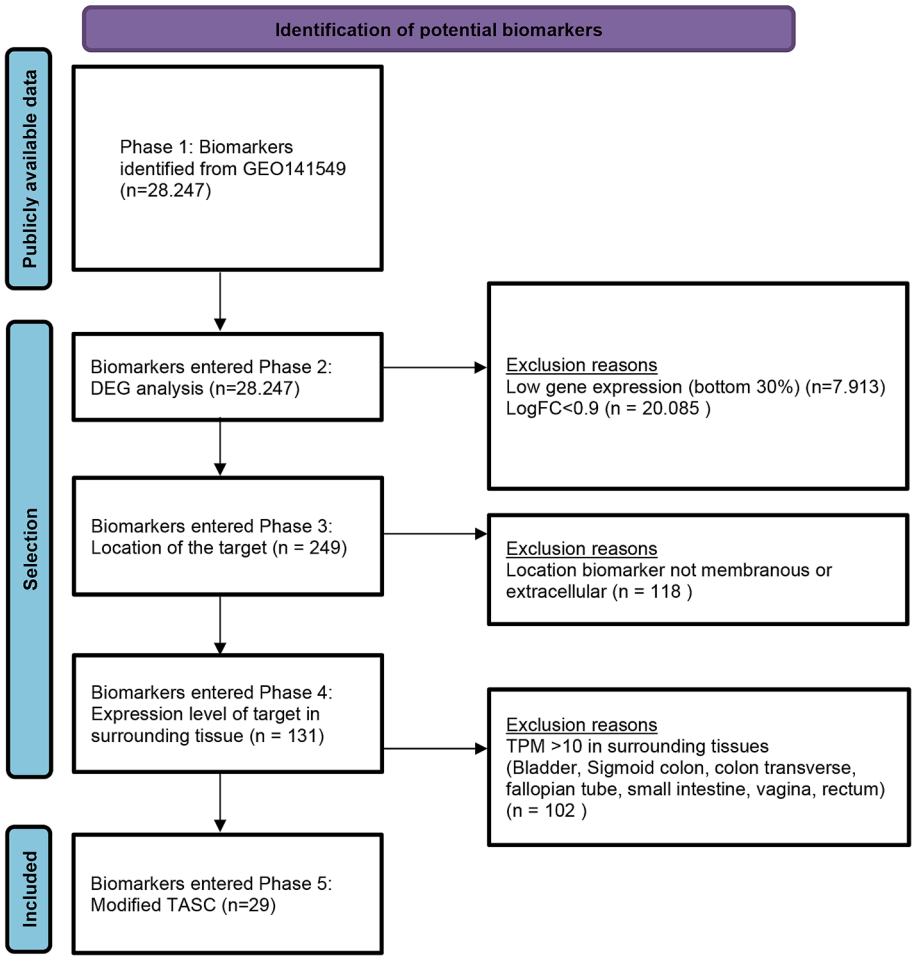

Fig. 1. Flowchart of identification of potential biomarkers for intra-operative fluorescent imaging of endometriosis. The flowchart shows the selection of biomarkers through five phases.

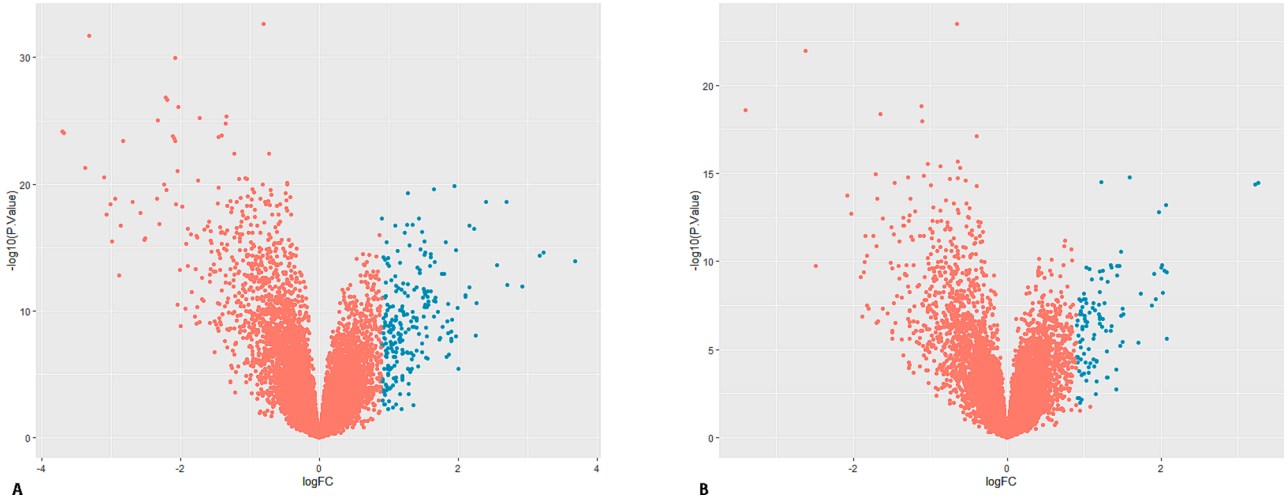

Fig. 2. Volcano plot showing the results of the Differentially Expressed Gene (DEG) analysis for deep endometriosis versus peritoneum (A) and superficial endometriosis versus peritoneum (B). X-axis shows logFC score and y-axis shows -log10 (P.Value), which is explained in the methods section. Dots represent biomarkers. Green dots indicate biomarkers with a logFC $\geq$ 0.9 and adjusted p. value $<$ 0.05, which were included for further analysis. Red dots indicate biomarkers with a logFC $<$ 0.9 or adjusted p. value $\geq$ 0.05, which were excluded from further analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

together with endometriosis (CXCL8) or fibrosis (CXCL8, IL1B). For 7 biomarkers, a clinical tracer (therapeutic, diagnostic, or imaging) is available, with only for FOLR1, a clinical fluorescent imaging tracer. Table 3 shows a summary of modified TASC ranking according to their potential for use as a target in fluorescent imaging. According to this ranking, FOLR1 shows the highest potential.

Table 2

Outcome of modified Target Selection Criteria (TASC) for all potential biomarkers for intra-operative fluorescent imaging of endometriosis. *All 29 biomarkers that entered phase 5, were scored according to the modified TASC score, as described in table I. Relevant references are shown. Citations in column titles refer to foodnotes from* Table 1.

Modified TASC	Biological characteristics										Feasibility for clinical translation			Score		
Biomarkers	**Location[1]**	**LogFC[2]**	**Endometriosis subtype[3]**	**Endometriosis association[4]** (nr. of references)	**Fibrosis association[5]** (nr. of references)	**Transcripts per Millions (TPM) surrounding tissue[6]**	**Proof of protein expression in endometriosis[7]**	**Protein expression in endometriosis among patients (%)[8]***	**Level of protein expression endometrium[9]***	**Level of protein expression surrounding tissue[10]***	**Previously imaged in vivo[11]**	**Clinically available tracer[12]**	**Gene encodes for receptor[13]**	**Total score**	**Maximum possible score**	**Scoring percentage (%)** (Total score/ maximum possible score)
ANO4	5	1	1	0	0	3	0	NA	NA	NA	0	0	0	10	31	32
ASIC2	5	1	1	0	1	3	0	NA	0	3	0	0	0	14	37	38
C1QTNF6	3	1	1	0	1	1	0	NA	2	2	0	0	0	11	37	30
CDH2	5	1	1	2	3	1	3 [57]	3 [57]	1	1	0	0	0	21	43	49
CLDN10	5	2	2	0	1	3	0	NA	1	3	0	0	0	17	37	46
COL10A1	5	1	1	0	1	3	0	NA	NA	NA	0	0	0	11	31	35
CXCL8	3	2	2	3	4	1	3 [58–66]	6 [65,66]	0	3	0	2 [67,68]	0	29	43	67
FOLR1	5	1	1	1	0	2	3 [32,69]	6 [32]	NA	NA	2 [56]	2 [56]	2	25	37	68
GNLY	3	2	2	1	1	1	0	NA	1	3	0	0	0	14	37	38
HAMP	3	2	1	1	2	1	0	NA	NA	NA	0	2 [70,71]	0	12	31	39
HOMER2	5	2	1	0	1	2	0	NA	3	0	0	0	0	14	37	38
HTR2B	5	1	1	0	2	3	0	NA	3	1	0	2 [72]	2	20	37	54
IGFBP1	3	2	2	2	2	3	3 [73]	NA	0	3	0	0	0	20	37	54
IL1B	5	1	1	2	4	1	3 [74]	NA	NA	NA	0	2 [75]	0	19	31	61
MMP10	5	2	1	1	2	3	3 [76]	NA	NA	NA	0	0	0	17	31	55
MMP11	5	2	2	1	1	1	3 [77]	NA	1	2	0	0	0	18	37	49
MMP3	5	1	1	2	3	3	3 [77]	NA	NA	NA	2 [78]	0	0	20	31	65
MMP7	5	4	2	2	3	2	3 [79]	1 [79]	1	2	2 [80]	0	0	27	43	63
NKD2	5	2	2	0	1	1	0	NA	NA	NA	0	0	0	11	31	35
PAEP	3	1	1	2	1	3	3 [81]	1 [81]	1	3	0	0	0	19	43	44
RGS4	5	1	1	0	1	2	0	NA	NA	NA	0	0	0	10	31	32
SCGB1D2	3	1	1	0	0	1	0	NA	0	3	0	0	0	9	37	24
SELE	5	2	1	1	2	1	3 [82]	0 [82]	0	2	2 [83]	2 [84]	0	21	43	49
STRA6	5	2	1	1	1	3	0	NA	1	2	0	0	2	18	37	49
TNFAIP6	3	2	1	1	2	2	0	NA	0	3	0	0	0	14	37	38
VCAN	5	2	2	1	2	1	0	NA	1	2	0	0	0	16	37	43
VTCN1	5	2	2	1	1	3	0	NA	1	1	0	2 [85]	0	18	37	49
WNT4	5	2	1	2	1	1	3 [86]	1 [86]	NA	NA	0	0	0	16	37	43
WTN7A	5	1	2	1	1	3	3 [87]	NA	0	3	0	0	0	19	37	51

Table 3
Summary of the outcome of the modified Target Selection Criteria (TASC) for intra-operative fluorescent imaging of endometriosis. *Biomarkers are ranked from the highest score, indicating the highest potential for usage as a target for fluorescent imaging, to the lowest score.*

Biomarker	Total score	Maximum possible score	Scoring percentage (%)
FOLR1	25	37	68
CXCL8	29	43	67
MMP3	20	31	65
MMP7	27	43	63
IL1B	19	31	61
MMP10	17	31	55
HTR2B	20	37	54
IGFBP1	20	37	54
WNT7A	19	37	51
CDH2	21	43	49
SELE	21	43	49
MMP11	18	37	49
STRA6	18	37	49
VTCN1	18	37	49
CLDN10	17	37	46
PAEP	19	43	44
VCAN	16	37	43
WNT4	16	37	43
HAMP	12	31	39
ASIC2	14	37	38
GNLY	14	37	38
HOMER2	14	37	38
TNFAIP6	14	37	38
COL10A1	11	31	35
NKD2	11	31	35
ANO4	10	31	32
RGS4	10	31	32
C1QTNF6	11	37	30
SCGB1D2	9	37	24

Discussion

In this study, we successfully showed a new approach, using transcriptomic analysis, to identify biomarkers as candidate targets for intra-operative fluorescent imaging of endometriosis. 29 biomarkers were identified as candidate targets. Additionally, a ranking of these biomarkers was provided to identify the most potential candidates to use for clinical fluorescent imaging.

A unique new approach was used, to broaden the search for candidate targets. Usually, available protein-based experiments in the researched disease are analyzed to identify targets. These, sometimes scarce, protein-based experiments include pre-selected targets, often for other indications than fluorescence guided surgery. Therefore, it is questionable if targets identified via the conventional approach are the most suitable for fluorescence guided surgery and also if the most potential targets might be missed. Additionally, the development of a tracer that sufficiently targets a biomarker is challenging. Therefore, it is a precondition that the search is performed as unbiasedly as possible and that all potential targets are considered. We believe that our approach is a good step towards an unbiased search.

To use this new approach, the availability of RNA expression data is essential. As performing these experiments is a resourceful process, fortunately, datasets are increasingly published on public databases. Data for endometriosis and especially including the surrounding tissues of the same patients was, however, still limited. Additional relevant datasets to validate our findings and datasets including all these tissues would be of great added value and could add to the search for candidate targets in the future. However, even with only one available dataset, our study shows the potential of the use of publicly shared datasets for this research field. This dataset was a reflection of the endometriosis population, including women with and without hormonal treatment, all subtypes and locations of endometriosis lesions and various rAFS scores. By including this heterogenous dataset, we aimed to find a target applicable for the whole population.

In the absence of data on many relevant surrounding tissues in the chosen publicly available dataset, we combined information from the DEG analysis with TPM values of the GTEx and TCGA. In an optimal situation, a DEG analysis was performed on a dataset including all the relevant tissues. As a result, we set a cut-off TPM of 10 for the remaining surrounding tissues. This could have resulted in a relatively strict cut-off, as a relative expression compared to endometriosis is more important than the absolute expression level. Therefore, a TPM of 10 might also result in relatively high expression levels for specific targets of surrounding tissue compared to endometriosis, with the potential of small clinical fluorescent signal differences intra-operatively. Despite these limitations, our approach is a practical and promising solution considering the limited available possibilities.

Role of candidate targets in pathophysiology of endometriosis

The candidate targets identified in this study show interesting and different characteristics, of which several can be related to the underlying biology of endometriosis. The varying functions and roles of the potential targets, as described by UniProt, could be associated with the various described cellular components and pathophysiology of endometriosis lesions. Within the candidate targets, four matrix metalloproteins were included (MMP3, MMP7, MMP10, and MMP11), which are involved in the breakdown of extracellular matrix (ECM) in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling. This is interesting, as fibrosis, with ECM as an essential component, is suggested to play an important role in endometriosis [46]. Some included biomarkers are associated with inflammation (CXCL8, IL1B, SELE, TNFAIP6) or immune response (VTCN1), both playing a vital role in endometriosis [47–49]. CXCL8 is also associated with angiogenesis, which has a critical role in the pathogenesis of endometriosis [50]. SCGB1D2 is involved in androgen binding, while other targets are involved in cell adhesion (CDH2, CLDN10, VCAN), which may play a role in the interaction of endometrium with peritoneum [51]. PAEP is the main protein synthesized and secreted in the endometrium from the mid-luteal phase. The remaining biomarkers show no clear relation with the pathophysiology of endometriosis [52].

Future perspectives and clinical implications

To evaluate this approach, the evaluation of protein expression is important. This relates to the fact that one should be aware of the variable correlation between RNA and protein expression [53]. Detecting protein expression levels will enhance the potential of some biomarkers, and concurrently weaken the potency of others. Additionally, protein detection may confirm or disprove the predicted location in the transcriptomic analysis. A necessary first next step in the identification of the best target for clinical use is immunohistochemical evaluation of protein expression of interesting candidate targets. This could also elaborate on potential differences in protein expression based on subtypes of endometriosis, location, and hormonal therapy use. The role of hormonal therapy on target expression is not yet clear. No hormonal receptors were selected as potential target, however hormonal therapy might still have an effect on target expression of the potential targets [54,55]. Future studies will need to evaluate this effect to further differentiate between potential targets, with the aim to find an optimal target suitable for all patients.

Subsequently, for potential targets without already available clinical fluorescent tracers, new tracers need to be developed, or need to be further developed with clinical diagnostics or therapeutic tracers as a base. These tracers should be tested in (pre)clinical models of endometriosis. If clinical fluorescent tracers are already available, like OTL-38 for folate receptor alpha [56], clinical studies could be designed. Clinical studies will also shed a light on the fluorescent characteristics of endometriosis itself, taking into account the diverse compounds of

endometriosis, including fibrosis. As fibrosis is thought to be present in both peritoneal and deep endometriosis [46], fibrosis was also present in the samples from the used dataset. Therefore, fibrosis was already included in this first step towards a targeted fluorescent tracer.

Conclusion

In this study, a new promising approach to broaden the identification of candidate targets for fluorescence-guided surgery was shown. A transcriptomic analysis, combined with target selection criteria, resulted in 29 candidate targets for endometriosis. Future preclinical and clinical studies will show their value as a target for a tracer for intra-operative fluorescent imaging of endometriosis.

Summary points

- Irradical endometriosis surgery is a risk factor for recurrence. Optimized intra-operative imaging with targeted fluorescent imaging could add to better surgical results.
- This study shows a new approach identifying potential targets which can be translated to other surgical fields.
- A unique five phase approach is used, using publicly available data.
- The approach includes differentially expressed genes analysis, predicted location of the target, low expression levels and modified Target Selection Criteria.
- This study shows the first step towards intraoperative targeted fluorescent imaging of endometriosis, identifying 29 potential biomarkers.

Author Contributions

FHMPT, FWJ, ALV, and PJKK contributed to the study design. FT, MKB, and PJKK contributed to data analysis, data interpretation, and manuscript writing. MDB, FWJ, ALV, and PJKK contributed to data interpretation and critical manuscript revision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethical conduct of research statement

Only publicly available data was used for this study therefore no IRB Review was needed. All used datasets are mentioned in the manuscript.

Data sharing statement

Only publicly available data were used in this study. All used datasets are mentioned in the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejogrb.2023.07.007.

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