Assessment of Serum MicroRNA Biomarkers for Differentiating Between Indolent and Aggressive Prostate Cancer in Active Surveillance Patients

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Laboratory Medicine and Pathobiology

University of Toronto

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Abstract

A significant portion prostate cancer (PCa) patients on active surveillance (AS) eventually reclassifies to a higher risk-status due to occult, aggressive tumours or rapid disease progression. Reclassified patients receive delayed treatment, while patients with indolent PCa are subjected to unnecessary and invasive AS procedures. We assessed nine candidate serum microRNAs in two AS cohorts for prediction of patient reclassification. A “3-miR score” (miR-223, -24, -374) was significant for predicting reclassification (training OR 2.271, 95% CI 1.499-4.939; validation OR 5.512, 95% CI 1.502-20.229) and was independent of clinical markers in multivariable models. A 3-miR score + prostate-specific antigen (PSA) cutoff derived from the training cohort achieved an excellent negative predictive value of 89% and specificity of 81% in the validation cohort. The 3-miR score may be used in concert with PSA as a non-invasive biomarker panel to identify patients who can be safely monitored with a less intensive AS protocol.
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Abbreviations

%core % of cores involved with cancer
4K 4 Kallikrein
95% CI 95% confidence interval
AJCC American Joint Committee on Cancer
Akt Protein kinase B
APC Adenomatous polyposis coli
AR androgen receptor
AUC area under the curve
BCAR1 Breast Cancer Anti-Estrogen Resistance 1
BCL2L11 Bcl-2-like protein 11
BCR Biochemical recurrence
BPH benign prostate hyperplasia
CAPRA Cancer of the Prostate Risk Assessment
CCP cell cycle progression
CRPC castration resistant prostate cancer
CSC cancer stem cell
Ct cycle threshold
DGCR8 DiGeorge critical region 8
DRE digital rectal exam
EMT epithelial to mesenchymal transition
ERG Erythroblast transformation-specific related gene
ERSPC European Randomized Study of Screening for Prostate Cancer trial
fPSA free prostate-specific antigen
GC genomic classifier
GG Gleason grade
GP Gleason pattern
GPS genomic prostate score
GS Gleason score
GSTP1 Glutathione S-transferase P
HOXB3 Homeobox protein Hox-B3
IGF-1R insulin-like growth factor 1 receptor
ISUP International Society of Urological Pathology
ITGA3 Integrin alpha-3
ITGB1 Integrin beta-1
LNA  locked nucleic acid
LPC  localized prostate cancer
MET  mesenchymal to epithelial transition
miRNA  microRNA
mPCa  metastatic prostate cancer
MRI  magnetic resonance imaging
mRNA  messenger RNA
mTOR  mechanistic target of rapamycin
NCCN  National Comprehensive Cancer Network
NPV  negative predictive value
OR  odds ratio
p53  Tumor protein p53
p70S6K  P70-S6 Kinase 1
PCa  prostate cancer
PCA3  Prostate cancer antigen 3
PCOS  Prostate Cancer Outcomes Study
PHI  Prostate Health Index
PLCO  Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial
PPV  positive predictive value
PRIAS  Prostate Cancer Research International Active Surveillance study
PRKCH  Protein kinase C eta type
ProTecT  Prostate Testing for Cancer and Treatment Trial
PSA  prostate-specific antigen
PSAD  prostate-specific antigen density
PTEN  Phosphatase and tensin homolog
RASSF1A  Ras association domain-containing protein 1A
RISC  RNA induced silencing complex
ROC  receiver operator characteristic
RP  radical prostatectomy
SB  Sunnybrook Health Sciences Centre
TAN  tumour adjacent normal
TMPRSS2  Transmembrane protease, serine 2
TNM  tumour, node, metastasis
TPM  template prostate mapping
tPSA  total prostate-specific antigen
TRUS  transurethral ultrasound
Chapter 1 Introduction

1.1 Epidemiology of prostate cancer

Prostate cancer (PCa) is the abnormal cell growth in prostate tissue with the potential to invade or spread to other regions of the body\(^1\). It is classified as an adenocarcinoma, cancer of the glands. Prostate cancers are relatively slow-growing and may not cause any noticeable symptoms until advanced stages of disease progression. Prostate cancer is the most commonly diagnosed type of cancer in men worldwide\(^2\). Among Canadian men, PCa accounts for 21% of all new cancer cases, and 1 in 7 will be diagnosed with PCa in their life time\(^3\). In the year 2017, approximately 21,300 men will be diagnosed with PCa in Canada and 4,100 men will die from the disease\(^3\).

Risk factors associated with PCa includes age, family history, ethnicity, and environmental factors\(^4\). Among men diagnosed with PCa, approximately 0.1% are under the age of 50 and 85% are over the age of 65\(^4\). A meta-analysis of 29 studies that reported incidental PCa found upon autopsy showed association of PCa risk was associated with increasing age odds ratio (OR) 1.7 per decade\(^5\). There is also substantial hereditary contributions to PCa development, as the patients with multiple diagnosed male relatives may be up to five time likely to develop PCa\(^6\). The incidence of PCa varies depending on geographic areas and ethnicity. Statistics from Asian countries have reported the lowest prevalence of PCa\(^2,7\). In contrast, the highest PCa rates are reported in Europe, North America, and Australia, though this may be partly due to widespread prostate-specific antigen (PSA screening in these regions\(^2\). Among North Americans, those of African descent have reported higher incidence of PCa and higher-grade PCa\(^8\).

1.2 Grading and risk stratification

1.2.1 Gleason score

Prostate cancers are classified with the Gleason grading system, developed by Dr. Donald F. Gleason in 1966\(^9\). It is still widely accepted as a method to evaluate the prognosis of PCa. Gleason
grading is determined based on histologic examination of prostate tissue\textsuperscript{10,11}. First, Gleason patterns (GP), which range from 1 – 5, are assigned based on tissue appearance and pathologic characteristics. GP1 represent prostate tissue which contains mainly well differentiated glands (unlikely to be aggressive PCa), whereas GP5 represents very poorly formed glands, which indicate aggressive disease (Figure 1.1). There is often more than one GP observed in a tissue sample. The primary GP consists of greater than 50% of the tumour area and the secondary GP consists of less than 50% but at least 5% of the tumour area. A tertiary GP may also be present, which consists of a small component of the tumour (less than the primary or secondary grade) but generally more aggressive. The sum of the primary and secondary GP observed in a prostate tissue sample then make up the Gleason score (GS) of the PCa, ranging from 2-10. If there is a tertiary GP reported, then the GS consists of the sum of the primary GP plus the higher of the secondary and tertiary GP, which may result in a GS that is the sum of the primary and tertiary GP\textsuperscript{10}.

For prognostication of PCa, tumours which are GS ≤ 6 are considered low-grade and are unlikely to progress significantly\textsuperscript{10,11}. Next, GS7 tumours are intermediate-grade, which have widely varying outcomes. Finally, GS ≥ 8 tumours are high-grade and are associated with poor prognosis.
A new Gleason grading system was proposed at the 2014 International Society of Urological Pathology (ISUP) to more accurately stratify PCa\textsuperscript{12}. In the new system, GS 1-6 tumours are reassigned into Gleason grade (GG) group 1, GS 3+4 tumours into GG group 2, GS 4 + 3 tumours into GG group 3, GS 8 tumours into GG 4, and GS 9-10 tumours into GG group 5\textsuperscript{12}. This change was motivated by evidence which showed distinctive recurrence outcomes of the 5 proposed GG groups after treatment\textsuperscript{13}. The changes also highlighted the differences between prognoses of GS 3 + 4 versus GS 4 + 3 PCa, which have been independently observed by various groups\textsuperscript{12,14,15}. Furthermore, patients may infer, under the previous Gleason grading system, that GS 6 cancers are at an intermediate level of aggressiveness, when in fact it is most likely to be insignificant PCa\textsuperscript{12}. 

\textbf{Figure 1.1 Gleason patterns.} A) original Gleason pattern (1966); B) modified Gleason pattern (2014). Adapted from Epstein \textit{et al.} The 2014 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma. Am J Surg Pathol 2016 Feb; 40 (2): 244-52.
Thus, grouping GS 1-6 tumours into GG group 1, the lowest risk group, makes the grading system easier to interpret accurately for those without a medical background.

Figure 1.2 Typical histologic appearance of each Gleason pattern. Column A: old Gleason grading system; column B: new Gleason grading system. Adapted from Chen N and Zhou Q. The Evolving Gleason grading system. Chin J Cancer Res, 2016 Feb 28(1):58-64.

1.2.2 Clinical staging

Prostate cancer is also categorized with the tumour-node-metastasis (TNM) at the clinical (cTNM) stage or pathologic (pTNM) stage. This system was developed by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) in 1992 to aid risk stratification. The cTNM is based on digital rectal exam (DRE) and imagining information prior to surgery, while pTNM also includes information gained from examination of prostate tissue after removal via radical prostatectomy (RP). The T stage describes involvement of the tumour within
the prostate or extension to surrounding structures, while N and M stage describes the lymph node involvement and distant metastasis status, respectively (Table 1.1).

**Table 1.1 Clinical and pathological staging of prostate cancer**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Clinical</th>
<th>Pathological</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tx</td>
<td>Primary tumour not assessed</td>
<td>-</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumour</td>
<td>-</td>
</tr>
</tbody>
</table>
| T1 (a-c) | Cannot be detected with DRE or imaging  
- T1a: Tumour found incidentally during TURP and is < 5% of resected tissue  
- T1b: Tumour found during TUPR and is > 5% of resected tissue  
- T1c: Tumour upon needle biopsy performed due to elevated PSA | Tumour is confined to the prostate  
- T2a: Tumour is in less than half of one lobe  
- T2b: Tumour is in more than half of one lobe  
- T2c: Tumour has spread to both lobes |
| T2 (a-c) | Tumour can be detected by DRE or imaging but appears to be confined to prostate  
- T2a: Tumour is in less than half of one lobe  
- T2b: Tumour is in more than half of one lobe  
- T2c: Tumour has spread to both lobes | Tumour has grown outside of the prostate  
- T3a: tumour has extended outside of the prostate but not the seminal vesicles  
- T3b: Tumour has invaded the seminal vesicles |
| T3 (a-b) | Tumour has grown outside of the prostate  
- T3a: tumour has extended outside of the prostate but not the seminal vesicles  
- T3b: Tumour has invaded the seminal vesicles | Tumour has invaded the rectum, levator muscle or pelvic wall  
- T3a: tumour has extended outside of the prostate but not the seminal vesicles  
- T3b: Tumour has invaded the seminal vesicles |
| T4    | - | Tumour has invaded the rectum, levator muscle or pelvic wall |
| Nx    | Regional Lymph nodes not assessed | Regional lymph nodes not sampled |
| N0    | No regional lymph node metastasis | No regional lymph node metastasis |
| N1    | Metastasis in regional lymph nodes | Metastasis in regional lymph nodes |
| M0    | No distant metastasis | No distant metastasis |
| M1 (a-c) | Distant Metastasis  
- M1a: tumour has spread to distant lymph nodes  
- M1b: tumour has spread to bones  
- M1c: tumour has spread to other organs | Distant Metastasis  
- M1a: tumour has spread to distant lymph nodes  
- M1b: tumour has spread to bones  
- M1c: tumour has spread to other organs |
1.2.3 Risk stratification

Several prognostic models based on clinical variables have been established and used for risk stratification of PCa, most take into account the GS, PSA, and clinical T stage. The most commonly used model is the D’Amico classification criteria reported by D’Amico et al. in 1998\(^\text{17}\). The D’Amico criteria was created based on risk of biochemical recurrence after definitive treatment associated with pre-operative clinical variables. It has also been shown to be a predictor of disease progression and survival after RP\(^\text{18,19}\). The National Comprehensive Cancer Network (NCCN) risk categories are also commonly used, which closely resembles the D’Amico model, but also includes two more categories, “very low-risk” and “very high-risk” (Table 1.2). Models developed or endorsed by other major cancer organizations are mostly consistent with the D’Amico criteria\(^\text{20}\).

**Table 1.2 Risk stratification criteria as defined in NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines) Version 3.2016**

| Very low risk                                                                 | Low risk                                                                 | Intermediate risk                                           | High risk                                                                 | Very high risk                                                                                          |
|---|---|---|---|---|---|
| T1c, GS ≤ 6, PSA < 10 ng/mL, < 3 biopsy cores positive, ≤ 50% cancer in any core, PSAD < 0.15 ng/mL/g | T1-T2a, GS ≤ 6, PSA < 10 ng/mL | T2b-T2c, or GS 7, or PSA 10–20 ng/mL | T3a, or GS 8–10, or PSA > 20 ng/mL | T3b-T4, or primary GP 5, > 4 cores with GS 8–10 |

A more contemporary model proposed by Cooperberg et al., called the Cancer of the Prostate Risk Assessment (CAPRA) score, estimates risk of recurrences after RP\(^\text{21,22}\). This model score may be more accurate than D’Amico and similar risk groupings, because the CAPRA score takes into consideration more clinical characteristics, including age, percent of biopsy cores involved with cancer, and perhaps most importantly, the primary GP, which allows the model to account for the differences in aggressiveness of GS 4 + 3 versus GS 3 + 4 tumours. A summary of the CAPRA score calculation is presented in Table 1.3.
Table 1.3 Calculation of CAPRA score for PCa risk stratification

<table>
<thead>
<tr>
<th>Clinical variable</th>
<th>Value</th>
<th>Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis</td>
<td>Under 50</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>50 or older</td>
<td>1</td>
</tr>
<tr>
<td>PSA at diagnosis (ng/ml)</td>
<td>less than or equal to 6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>between 6.1 and 10</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>between 10.1 and 20</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>between 20.1 and 30</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>more than 30</td>
<td>4</td>
</tr>
<tr>
<td>Gleason score of the biopsy (primary/secondary)</td>
<td>no pattern 4 or 5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>secondary pattern 4 or 5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>primary pattern 4 or 5</td>
<td>3</td>
</tr>
<tr>
<td>Clinical stage (T-stage)</td>
<td>T1 or T2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>T3a</td>
<td>1</td>
</tr>
<tr>
<td>Percent of biopsy cores involved with cancer (positive for cancer)</td>
<td>less than 34 percent</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>34 percent or more</td>
<td>1</td>
</tr>
</tbody>
</table>

CAPRA score is equal the sum of points assigned for each clinical variable. Low risk: 0-2, intermediate risk: 3-5, and high risk: 6-10\(^2\). Adapted from reference 1.3.

1.3 Prostate cancer screening and diagnosis

Men over the age of 50 are recommended to receive periodic screening for PCa via digital rectal exams (DRE) and prostate-specific antigen (PSA) tests\(^2\). Prostate-specific antigen is secreted by prostate epithelial cells\(^2\). Increased proliferation of prostate epithelial cells and disruption of the prostate epithelium leads to a detectable increase in blood PSA levels\(^2,5\). If abnormal results are found in DRE and PSA tests, a prostate biopsy is recommended to patients\(^2\). Biopsies extract small cores of prostate tissue for histopathological examination by a pathologist to determine whether there are cancerous tissues present. Currently, a prostate biopsy is needed for confirmation of a PCa diagnosis\(^2\).
1.3.1 Digital rectal exam

During a DRE, the physician will palpate the prostate of the patient through the wall of the rectum\textsuperscript{27}. In this process, a tumour may be felt as a hard lump. The sensitivity of this procedure is low, and early stage PCa often cannot be detected by DRE\textsuperscript{27}. Furthermore, the thoroughness and interpretation of the DRE may vary between physicians. Nonetheless, DRE is still routinely performed for clinical T staging, which contributes to risk assessment models, such as the D’Amico criteria\textsuperscript{17}.

1.3.2 Prostate-specific antigen

PSA is a serine protease encoded by the \textit{Kallikrein 3} gene\textsuperscript{28}. It is primarily produced by prostatic epithelial cells and aids liquefaction of semen by cleaving semenogelin I and II\textsuperscript{28}. Early studies observed that PSA is found at a low level in circulation of healthy men, but often elevated in PCa patients. PSA testing alongside DRE significantly improved the sensitivity for detecting PCa compared to DRE alone, which eventually led to implementation of PSA testing in routine screening procedures\textsuperscript{29,30}.

The conventional threshold for suspicion of PCa is a serum PSA level above 4ng/mL\textsuperscript{31-33}. However, men with PSA below 4ng/mL may still have PCa and a significant portion of men with PSA above 4ng/mL do not have PCa. A study which examined a large cohort of men with PSA under 4ng/mL and no abnormal DRE findings reported that approximately 15% of these men harboured PCa\textsuperscript{31}. Furthermore, elevated PSA may be associated with a variety of other factors, such as benign prostate hyperplasia (BPH), prostatitis, ethnicity, and age\textsuperscript{32}. Only 27-44% of patients screened using the 4ng/mL threshold are found to harbour PCa\textsuperscript{34-36}. PSA became widely adopted in clinical practice for PCa screening in the early 1990’s and drastically increased PCa incidence\textsuperscript{37}. However, newly diagnosed PCa cases were mostly localized or regional\textsuperscript{38}. Incidence of distant-metastatic PCa, which is the most clinically significant category, incidence remained similar to pre-1986\textsuperscript{38}. As such, the stage and grade of new PCa diagnoses in the last two decades has shifted heavily towards low-risk tumours. There is generally a consensus that low-risk PCa is overdiagnosed and
overtreated in contemporary practice\textsuperscript{39-42}. As such, PSA screening is currently not recommended by the United States Preventative Services Task Force, with a grade scale of “D,” which states that there is moderate or high certainty that PSA testing has no net benefit or that the harms outweigh the benefits.

Two major randomized control trials have reported long-term outcomes of PSA screening. The Prostate, Lung, Colorectal, and Ovarian Cancer Screening (PLCO) trial randomly assigned patients to either the intervention group (annual PSA screening) or control group (usual care, including opportunistic PSA testing)\textsuperscript{36,40,43}. Over 13 years, there was a 12% increase in PCa detection in the intervention group of the PLCO trial\textsuperscript{43}. Despite this finding, the PCa-specific survival did not significantly differ between the two groups, which led the authors to conclude that there was no survival benefit in annual PSA screening protocols. The European Randomized Study of Screening for Prostate Cancer (ERSPC) also aimed to determine the benefit of PSA screening. In this study, patients were randomly assigned into the PSA screening group (PSA testing every 4 years) or control group (no PSA testing)\textsuperscript{44-46}. In contrast to the PLCO study, the ERSPC study found a significant benefit to PSA screening. The ERSPC trial authors, after 13 years of followup, reported that the incidence of PCa-specific death was 21% lower in the PSA screening group and estimated one PCa-related death would be prevented for each 781 men screened and 27 PCa cases detected\textsuperscript{44}.

1.3.2.1 PSA isoforms

Total PSA (tPSA or just PSA) consists of complexed and free PSA (fPSA)\textsuperscript{28}. Complexed PSA are bound to proteins, while fPSA are not. Complexed PSA account for approximately 70% of tPSA in circulation\textsuperscript{47}. fPSA can be further categorized into three common isoforms, proPSA, BPH-associated PSA and intact PSA\textsuperscript{48}. The ratio of free-to-total PSA has been shown to aid diagnosis of PCa patients with moderately elevated PSA, between 4-10ng/mL\textsuperscript{49}. Various predictive models and biomarker tests have incorporated these PSA derivatives in multivariable models to improve cancer detection. For example, the Prostate Health Index (PHI) utilizes a formula of [
2) proPSA/fPSA x vTtPSA to derive a score which has better sensitivity and specificity for PCa detection compared to PSA alone\textsuperscript{50}.

### 1.3.2.2 Age-adjusted PSA and PSA density

Studies have demonstrated a positive correlation between age and PSA. This is likely due to the increase in prostate size of older men\textsuperscript{51}. As such, elderly men may have overall higher baseline PSA, and this factor should be taken into consideration when performing PSA screening in an older population. Age-adjusted PSA cutoffs have been shown to increase the specificity of PCa detection and reduce unnecessary biopsies\textsuperscript{52}.

Another way to account for increasing prostate size is calculating the PSA density (PSAD), which normalizes PSA to the prostate volume. This variable was initially developed to discriminate between BPH and PCa patients. However, this marker has not been extensively utilized due to the difficulty of obtaining prostate volume measurements during biopsy\textsuperscript{53}.

### 1.3.3 Prostate biopsy

A prostate biopsy followed by histologic examination is required to confirm a PCa diagnosis\textsuperscript{26}. Transurethral ultrasound (TRUS) guided biopsies are routinely performed for this purpose. In this procedure, 6-12 tissue cores are extracted with a needle and help of ultrasound imaging to estimate the locations of tumours\textsuperscript{26}. There are several shortcomings to this process. First, only a very small portion of the entire prostate volume is sampled, and cancerous tissue or high-grade tumours may be missed, leading to false negatives and undergrading of tumours, respectively. In an individual TRUS-guided biopsy, the false-negative rate is estimated to be 19-23\textsuperscript{45}, and based on pathologic upgrading between biopsy and RP, approximately 30% of cases are undergraded at the biopsy stage\textsuperscript{54}. Secondly, the pathologist cannot always confidently distinguish between benign and malignant tissue based on the biopsy sample and the tissue is labeled as atypia, in which case, another biopsy is required\textsuperscript{26}. Finally, interobserver variability between pathologists may also lead to undergrading or overgrading\textsuperscript{55}. 


Cores can also be taken in a transperineal fashion, with template prostate mapping (TPM) biopsy. During TPM biopsies, the cores are extracted according to a template of varying number of cores depending on thoroughness. Typically, a much larger number of cores are taken with this method, which improves the cancer detection rate, but also requires a general or spinal anesthetic.\textsuperscript{56,57}

In recent years, magnetic resonance imaging (MRI)-guided biopsies have been gaining traction as a more efficient alternative to TRUS-guided biopsies. Studies have demonstrated that using it in combination with ultrasound for guiding biopsy improves sensitivity and specificity for detecting PCa\textsuperscript{58-60}. It has also been proposed as a triage protocol for deciding whether men suspected of having PCa should even undergo a primary biopsy, owing to its excellent negative predictive value (NPV) for ruling out clinically significant PCa\textsuperscript{57}. Ahmed \textit{et al.} recently demonstrated that using a Prostate Imaging Reporting and Data System (PIRADS) score of 1 (most probably benign) or 2 (probably benign) as indication for insignificant PCa, MRI have a NPV of 89% and specificity of 41\%\textsuperscript{57}.

1.4 Treatment of localized prostate cancer

In contemporary practice, definitive treatment of localized prostate cancer involves RP or radiotherapy\textsuperscript{42}. Radical prostatectomy is the surgical removal of the prostate and surrounding tissues to ensure negative surgical margins\textsuperscript{41}. Radiotherapy, including external beam radiation therapy and brachytherapy, is the usage of ionizing radiation directed to control or kill malignant cells\textsuperscript{41}. Both methods are effective in treating localized PCa, but are associated with significant risks and complications at the short and long term. The Prostate Cancer Outcomes Study (PCOS) followed 1164 men who underwent RP and 491 men who received radiotherapy, and reported that a significant proportion of patients experience urinary incontinence, sexual dysfunction, and decreased bowel functions (Table 1.4)\textsuperscript{61}. 
Followup of patients who received RP (n=1164) or radiotherapy (n=491) treatment at 2, 5, and 15 years. The majority of patients in both treatment groups experienced sexual dysfunction. Patients who received RP suffered less loss of bowel function, but more urinary incontinence compared to patients who underwent radiotherapy. Proportions of all complications increased over time. Adapted from Resnick, M.J., et al., Long-term functional outcomes after treatment for localized prostate cancer. N Engl J Med, 2013. 368(5): p. 436-45.

With the increasing evidence that localized PCa, especially cases which fit the low-risk criterion, are overtreated, there is a shift in practice towards monitoring of localized PCa to minimize the side effects of definitive treatment. The ProtecT trial did indeed report that monitoring with the option of delayed treatment is not associated with significantly increased long-term PCa-specific deaths compared to treatment by either RP or radiotherapy, although monitoring was associated with higher incidence of metastasis.

1.5 Active surveillance for prostate cancer

The advent of PSA screening has greatly increased PCa incidence, yet most new PCa patients are diagnosed with localized, low-grade disease that is likely insignificant. Early detection and treatment for men with high-risk disease is associated with lower PCa-specific mortality rates.
However, for low-risk patients, the benefits of treatment are not substantial and treatment morbidities outweigh any potential benefits\textsuperscript{41}. Active surveillance (AS) is a management option for low-risk PCa patients which aims to mitigate overtreatment by closely monitoring patients for cancer progression, and only recommending treatment for patients who show disease progression\textsuperscript{42}. Several AS programs in North America and Europe have reported long-term outcomes and concluded that AS monitoring is safe for low-risk patients (i.e. no PCa-specific survival differences between immediate treatment versus AS and potential delayed treatment)\textsuperscript{62-70}. Thus, AS is employed as a management option to combat overtreatment of men with low-risk PCa. This approach has become increasingly utilized in recent years and is recommended as a management strategy for newly diagnosed, low-risk PCa patients\textsuperscript{42,71}. According to NCCN guidelines, AS should be offered to low-risk and very-low-risk PCa patients\textsuperscript{72}. Patients are monitored through periodic tests and reclassified upon discovery of occult, aggressive tumours or disease progression\textsuperscript{42}. Low-risk patients on AS are then able to delay or avoid definitive treatment, minimizing treatment morbidities and retain better quality of life.

1.5.1 Active surveillance protocols

Various institutions have reported their experiences with AS for PCa patients, but there is not yet a consensus on what is the best protocol, as reflected by the varied selection, monitoring, and reclassification criteria across different AS programs (Table 1.5).
Table 1.5 Selection criteria, monitoring protocol, and triggers for reclassification of various active surveillance programs.

<table>
<thead>
<tr>
<th>Institution</th>
<th>Selection criteria</th>
<th>Monitoring protocol</th>
<th>Triggers for reclassification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sunnybrook</td>
<td>GS≤6, PSA≤10, &lt;3 positive cores, or if LE&lt;10yrs: GS≤3+4, PSA≤20</td>
<td>PSA every 3 months for 2 years, then every 6 months confirmatory biopsy within 6-12 months, then every 3-4 years</td>
<td>GS upgrading, PSA-DT&lt;3 years*</td>
</tr>
<tr>
<td>UCSF</td>
<td>GS≤6, PSA&lt;10, ≤T2, &lt;33% of total cores involved, ≤50% of any core involved</td>
<td>PSA every 3 months, biopsy every 6-12 months, Biopsies repeated every 1-2yrs</td>
<td>GS &gt;6, &gt;33% of any core involved, PSAV &gt;0.75</td>
</tr>
<tr>
<td>Royal Marsden</td>
<td>GS≤6, ≤T2, ≤50% of total cores involved, or if age&gt;65, GS≤3+4, PSA&lt;15</td>
<td>PSA every 3 months for 1st year, 4 months for 2nd year, then every 6 months, confirmatory biopsy within 18-24 months, then every 2 years</td>
<td>GS&gt;3+4, &gt;50% of any cores involved, PSAV&gt;1</td>
</tr>
<tr>
<td>Göteberg</td>
<td>GS≤6, PSA&lt;10, ≤T2a</td>
<td>PSA 3-6 months, biopsy every 2-3 years</td>
<td>PSA, stage, or grade progression (no explicit definition)</td>
</tr>
<tr>
<td>Copenhagen</td>
<td>GS≤6, PSA≤10, ≤T2a, ≤50% of any cores involved and ≤3 positive cores</td>
<td>PSA 3 months, confirmatory biopsy within 12 months, then periodically</td>
<td>PSA-DT&lt;3 years, GS&gt;3+4, &gt;3 cores involved, PSA-DT&lt;3 years</td>
</tr>
<tr>
<td>PRIAS</td>
<td>GS≤6, PSA≤10, ≤T2, PSAD&lt;0.2, ≤2 cores involved</td>
<td>PSA 3 months for 2 years, then every 6 months, biopsies repeated at 1,4 and 7 years (or yearly if PSA-DT &lt;10 years)</td>
<td>GS&gt;6, &gt;T2, PSA-DT&lt;3 years, &gt;2 cores involved</td>
</tr>
<tr>
<td>University of Miami</td>
<td>GS≤6, PSA≤10, ≤T2, ≤20% of any cores involved, ≤2 positive cores</td>
<td>PSA 3-4 months for 2 years, then every 6 months, confirmatory biopsy within 9-12 months, then every year</td>
<td>GS&gt;6, &gt;2 cores involved, &gt;20% of any cores involved</td>
</tr>
</tbody>
</table>
### 1.5.2 Active surveillance candidates

Patients whom should be recommended for AS can range from very low-risk to intermediate-risk (NCCN risk categories). Table 1.5 summarizes the selection criteria of various major AS programs. Most programs include only low-risk patients (GS ≤ 6, PSA ≤ 10ng/mL) with the exception of Sunnybrook and Royal Marsden, which also include intermediate-risk patients. Sunnybrook includes patients who have GS 3+4 PCa, PSA ≤ 20ng/mL, and life expectancy of less than 10 years. Royal Marsden includes patients who have GS 3+4 PCa, positive biopsy cores ≤ 50%, PSA < 15ng/mL, and age > 65 years. Aside from GS and PSA, most programs also take into consideration the number or percentage of positive biopsy cores, with the exception of Sunnybrook and Goteborg. About half of the programs also considers the maximum percent of cancer found in any biopsy core and two programs considers PSAD (John Hopkins PSAD < 0.15 and PRIAS PSAD < 0.20).

### 1.5.3 Monitoring regimen

Monitoring protocols also vary between different AS programs. All program protocols include periodic biopsies and PSA measurements, ranging from every 3-6 months for PSA tests and 1-5 years for biopsies. A confirmatory biopsy is required within one year of diagnosis for all programs with the exception of Royal Marsden (required within 2 years) and Goteborg (not required).

MRI is becoming increasingly utilized in contemporary AS programs. Most programs have yet to defer to MRI alone for reclassifying patients, but those who show adverse disease upon imaging, PIRADS score 4 (probably malignant) or 5 (most probably malignant), are monitored more closely or undergo repeat biopsy to confirm high-grade disease. MRI may also be used in combination
with TRUS biopsies to improve detection of significant tumours. The high NPV of MRI may be used for identifying patients who are very unlikely to have clinically significant PCa\textsuperscript{57}.

1.5.4 Triggers for reclassification

Several triggers have been proposed as indication of aggressive PCa. The most reliable indication of aggressive disease is GP 4 or 5 upon repeat biopsies. All AS programs employ either GS upgrading or GS > 6 as a criterion for reclassification (Table 1.5).

There is increasing evidence that suggest true GP 3 tumours are insignificant and have extremely low potential to metastasize\textsuperscript{80-82}. Despite these, all programs still consider increasing prostate volume, as detected by number of biopsy cores positive or percent of any core involved with cancer, to be a trigger for reclassification, with the exception of Sunnybrook and Goteborg. Finally, all programs consider adverse PSA kinetics (either doubling time or velocity) to be grounds for reclassification except University of Miami and John Hopkins. PSA kinetics as an indicator of significant PCa was first reported by Choo \textit{et al.}, who found that PSA-DT < 3yrs was a predictor of disease progression\textsuperscript{75}.

1.5.5 Shortcomings of active surveillance

Though AS has been very successful in reducing unnecessary treatments, it has certain limitations. A subset of AS patients eventually reclassify due to detection of aforementioned triggers, which indicate occult, aggressive PCa or true disease progression. Upon reclassification, patients are recommended definitive intervention\textsuperscript{42}. An estimated 25-35\% of patients are reclassified within 5 years of AS enrolment\textsuperscript{42,62,64}. This subset of patients may benefit from immediate treatment, rather than delayed treatment upon reclassification. Conversely, patients with indolent PCa who will not progress are subjected to unnecessary serial biopsies. Though much preferred to definitive treatment, tissue biopsies are still invasive. Repeat biopsies can be painful and side effects may include hematuria, infection that may lead to hospitalization, and increased risk of erectile dysfunction\textsuperscript{83,84}. Conventional clinical markers cannot accurately distinguish between AS patients with indolent versus aggressive PCa. As a significant portion of low-risk PCa patients are being
managed with AS, it is important to address these problems so that patients with aggressive PCa can receive treatment with less delay and patients with indolent PCa may avoid invasive monitoring procedures.

1.6 Current landscape of prostate cancer biomarkers

Due to shortcomings of current clinical variables in diagnosis and prognosis of PCa, there has been extensive research conducted to develop novel biomarkers. Biomarkers are the biological factors that can be measured to indicate disease states, which in turn aids the diagnosis and prognosis of illnesses. Various genetic and epigenetic changes have been demonstrated to be associated with PCa or PCa progression, some of which have been approved by the Food and Drug Administration (FDA) or Clinical Laboratory Improvement Amendments (CLIA) for use as PCa biomarkers.

1.6.1 Prostate cancer associated antigen 3 (PCA3)

PCA3 is a prostate-specific, long non-coding RNA commonly overexpressed in PCa patients. Progensa (Hologic, Bedford, MA, USA) PCA3 test is FDA approved for men greater than 50 years of age in consideration for repeat biopsy. The test measures the ratio of PCA3 to PSA in post-DRE urine samples, and studies have shown PCA3 to have diagnostic ability of area under the curve (AUC) = 0.678-0.693\textsuperscript{85,86}. However, PCA3 has not been consistently demonstrated to be associated with high-grade PCa such that it can be used as a prognostic biomarker.

1.6.2 4 Kallikrein (4K) score

The 4K score (OPKO Laboratory, Nashville, TN, USA) is a formula which takes into account four forms of Kallikrein proteins (tPSA, fPSA, intact PSA, and human Kallikrein 2), as well as DRE and previous biopsy findings. The 4K algorithm was conceived based on findings from the ERSPC study. This biomarker test is CLIA-certified and identifies clinically significant PCa in previously undiagnosed patients. Parekh et al. conducted a multi-centred study which showed the 4K score was able to discriminate between benign (n = 542) or low-grade PCa (n = 239) versus high-grade PCa (n = 231)\textsuperscript{87}. 
1.6.3 Prostate Health Index (PHI)

The PHI test (Beckman Coulter, Brea, CA, USA) is an algorithm consisting of tPSA, fPSA, and p2PSA in the following formula: \( \text{phi} = (p2\text{PSA}/f\text{PSA}) \times \sqrt{t\text{PSA}} \). Prostate Health index was initially shown to be a biomarker for PCa diagnosis, with AUC up to 0.75 for distinguishing between normal and PCa patients\(^88,89\). A recent multi-center study showed that PHI can have prognostic ability as well\(^90\). Heidegger \textit{et al.} examined 112 GS6 patients, who were scheduled for radical prostatectomy. The PHI had OR of 1.039 (p = 0.004) for predicting GS upgrading after surgery. However, in the subset of patients which fits the NNCN criteria of AS, the PHI was not a significant predictor for GS upgrading after surgery (OR=1.06, p=0.412)\(^90\). PHI test is FDA-approved for aiding PCa diagnosis in men greater than 50 years of age and have PSA between 4-10ng/mL.

1.6.4 Confirm MDx

DNA methylation aberrations of certain genes are associated with PCa. Confirm MDx (MDxHealth, Irvine, CA, USA) is a test which measures the methylation level of \textit{GSTP1}, \textit{APC}, and \textit{RASSF1A} in PCa-negative biopsy tissues\(^91\). These genes have been shown to be hypermethylated in tumour adjacent normal (TAN) tissues due to field cancerization. Thus, biopsies which missed existing PCa tumours but sampled TAN tissues in close proximity to the tumour may still be deemed suspicious by a Confirm MDx test and warrant a repeat biopsy. In a cohort of 423 patients, Stewart \textit{et al.} found Confirm MDx had a negative predictive value (NPV) of 90% with 64% specificity\(^91\). Confirm MDx is currently CLIA-certified.

1.6.5 OncoType DX

The OncoType DX prostate test (Genomic Health, Redwood City, CA, USA) measures the expression levels of 5 references genes and 12 cancer-related genes involved in stromal response, androgen signaling, and cellular organization. Based on this analysis, a proliferation and a genomic prostate score (GPS), ranging from 0-100, is calculated\(^92\). The test is marketed for improving selection of AS patients by predicting GS 4+3 or worse PCa. The test shows an OR of 2.3 associated with adverse pathology for each 20-point GPS increase\(^92\).
1.6.6 TMPRSS2:ERG fusion protein

A gene fusion between transmembrane protease, serine 2 (TMPRSS2) and erythroblast transformation specific related gene (ERG) is observed in about 50% of PCa cases and occurs due to abnormal chromosomal rearrangement\(^9^3\). TMPRSS2:ERG fusion has been demonstrated as both a diagnostic and prognostic biomarker. An early study of urinary sediments demonstrated TMPRSS2:ERG to be a potential biomarker for PCa with high specificity (93%) but limited sensitivity (37%)\(^9^3\). However, sensitivity was greatly improved when paired with PCA3 (73%). Later, a large cohort study demonstrated that urinary TMPRSS2:ERG can predict GS > 6 PCa on biopsy with AUC = 0.729, and the AUC further improved to 0.772 when PCA3 and PSA were incorporated along with TMPRSS2:ERG\(^9^4\). There is a biomarker test currently under development, which measures the TMPRSS2:ERG and PCA3 isolated from urinary exosomes, called ExoDx Prostate IntelliScore assay (Exosome Diagnostics, Cambridge, MA, USA). A study of 774 patients showed this upcoming biomarker test was able to differentiate between GS ≥ 7 versus GS ≤ 6 and non-PCa with AUC=0.74\(^9^5\).

1.6.7 Decipher

Decipher (GenomeDx Biosciences, San Diego, CA, USA) is a CLIA-certified test which measures the expression of 22 genes involved in cell proliferation, migration, tumour motility, androgen signaling, and immune system evasion\(^9^6\). A genomic classifier (GC) risk score is subsequently calculated. This test is marketed for predicting recurrence after treatment. Den et al. found that in RP patients with pT3 PCa or positive margin after surgery, higher GC risk score was associated with higher rates of recurrence\(^9^7\). Klein et al. demonstrated that the Decipher test on biopsy tissue samples along with NCCN risk stratification information can predict metastasis at 10 years after RP with a hazard ratio (HR) of 1.72\(^9^6\).

1.6.8 Prolaris

Prolaris (Myria Gnenetics, Salt Lake City, UT, USA) test measures the expression of 31 cell cycle progression (CCP) genes and is used to predict outcomes after treatment. In a retrospective study,
Cuzick et al. found that patients with high CCP score had a HR of 1.89 for biochemical recurrence after RP and when combined with the CAPRA score, it can also estimate the 10-year PCa-specific mortality risk. Prolaris is FDA-approved for use in men with low-risk (NCCN criteria) and post-RP patients to guide management options.

1.7 microRNA

MicroRNAs (miRNA) are small, single stranded non-coding RNAs 18 - 22 nucleotides in length. They function through binding to the 3’ untranslated region of target messenger RNAs (mRNA) to inhibit translation. Up to 60% of the human genome is estimated to be targets of miRNAs. The first miRNA, lin-4 in Caenorhabditis elegans, was discovered in 1993. However, the role of miRNAs was not fully appreciated until the early 2000’s, when multiple studies indicated that miRNAs should be considered a distinct class of regulatory molecules due to their extensive involvement in a wide array of biological processes in many plant and animal species. Since then, over 1000 human miRNA genes have been identified. An individual miRNA may target hundreds or thousands of genes, and conversely, each gene may be targeted by multiple miRNAs. Changes in gene expression due to regulation by miRNAs is considered an epigenetic phenomenon, since it does not cause changes in the DNA sequence. As such, these transient changes hold great potential not only as biomarkers, but therapeutic targets as well.

1.7.1 Biosynthesis

MiRNA genes are transcribed by RNA polymerase II to produce primary miRNA (pri-miRNA). Approximately 30% of miRNAs are transcribed from introns of protein coding regions. In the nucleus, pri-miRNA is cleaved by a RNase III enzyme, DROSHA, and its cofactor, DiGeorge syndrome critical region 8 (DGCR8), into pre-miRNA. Pre-miRNAs have a double stranded stem-loop structure approximately 70 nucleotides in length, which contains the mature miRNA in the double stranded region. The pre-miRNA is transported out of the nucleus by the Exportin-5 protein and further processed by DICER ribonuclease. In the cytoplasm, DICER cleaves the 5' and 3' ends of the pre-miRNA hairpin to produce the mature miRNA duplex with 2-nucleotide
overhangs on the 3’ end\textsuperscript{101,105}. DICER recruits transactivation-responsive RNA-binding protein (TRBP), which bridges DICER with Argonaute proteins to the miRNA induced silencing complex (MiRISC)\textsuperscript{102}. One strand from the mature miRNA duplex associate with Argonaute proteins and “guides” the miRISC to mediate base-pairing of miRNAs and target mRNAs\textsuperscript{101,105}. Mature miRNAs recognize seed sequences in the 3’ untranslated region of mRNAs\textsuperscript{101,105}.

**Figure 1.3 Overview of miRNA biosynthesis.** Adapted from Winter, J., et al., Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol, 2009. 11(3): p. 228-34.
1.7.2 Role of miRNAs in prostate cancer development

Each miRNA may act as an oncomiR (promotes carcinogenesis) through downregulation of tumour suppressor genes or tumour suppressor miRNA (resists carcinogenesis) through downregulation of oncogenes\textsuperscript{106}. MiRNAs have been implicated in various cellular processes involved in prostate cancer development.

Cell cycle dysregulation: Dysregulation of the cell cycle leads to uncontrolled cell division, a hallmark of cancer. OncomiRs can downregulate cell cycle regulators. For example, Lewis et al., have previously reported miR-888 inhibits RBL1, which blocks the first gap phase to synthetic phase during cell cycle progression, and this led to increased colony formation of prostate cancer cell during in vitro experiments\textsuperscript{107}.

Apoptosis resistance: Programmed cell death is required for structured development of organisms and destruction of damaged or infected cells. Another hallmark of cancer is the evasion of programmed cell death. MiR-21 has been demonstrated to target PTEN, a tumour repressor gene which negatively regulates the Akt/PKB singling pathway that promotes cell survival\textsuperscript{108}. In PCa cell lines, inactivation of miR-21 resulted in sensitivity to apoptosis\textsuperscript{109}.

Epithelial to mesenchymal Transition (EMT) and mesenchymal to epithelial transition (MET): In many late-stage tumours, epithelial cells lose their cell polarity and assume a mesenchymal cell-type, which possess migratory and invasive properties\textsuperscript{110}. Cells that have undergone EMT may invade surround tissues through the extracellular matrix and metastasize to other parts of the body through the circulatory system\textsuperscript{111}. Metastasized cells can also undergo MET to revert back to epithelial-type cells and acquire characteristics similar to cells in the primary tumour\textsuperscript{110}. MiRNAs have been found to regulate expression of genes key in EMT and MET processes. The miR-200 family members regulate EMT by directly targeting mesenchymal markers, such as vimentin, and upregulating epithelial markers, such as E-cadherin, through repression of ZEB\textsuperscript{2}\textsuperscript{112}.

Androgen signaling: The androgen receptor (AR) is an important oncogenic pathway for PCa progression. Patients who develop resistance to hormone therapy are termed to have castration
resistant PCa (CRPC), and AR play a determinant role in the progression to CRPC. Several miRNAs have been shown to regulate AR, including miR-21, -31, -34, and -124, and several miRNAs are under the regulation of AR, including miR-21, -27a, -34, -125b, -221, and let-7\textsuperscript{113}.

Cancer stem cells (CSC): A subset of cancer cell population in tumours possess the ability to self-renew and differentiate into multiple cell types to create new tumours. These cells can cause relapse after treatment and metastasis. MiRNAs in the miR-34a and let-7 families have been shown to be involved in the regulation of prostate CSC populations\textsuperscript{114,115}.

1.7.3 miRNA as a biomarker for prostate cancer

In 2006, Volinia \textit{et al.} first reported miRNA profile alterations of PCa patients through analysis of 228 miRNAs in 56 PCa and six normal prostate tissue samples\textsuperscript{116}. The authors found 39 miRNAs were upregulated and six miRNAs were downregulated in PCa tissue. Due to stability of miRNAs in biofluids (blood, urine), research quickly expanded to these sample types as well to explore non-invasive biomarkers. In human plasma samples, endogenous miRNAs have been shown to resist degradation and remain stable after 8 freeze-thaw cycles or incubation at room temperature for 24 hours\textsuperscript{117}. The cause of circulating miRNA dysregulation observed in cancer patients is not well elucidated. MiRNAs can be actively released into the blood stream as a part of exosomes\textsuperscript{118,119} or protein complexes\textsuperscript{120}. Passive release of Argonaute-protein-bound miRNA can occur during cell apoptosis or necrosis\textsuperscript{121}. It has also been suggested that circulating miRNA alterations may be a consequence of an immune response by the adjacent tissue or immune cells\textsuperscript{122}.

Numerous circulating miRNAs have been shown to have potential as prognostic PCa biomarkers\textsuperscript{123,124}. Past studies on circulating miRNAs typically examined either serum or plasma, and less commonly whole blood. The miRNA expression profiles of serum and plasma has been demonstrated to be similar, but RNA isolated from whole blood samples will also contain cellular miRNA\textsuperscript{123}. The first report of aberrant circulating miRNA signatures in human PCa patients was in 2008 by Mitchel \textit{et al.}, who observed that there was a significant difference in between serum levels of miR-141 in metastatic PCa patients versus healthy controls\textsuperscript{117}. Since then, many more
circulating miRNAs have been found to be associated with PCa, disease progression, or treatment outcome. Peer-reviewed studies which examined circulating miRNAs in PCa patients, ordered by year of publication, are summarized in Table 1.6.

Despite more than 30 reports on the subject, there is limited concordance between studies. Significance of proposed miRNA biomarkers has not been consistently demonstrated between studies. MiRNAs have also been reported to be dysregulated in opposite directions and reliability of detection may vary drastically between studies\textsuperscript{123}. Discordant findings between studies may be attributed to inconsistent processing techniques, platform used for analysis of miRNAs, or means of normalization\textsuperscript{123,124}. Preanalytical factors, such as protocols of sample collection, handling, and storage may influence levels of miRNAs. For example, hemolysis of red blood cells prior to serum isolation may contaminate serum samples with cellular RNA\textsuperscript{125} and certain extraction techniques have been found to bias the miRNA makeup\textsuperscript{126}. Mestdagh \textit{et al.} performed a comprehensive comparison of 12 miRNA detection platforms, including seven qPCR-based, three hybridization-based, and two sequencing-based techniques, and found there was low concordance (average 54.6\%) of differentially expressed miRNAs generated between platforms\textsuperscript{127}. In circulating miRNA studies, results may be commonly skewed due to normalization methods. About 1/3 of studies summarized in Table 1.6 used a small RNA, such as U6, for normalization of circulating miRNA levels, despite previous reports in wide variability of this class of RNAs in circulation\textsuperscript{128}. 
### Table 1.6 Summary of circulating miRNA studies in PCa

<table>
<thead>
<tr>
<th>Author</th>
<th>Journal, year published</th>
<th># miRNA screened</th>
<th>Sample size and patient characteristics</th>
<th>Upregulated miR</th>
<th>Downregulated miR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitchell et al.</td>
<td>Proc Natl Acad Sci, 2008</td>
<td>Candidate approach</td>
<td>25 mPCa, 25 healthy patients</td>
<td>mPCa vs healthy miR-141</td>
<td></td>
</tr>
<tr>
<td>Brase et al.</td>
<td>Int J Cancer, 2011</td>
<td>667 miR, 5 selected for validation</td>
<td>Screening: 7 mCRPC, 14 LPC; Validation 1: 15 GS6, 21 GS7, 9 GS8 patients, Validation 2: 25 GS8, 42 GS7 patients</td>
<td>Correlated with high GS: miR-375, 9*, 141, 200b, 516a-3p</td>
<td></td>
</tr>
<tr>
<td>Moltzahn et al.</td>
<td>Cancer Res, 2011</td>
<td>384 miR</td>
<td>12 low risk, 12 intermediate risk, 12 high risk (CAPRA), 12 healthy patients</td>
<td>PCa vs. healthy: miR-874, -1274a, -1207-5p, -93 and -106a, high risk vs. healthy group: miR-451</td>
<td>PCa vs. healthy group: miR-223, -26b, -30c and -24</td>
</tr>
<tr>
<td>Zhang et al.</td>
<td>Prostate, 2011</td>
<td>Candidate approach</td>
<td>20 LPC, 20 androgen dependent PC, 10 Hormone refractory PC, 6 BPH patients</td>
<td>miR-21 correlated with PSA level in ADPC and HRPC, miR-21 higher in patients resistant to docetaxel chemotherapy</td>
<td></td>
</tr>
<tr>
<td>Mahn et al.</td>
<td>Urology, 2011</td>
<td>Candidate approach</td>
<td>20 healthy, 18 BPH, 37 LPC, 8 mPCa patients</td>
<td></td>
<td>miR-195 and let-7i negatively correlated with GS</td>
</tr>
<tr>
<td>Yaman Agaoglu et al.</td>
<td>Tumour Biol, 2011</td>
<td>Candidate approach</td>
<td>25 mPCa, 26 PC, 20 healthy patients</td>
<td>mPCa vs LPC: miR-21, -141, -221</td>
<td></td>
</tr>
<tr>
<td>Gonzales et al.</td>
<td>Clin Genitourin Cancer, 2011</td>
<td>Candidate approach</td>
<td>21 PCa patients</td>
<td>miR-141 correlated with clinical course in longitudinal followup</td>
<td></td>
</tr>
<tr>
<td>Bryant et al.</td>
<td>Br J Cancer, 2012</td>
<td>742 miR, 12 selected for validation</td>
<td>plasma: 78 PC, 28 healthy patients; serum: 72 no recurrence, 47 metastatic patients</td>
<td>mPC vs non-recurrent PC: miR-107, -130b, -141, -2100, -301a, -326, -331-3p, -432, -484, -574-3p, -625</td>
<td>mPC vs non-recurrent PC: miR-181a-2*</td>
</tr>
<tr>
<td>Chen et al.</td>
<td>Prostate, 2012</td>
<td>1146 miR, 8 selected for validation</td>
<td>Screening set: 17 BPH and 25 PCa patients, Validation set: 44 BPH, 54 healthy, and 80 PCa patients</td>
<td>PCa vs PBH and PC vs healthy: miR-622 and -1285</td>
<td>PCa vs PBH and PC vs healthy: let-7e, let-7c, and miR-30c</td>
</tr>
<tr>
<td>Shen et al.</td>
<td>Prostate, 2012</td>
<td>Candidate approach</td>
<td>38 low risk, 27 intermediate risk, 17 high risk (D’Amico score) patients</td>
<td>high risk vs low risk: miR-21, -145, T3 vs T2: miR-20a</td>
<td></td>
</tr>
<tr>
<td>Authors</td>
<td>Journal, Year</td>
<td>Study Design</td>
<td>Results</td>
<td>Notes</td>
<td></td>
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<tr>
<td>Selth et al.</td>
<td>Int J Cancer, 2012</td>
<td>Candidate approach</td>
<td>25 healthy and 25 CRPC patients</td>
<td>CPRC vs. healthy patients: miR-141, miR-298, miR-346 and miR-37</td>
<td></td>
</tr>
<tr>
<td>Nguyen et al.</td>
<td>Prostate, 2013</td>
<td>669 miR, 4 selected for validation</td>
<td>28 low-risk, 30 high-risk, 26 CRPC patients</td>
<td>CRPC vs low risk MIr-375, -378*-141</td>
<td></td>
</tr>
<tr>
<td>Cheng et al.</td>
<td>PLoS One, 2013</td>
<td>365 miR, 9 selected for validation</td>
<td>Screening: 25 mCRPC and 25 healthy patients; Validation set: 21 mCRPC and 20 healthy patients</td>
<td>mPC vs healthy: miR-141, -200a, -200c, -210, -375, -210</td>
<td></td>
</tr>
<tr>
<td>Watahiki et al.</td>
<td>Int J Mol Sci, 2013</td>
<td>742 miR, 10 selected for validation</td>
<td>25 LPC and 25 mCRPC patients</td>
<td>metastatic vs. LPCa: miR-141, -375, -200c, -126, -21, -151-3p, -152 and -423-3p</td>
<td></td>
</tr>
<tr>
<td>Wang et al.</td>
<td>PLoS One, 2014</td>
<td>672 miR, 32 selected for validation</td>
<td>Screening: 48 low risk, 48 GS≥7 patients; Validation: 25 low risk, 35 GS≥7 patients</td>
<td>miR-19a, -19b</td>
<td></td>
</tr>
<tr>
<td>Lin et al.</td>
<td>Br J Cancer, 2014</td>
<td>46 (custom miRNA array)</td>
<td>97 mCRPC patients receiving docetaxel</td>
<td>-</td>
<td></td>
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<tr>
<td>Westermann et al.</td>
<td>Anticancer Res, 2014</td>
<td>Candidate approach</td>
<td>54 PC, 79 non-malignant patients</td>
<td>miR-141 is associated with high GS (9)</td>
<td></td>
</tr>
<tr>
<td>Kotb et al.</td>
<td>Tumour Biol, 2014</td>
<td>Candidate approach</td>
<td>10 PC and 10 BPH patients</td>
<td>PC vs BPH: miR-21 and miR-221</td>
<td></td>
</tr>
<tr>
<td>Singh et al.</td>
<td>Oncotarget, 2014</td>
<td>Candidate approach</td>
<td>62 patients without BCR after RP, 31 patients with BCR after RP</td>
<td>BCR vs. non-BCR: miR-125b, -222</td>
<td></td>
</tr>
<tr>
<td>Srivastava et al.</td>
<td>Tumour Biol, 2014</td>
<td>667 miR, 3 selected for validation</td>
<td>Screening: 12PC, validation: 40PCa and 32 age matched healthy patients</td>
<td>PC vs healthy: miR-25, -101, -628-5p</td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Title</td>
<td>Patient Details</td>
<td>Candidate approach</td>
<td>Findings</td>
<td>Notes</td>
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<tr>
<td>Santos et al.</td>
<td>Tumour Biol, 2014</td>
<td>45 PCa patients</td>
<td>Candidate approach</td>
<td>upregulated miR-7 and miR-221 prognostic for CRPC development</td>
<td>-</td>
</tr>
<tr>
<td>Huang et al.</td>
<td>Genet Test Mol Biomarkers, 2015</td>
<td>75 PCa and 75 healthy patients</td>
<td>Candidate approach</td>
<td>recurrence vs no recurrence miR-21</td>
<td>-</td>
</tr>
<tr>
<td>Kelly et al.</td>
<td>J Clin Med, 2015</td>
<td>75 PC and 27 non-PCa (negative biopsy) patients</td>
<td>Candidate approach</td>
<td>high risk vs low risk: miR-145, -155, PC vs non PCa: miR-141</td>
<td>high risk vs low risk: let-7a</td>
</tr>
<tr>
<td>Mihelich et al.</td>
<td>PLoS One, 2015</td>
<td>50 low risk, 50 high risk, 50 BPH patients</td>
<td>Candidate approach</td>
<td>-</td>
<td>high risk vs low risk &amp; BPH and BCR vs non BCR: Let-7a, mir-24, - 26b, -30c, -93, -103, -106a, - 107, -130b, -146a, -223, -451</td>
</tr>
<tr>
<td>Huang et al.</td>
<td>Eur Urol, 2015</td>
<td>RNA seq screening: 23 PCa patients, validation: 100 PCa patients on ADT</td>
<td>Candidate approach</td>
<td>poor vs better PSA survival patients after RP: mir-1290, -375</td>
<td>-</td>
</tr>
<tr>
<td>Wang et al.</td>
<td>Cancer Cell Int, 2016</td>
<td>149 PCa, 57 healthy, 121 BPH/ 65 GS&lt;7, 84 GS≥7 / 115 no recurrence, 34 poor prognosis patients</td>
<td>Candidate approach</td>
<td>G5a7 vs G5c7 and poor prognosis vs no BCR: miR-410-5p</td>
<td>-</td>
</tr>
<tr>
<td>Li et al.</td>
<td>Onco Targets Ther, 2016</td>
<td>50 PC (8 GS≤6, 23 GS7, 20 GS≥8), 40 healthy patients</td>
<td>Candidate approach</td>
<td>PCA vs. health patients, in high GS vs. low GS patients: miR-141</td>
<td>-</td>
</tr>
<tr>
<td>Sapre et al.</td>
<td>PLoS One, 2014</td>
<td>37 Low-risk and 33 high-risk patients</td>
<td>Candidate approach</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kachakova et al.</td>
<td>DNA Cell Biol, 2015</td>
<td>59 PCa and 27 benign patients</td>
<td>Candidate approach</td>
<td>-</td>
<td>PCa vs benign: let-7c, miR-30c, -375</td>
</tr>
</tbody>
</table>

mPCa: metastatic prostate cancer; LPC: localized prostate cancer; CRPC: castration resistant prostate cancer; BPH: benign prostatic hyperplasia; BCR: biochemical recurrence.
MiRNAs are also highly stable in urine samples. Because of the anatomical proximity of the prostate to the urethra, exfoliated cancer cells or secreted molecules are present in urine\textsuperscript{123}. Levels of urinary miRNA is typically low, but a prostate massage performed prior to urine collection enriches the number of prostatic cells\textsuperscript{158}. MiRNAs may be found in the urinary cell pellet or the cell-free supernatant after centrifugation\textsuperscript{107}. Though less extensively explored in PCa compared to serum, urinary miRNAs have also been reported to be associated with PCa and disease progression\textsuperscript{107,159,160}. Similar to circulating miRNA studies, there is often discordance in the significance reported in different studies, likely partially due to differences in sample collection, processing, normalization, and analysis.

1.8 Study rationale, objectives, and hypothesis

1.8.1 Study rationale

In AS programs, 25-35\% of patients are reclassified to higher risk-status and recommended for treatment within 5 years of enrolment due to disease progression or detection of occult, high-grade PCa\textsuperscript{62,64}. Triggers for reclassification commonly includes GS upgrading upon repeat biopsy, rapid PSA kinetics, or increased tumour volume\textsuperscript{42,62,64}. As such, these reclassified patients receive delayed treatment. Conversely, patients who harbour truly indolent PCa are subjected to repeat invasive biopsies, which can cause pain, bleeding, and infection\textsuperscript{84,161}. Conventional clinical markers used in AS programs, such as PSA, GS, and percent of cancer-positive cores (%core) at diagnosis cannot accurately distinguish between AS patients with indolent and aggressive PCa upfront.

Circulating microRNAs (miRNAs) have emerged as potential PCa biomarkers in recent years. MiRNAs are small, single stranded non-coding RNAs (18 - 22 nucleotides) which regulate gene expression through hybridization to the 3’ untranslated region of target mRNAs to inhibit translation\textsuperscript{102,104}. These molecules are stable and reliably detected in biofluids, thus they are amenable to serve as non-invasive biomarkers\textsuperscript{123,124}. Furthermore, there are multiple circulating miRNAs which have been shown to be associated with PCa progression or predict response to therapy\textsuperscript{123,124}. However, it is unknown whether these differences are detectable during the early
stages of PCa development, such that they can identify seemingly low-risk patients who are likely to experience disease progression. In this thesis, we explored the utility of serum miRNAs as a novel, non-invasive monitoring tool for AS patients.

1.8.2 Study hypothesis

We hypothesize that select miRNAs found in serum of PCa patients may be used as a prognostic biomarker for differentiating between AS patients with indolent versus aggressive disease. These miRNA signatures can be used to supplement current clinical variables for more efficient monitoring of AS patients.

1.8.3 Study objectives

- Identify serum miRNA biomarkers for prediction of reclassification in an AS cohort
- Validate serum miRNA biomarkers in an independent AS cohort
- Assess the clinical significance of miRNA biomarkers through comparison to and in combination with conventional clinical prognosticators
Chapter 2 Methods

2.1 Development of an optimized protocol for serum miRNA analysis

MiRNA expression profiles may be used as biomarkers in various diseases, including PCa, due to their involvement in a multitude of biological processes. As these molecules are stable in biofluids, they hold great potential for non-invasive testing, which would be preferred over invasive procedures, such as tissue biopsies, that are currently common-practice. However, due to the short lengths and high sequence similarity between miRNAs, accurate and reliable quantification is challenging. Furthermore, the amount of RNA found in serum or plasma is substantially less than tissue. To develop robust biomarkers, one must employ a highly sensitive assay for reproducible analysis of often limited clinical samples. Thus, we explored several RNA isolation kits, protocol modifications, and miRNA quantification platforms that have been reported to optimize the analysis of circulating miRNAs. Figure 2.1 summarizes the overall workflow of typical serum miRNA analyses and techniques at various steps we assessed to develop an optimal protocol.
2.1.1 Optimization of RNA extraction

2.1.1.1 Accrual of serum samples

Serum samples from 19 PCa patients (ID 18-20, 22-37) who visited the University Health Network or Sunnybrook for PSA testing were used for experiments in this section. All patients have had at least one positive prostate biopsy. These patients were not a part of either cohort, later described in section 2.2, that were used for assessment of miRNA biomarkers. Target miRNAs analyzed during optimization experiments were selected due to availability in the lab.

2.1.1.2 RNA extraction kits

We compared three commercially available extraction kits suitable for isolating total RNA, including miRNA, from biofluids:

1. miRNeasy serum/plasma kit (Qiagen, Germany)
2. mirVana PARIS kit (Thermo Fisher Scientific, Waltham, MA, USA)
3. Norgen Total RNA Isolation Kit (Norgen Biotek Corp, Thorold, ON, Canada)

Extractions were performed in duplicate for six samples with each kit from 200µL of serum, per manufacturer’s protocols. Spike-in RNA cel-miR-39 (Qiagen) was added at the lysis phase of each protocol for technical control. Isolated RNA was then quantified with qRT-PCR for spike-in RNA and endogenous RNAs miR-26a, let-7f, and small nucleolar RNA U6.

### 2.1.1.3 RNA extraction aids

RNA extractions for the remaining optimization experiments were performed using miRNeasy serum/plasma kit and 200µL of serum, unless otherwise stated. Cel-miR-39 or UniSP6 spike-in RNA was added during the lysis step for technical control. Manufacturer protocol was followed with the following modifications:

1. At sample lysis: glycogen (Invitrogen, Carlsbad, CA, USA), linear poly acrylamide (LPA, Thermo Fisher), or MS2 bacteriophage RNA (Hoffmann-La Roche Limited, Mississauga, ON, Canada) as carrier molecules to increase yield.
2. At phenol-chloroform centrifugation: MaXtract phase lock gel tubes (Qiagen) were used to facilitate phase separation or spin time was increased to 30 minutes (from 15 minutes).

Carrier molecules, including MS2 bacteriophage RNA, LPA, and glycogen, may improve nucleic acid isolation from low-yield samples to facilitate co-precipitation or binding. To determine the benefits of including carrier molecules in the extraction process, RNA extractions supplemented with MS2 (1µg), LPA (5µg), glycogen (5µg), or no carrier were performed for six patients’ serum samples. Three additional extractions were performed with miRNeasy mini kit and supplemented with MS2 RNA with the optional small RNA enrichment step for filtering out large RNAs (>200 nucleotides). Nanodrop was used to estimate RNA yield from each extraction. Two additional patient serum samples were extracted in duplicate, supplemented with MS2 or glycogen and qRT-PCR was subsequently performed to quantify the following endogenous miRNAs: let-7a, cel-miR-39, miR-141, -145, -21, -223, -24, -30c, -375.
Phenol-chloroform phase separation is a common step in most RNA extraction protocols. However, retrieving the upper aqueous layer, which contains RNA, without transferring any contaminants from the lower organic phase can be difficult. Thus, we assessed two protocol modifications to facilitate better phase separation. Three samples were extracted with an extended phenol-chloroform separation (centrifugation) step, usage of MaXtract phase lock gel tube, or no modified protocol. RNA yield from each method was quantified with Nanodrop.

2.1.2 Comparison of platforms for miRNA quantification

Post-extraction cleanup protocols were performed to further remove potential impurities that were eluted with the total RNA. Column based methods for post-extraction cleanup (Zymo and Amicon) were employed according to manufacturer’s protocol. Ethanol precipitation was performed as follows:

1. 400 µL of 100% ethanol, 14 µL of 3M NaOAc, and 15µL of linear acrylamide were added
2. Samples were precipitated at -80°C overnight.
3. Samples were centrifuge at 20000 x g in a microcentrifuge for 30 minutes at 4°C.
4. The supernatant was discarded, and the pellet was washed in 1 ml 4°C 80% ethanol and centrifuged for 2 minutes at 20000 x g.
5. Step 4 was repeated and the pellet was allowed to air-dry at room temperature.
6. The pellet was re-suspended in 20µL of RNase-free water.

Isolated RNA was then quantified with Nanodrop. Select samples were also analyzed with qRT-PCR for spike-in RNA and endogenous RNAs miR-26a, let-7f and small nucleolar RNA U6.

2.1.2.1 NanoString nCounter

NanoString nCounter (NanoString Technologies, Seattle, WA, USA) is a hybridization-based technique for high-throughput quantification of nucleic acids and proteins. Twelve RNA samples were sent for nCounter analysis (human v2.0 miRNA panel) to assess the feasibility of this technology for profiling serum miRNAs. Samples were not diluted due to lack of accurate quantification techniques available for low yields typically observed for biofluids. The process of nCounter is summarized in Figure 2.2. Similar to microarrays, the nCounter targets a large number
(~800) of known human miRNAs, including clinically relevant miRNAs, for quantification. MiRNA sequences for targeting in the assay were derived from miRbase, a large database of miRNA sequences and annotations.

We chose this platform because it is becoming established as a reliable method for biomarker research. A recent study demonstrated high sensitivity, reproducibility, and technical robustness of NanoString for analysis of formalin-fixed, paraffin-embedded tissue\textsuperscript{162}. Furthermore, NanoString is also FDA-approved for the Prosigna test for prognostication of breast cancer\textsuperscript{163}. Finally, two other ongoing projects in our group have used this platform to profile miRNA expression of PCa tissues and urinary cells. However, there have been very few studies that used NanoString to profile serum miRNAs in a PCa context. Thus, we hoped to employ a consistent, novel platform to identify robust miRNA signatures that may be detected in multiple sample types.
2.1.2.2 Taqman microRNA assay

Quantitative PCR is better established for detection of miRNA in serum compared to hybridization or sequencing methods\(^{127}\). We assessed two qRT-PCR platforms for candidate miRNA analysis as potential alternatives to NanoString profiling, first of which was the Taqman microRNA assay (Thermo Fisher). We analyzed 19 serum samples for endogenous (miR-26a, let-7f, U6) and spike-in (cel-miR-39) RNAs.
The process of Taqman miRNA analysis is summarized in figure 2.3. Briefly, total RNA equivalent to 10µL of serum was reverse transcribed with a stem looped primer partially specific to the mature miRNA sequence. The stem loop primer is subsequently extended with RNA polymerase. After denaturation, the stem loop primer is released into a linear structure. Due to the smaller number of nucleotides in a miRNA, there are limited options for primer design. However, the stem loop primers take advantage of the fact that it is much longer than a typical miRNA molecule, while is still specific to the target miRNA sequence.

The resulting cDNA equivalent to 5µL of starting serum volume is subsequently amplified using a miRNA-specific primer, a reverse transcription-specific primer, and a Taqman probe. The two primers flank the amplicon, while a Taqman probe is specific to a part of the miRNA sequence in the middle of the amplicon. The probe contains a fluorophore one the 5’ end and a quencher on the 3’ end. During amplification, DNA polymerase passes across the Taqman probe bound to the miRNA template and cleave the fluorophore. Without being in close proximity to the quencher, the fluorophore emits fluorescence. In our experiments, the fluorophore used was FAM, which emits fluorescence at 520nm.

Figure 2.3 Overview of qRT-PCR with Taqman microRNA assays. Adapted from https://genome.med.harvard.edu/documents/qpcr/TaqManMicroRNAProductBrochure.pdf.
2.1.2.3 Exiqon miRCURY assay

The second qRT-PCR platform we tested was the miRCURY assay (Exiqon, Denmark). Fourteen serum samples were analyzed in duplicates for endogenous (miR-103, -30e, -425) and spike-in (UniSp6) RNAs. Total RNA equivalent to 15µL of serum was reverse transcribed with the Universal cDNA Synthesis Kit per manufacturer protocols. Briefly, poly A tails are added to the mature microRNAs. Then a poly-T primer with 3’ degenerate anchor anneals to the mature microRNA and added poly A tail. Finally, the reverse transcriptase synthesizes the cDNA by adding nucleotides onto the 3’ degenerate anchor and using the mature microRNA sequence as a template.

Subsequent cDNA equivalent to 0.08µL of starting serum volume was amplified with ExiLENT SYBR® Green assay (Exiqon) per manufacturer’s protocols. Individual assays utilized miRNA-specific primers designed and optimized by Exiqon (https://www.exiqon.com/mirna-pcr-primer) and are only with compatible with ExiLENT SYBR® Green reactions. Briefly, miRNA specific LNA forward and reverse primers facilitate exponential amplification of the template cDNA. SYBR green dye binds to the minor groove of double stranded DNA and emit fluorescence. The intensity of fluorescent signal is detected by the qPCR equipment.

Exiqon primers incorporates locked nucleic acids (LNA), which increases the affinity of the primer for its target, resulting in a marked improvement in sensitivity and specificity. LNA is a RNA nucleotide which contains an extra covalent bond between the 2’ oxygen and 4’ carbon of the ribose molecule (Figure 2.4)\textsuperscript{164,165}. This bridge “locks” the ribose in a 3’-endo conformation, which enhances base stacking and backbone pre-organization and significantly increases the melting temperature of oligonucleotides\textsuperscript{164,165}. Due to the smaller number of nucleotides in a miRNA, there are limited options for primer design. Furthermore, miRNAs in the same family are highly similar, often differing in only one nucleotide. As such, achieving optimal melting temperature and specificity are major challenges in qPCR detection of miRNAs. Chemical properties of LNA have been exploited to overcome both problems. LNA nucleotides can be incorporated into a primer to optimize the melting temperature for qPCR reactions, which in turn improves the sensitivity\textsuperscript{164,165}. 
Furthermore, a single-nucleotide mismatch flanked by LNA nucleotides greatly increases specificity compared to conventional oligonucleotides\textsuperscript{164,165}.

\begin{center}
\includegraphics[width=0.3\textwidth]{LNAstructure.png}
\end{center}

\textbf{Figure 2.4 Locked nucleic acid structure.} Adapted from https://en.wikipedia.org/wiki/Locked_nucleic_acid#/media/File:LNASchem.svg.

2.1.3 Normalization of miRNAs

In circulating miRNA studies, results may be skewed due to normalization methods. For example, many contemporary studies of circulating miRNA still utilize small RNA, such as U6, for normalization of miRNA levels, despite previous findings of wide variability of this class of RNAs in circulation\textsuperscript{128}. To select an appropriate normalization control, we compared the a spike-in RNA and two endogenous miRNAs previously proposed as a normalizing control from circulating miRNA studies in PCa patient samples\textsuperscript{151,153} in 14 serum samples.

\textbf{2.2 Assessment of serum miRNAs for prediction of patient reclassification}

An optimized workflow was utilized to analyze candidate miRNAs, briefly summarized in Figure 2.5.
Figure 2.5 Optimized workflow for serum miRNA analysis. Methods incorporated in this protocol are based on findings of optimization experiments performed in section 2.1.

2.2.1 Patients and specimens

Serum samples were collected, with informed consent, from AS patients at two independent healthcare centres for training and validation of candidate miRNA biomarkers. All patients in the study were diagnosed with GS6 PCa and were treatment naïve. All samples were collected prior to potential reclassification. Approval was obtained from institutional research ethics boards.

2.2.1.1 Training cohort

One hundred and ninety-six (196) patients were retrospectively enrolled between May 2009 and June 2016 from the Sunnybrook Health Sciences Centre (SB), Toronto, Canada. All patients are a part of a larger cohort detailed previously. Blood samples were collected in SST™ Serum Separation Tubes (Fisher Scientific Co., Markham, ON, Canada) and allowed to clot for at least 30 minutes. Processing was performed within 2 hours of blood collection. Whole blood was spun for 15 minutes at 1211 x g. After centrifugation, the serum (upper phase) was collected and stored at -80°C.

2.2.1.2 Validation cohort

One hundred and ten (110) patients were prospectively enrolled between December 2015 and February 2017 from the University Health Network (UHN), Toronto, Canada. Blood samples were
collected in SST™ Serum Separation Tubes and allowed to clot for at least 30 minutes. Processing was performed within 2 hours of blood collection. Whole blood was spun for 10 minutes at 1300 x g. The upper phase was transferred to 15mL Falcon™ tube and spun for 10 minutes at 1300 x g to separate any possible red blood cells that were transferred. The isolated serum was stored at -80°C.

2.2.1.3 Monitoring regimen

Sunnybrook: PSA was measured every 3 months for 2 years, then every 6 months. A confirmatory biopsy was performed within 12 months of the initial biopsy and then repeat biopsies were performed every 3 to 4 years.

University Health Network: PSA was measured every 3 months for 2 years, then every 6 months. A confirmatory biopsy was performed within 12-18 months of diagnosis and repeat biopsies were performed every 1-3 years. DREs were performed every 6-12 months.

2.2.1.4 Triggers for reclassification

Triggers for reclassification included detection of any one or more of the following: GS upgrading on repeat biopsy, PSA-DT less than 3 years, or PIRADS 4/5 detected via MRI. Since this study pertains specifically to the context of AS, clinically significant disease was defined as positive reclassification status.

2.2.2 Selection of miRNA candidates

A literature search of the MEDLINE database was conducted to assess published evidence of miRNA biomarker potential for PCa progression, using key words: “prostate cancer” AND “microRNA OR miRNA” AND “serum OR plasma OR circulating OR blood” Abstracts were reviewed to determine relevance of study. Review articles, editorials, and studies solely in cell lines or animal models were excluded from consideration. All relevant, peer-reviewed studies published prior to June 2016 were critically reviewed. The following information, if available, were extracted from each article and compiled for further comparison and review:
A panel of 9 miRNAs (miR-141, 375, 21, 30c, 145, 26b, 223, 24, and let-7a) were selected based on literature review of miRNA studies in serum or plasma samples from PCa patients. MiRNAs were selected on the basis of association with PCa progression and validation in independent cohorts (Table 2.1).
Table 2.1 Summary of candidate miRNAs

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Sample type</th>
<th>Dysregulation in prostate cancer</th>
</tr>
</thead>
</table>
| miR-141 | serum, plasma, whole blood | ↑ mPCa vs. LPC\(^{129,142,155}\) 133,141  
↑ CRPC vs. low risk (D’amico)\(^{139}\)  
↑ Recurrence after treatment\(^{135}\)  
↑ PCa vs. benign\(^{129,140,151,155}\)  
↑ Increasing GS\(^{155}\)                                                                                     |
| miR-375 | serum, plasma              | ↑ mPCa vs. LPC\(^{129,141,142}\)  
↑ CRPC vs. low risk (D’amico)\(^{139}\)  
↑ Recurrence after treatment\(^{135,153}\)  
↑ PCa vs. benign\(^{129,140}\)                                                                                   |
| miR-21  | serum, plasma              | ↑ high risk vs. low risk PCa (D’amico)\(^{137}\)  
mPCa vs. LPC\(^{133,141}\)  
↑ Recurrence after treatment\(^{150}\)  
↑ PCa vs benign\(^{146}\)                                                                                         |
| miR-30c | serum, plasma              | ↓ high-grade vs. low grade PCa\(^{152}\)  
↓ PCa vs. benign\(^{136,151}\)                                                                                   |
| let-7a  | serum, whole blood         | ↓ high-grade vs. low grade PCa\(^{152}\)  
↓ PCa vs. benign\(^{151}\)                                                                                       |
| miR-145 | plasma, whole blood        | ↑ high risk vs. low risk PCa (D’amico)\(^{137}\)  
↑ PCa vs. benign\(^{151}\)                                                                                       |
| mi-26b  | serum                      | ↓ high-grade vs. low grade PCa\(^{152}\)  
↓ PCa vs. benign\(^{130}\)                                                                                       |
| miR-223 | serum                      | ↓ high-grade vs. low grade PCa\(^{152}\)  
↓ PCa vs benign\(^{130}\)                                                                                       |
| miR-24  | serum                      | ↓ high-grade vs. low grade PCa\(^{152}\)  
↓ intermediate risk vs. low risk PCa (CAPRA)\(^{130}\)                                                           |

↑ upregulation; ↓ downregulation. mPCa: metastatic prostate cancer, LPC: localized prostate cancer, GS: Gleason score, CRPC: castration resistant prostate cancer.

2.2.3 RNA extraction

Serum samples were randomly coded and blinded prior to RNA extraction. Total RNA was extracted from 50µL of serum with miRNAeasy serum/plasma kit per manufacturer’s protocols with minor modifications. Glycogen as a carrier molecule (5µg) and cel-miR-39 spike-in RNA
(6.4x10^8 copies) were added during the initial lysis step. MaXtract gel phase lock tubes were used during this step to facilitate better phase separation. Total RNA was eluted in 40µL of RNase-free water.

2.2.4 Reverse transcription and qPCR

Total RNA equivalent of 15µL of serum volume was reverse transcribed with the miRCURY Universal cDNA Synthesis Kit. Subsequent cDNA equivalent of 0.08µL of serum volume was analyzed with miRCURY ExiLENT SYBR® Green assay. RT-qPCR was performed per manufacturer’s protocols.

All qPCR reactions were performed on QuantStudio 6 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Reactions with threshold cycle (Ct) > 35 were deemed to be non-specific amplification. Samples were run in single reactions. To ensure reproducibility of miRNA qPCR signals and assess potential batch effects, each miRNA assay was run in duplicate reactions for up to 6% of randomly selected samples.

2.2.5 Statistical analysis

Relative quantities of candidate miRNAs were calculated by normalization to cel-miR-39 spike-in control RNA with a modified delta Ct method: 2^{(\text{Ct}_{\text{cel-miR-39}} - \text{Ct}_{\text{miRNA}})}. Relative quantities from duplicate assays were averaged. To compare the clinical variables between the SB and UHN cohort, the following tests were performed: Mann-Whitney U test for PSA, %core, and prostate volume, Student’s t-test for age, and Chi-squared test for clinical stage and reclassification status. Univariable logistic regression was performed to measure miRNA and clinical variables as predictors of reclassification. Backward stepwise logistic regression was performed to construct multivariable models of candidate miRNAs for predicting reclassification. Receiver operator characteristic (ROC) curves were constructed to quantify classification ability of assessed markers. Two by two contingency tables were used to calculate the sensitivity, specificity, PPV, and NPV for biomarker cutoffs. For all analyses, a p-value of 0.05 or less was considered significant. All statistical tests were performed with statistical software program IBM SPSS (version 23.0).
Chapter 3 Results

3.1 Development of an optimized protocol for serum miRNA analysis

3.1.1 Optimization of RNA extraction

We compared the three commonly used RNA isolation kits: miRNeasy serum/plasma kit, mirVana PARIS kit, and Norgen total RNA isolation kit. Endogenous RNAs (U6, miR-26a, and let-7f) and spike-in RNA (cel-miR-39) were reliably detected in all samples through RT-qPCR. Comparing the Ct values of RNAs, samples isolated using miRNeasy serum/plasma kit showed the lowest Ct values (i.e. highest miRNA levels). MiRNeasy serum/plasma kit extraction was subsequently used for all RNA isolation from serum.

Figure 3.1 qRT-PCR analysis of RNA isolated with various extraction kits. Error bars represent standard deviation of Ct values. Extractions were performed in duplicate for six samples. Taqman qRT-PCR assays were used to quantify target miRNAs. Lower Ct corresponds to higher RNA levels. Average Ct values of each sample can be found in Supplementary Table 1.
3.1.1.1 RNA extraction aids

We tested various modifications to the miRNeasy serum/plasma protocol to further optimize RNA isolation. In a comparison of these carrier molecules, it was found that the glycogen and MS2 resulted in higher RNA yield compared to the unmodified protocol (Table 3.1).

Table 3.1 RNA extractions supplemented with various carrier molecules

<table>
<thead>
<tr>
<th>Description</th>
<th>Average RNA recovered (ng)</th>
<th>260/230 range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard protocol</td>
<td>219.40</td>
<td>0.06-0.52</td>
</tr>
<tr>
<td>Glycogen-supplemented</td>
<td>257.83</td>
<td>0.31-0.53</td>
</tr>
<tr>
<td>MS2-supplemented</td>
<td>3400.13</td>
<td>1.13-1.76</td>
</tr>
<tr>
<td>LPA-supplemented</td>
<td>205.75</td>
<td>0.07-0.44</td>
</tr>
</tbody>
</table>

Extractions were performed for six serum samples. Nanodrop was used to quantify isolated RNA. Quantities observed for individual reactions can be found in Supplementary Table 2.

Extracted RNA samples had poor 260/230 ratio (range 0.07-0.52), with the exception of MS2-supplemented samples (range 1.34-1.76). Due to the large amount of RNA recovered in MS2-supplemented extractions, it is highly likely that the MS2 RNA was not filtered out in the extraction process. We examined MS2 and glycogen further by performing triplicate RNA extractions in two additional serum samples and quantified endogenous miRNAs with qRT-PCR. Glycogen-supplemented extractions were highly reproducible, whereas RNA extracted with MS2 carrier molecules showed large variations between replicates for all miRNA assayed (Figure 3.2). This variation may be due to interference by MS2 RNA left in the isolated RNA sample.
Figure 3.2 Comparison of MS2 and glycogen as carrier molecules for RNA isolation. A) RNA extraction performed with glycogen; B) RNA extraction performed with MS2 RNA. Extractions were performed in triplicate for two samples, results from one sample shown here.

Due to potential interference of MS2 RNA in the PCR reaction, we attempted to remove MS2 with a the miRNeasy mini kit with an enrichment step for small RNAs. In six serum samples processed
with this protocol, the RNA yield was still unusually high, which indicated that MS2 RNA was not filtered out (Supplementary Table 3).

To better facilitate the phenol-chloroform phase separation, we performed two protocol modifications, usage of a phase-lock gel tube and extending the centrifugation time to 30 minutes. The phase-lock gel tube formed a gel layer between the aqueous and organic phases after centrifugation, which allowed easier extraction of the aqueous layer without accidentally disrupting the organic layer. The RNA yield was higher in extractions that used a phase-lock gel tube, whereas extending the spin time did not noticeably improve the process (Table 3.2).

**Table 3.2 Protocol modifications to facilitate better phase separation during phenol-chloroform centrifugation**

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Description</th>
<th>Conc. (ng/µL)</th>
<th>260/280</th>
<th>260/230</th>
<th>Volume (µL)</th>
<th>RNA (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>Standard protocol</td>
<td>20.69</td>
<td>1.53</td>
<td>0.54</td>
<td>15</td>
<td>310.4</td>
</tr>
<tr>
<td>25</td>
<td>Extended spin (30min)</td>
<td>21.16</td>
<td>1.48</td>
<td>0.34</td>
<td>15</td>
<td>317.3</td>
</tr>
<tr>
<td>25</td>
<td>Phase lock gel</td>
<td>33.99</td>
<td>0.97</td>
<td>0.12</td>
<td>15</td>
<td>509.9</td>
</tr>
<tr>
<td>26</td>
<td>Standard protocol</td>
<td>22.97</td>
<td>1.49</td>
<td>0.28</td>
<td>15</td>
<td>344.6</td>
</tr>
<tr>
<td>26</td>
<td>Extended spin (30min)</td>
<td>23.14</td>
<td>1.06</td>
<td>0.18</td>
<td>15</td>
<td>347.1</td>
</tr>
<tr>
<td>26</td>
<td>Phase lock gel</td>
<td>36.36</td>
<td>1.18</td>
<td>0.13</td>
<td>15</td>
<td>545.4</td>
</tr>
<tr>
<td>27</td>
<td>Standard protocol</td>
<td>30.53</td>
<td>1.43</td>
<td>0.41</td>
<td>15</td>
<td>458.0</td>
</tr>
<tr>
<td>27</td>
<td>Extended spin (30min)</td>
<td>22.64</td>
<td>1.49</td>
<td>0.06</td>
<td>15</td>
<td>339.6</td>
</tr>
<tr>
<td>27</td>
<td>Phase lock gel</td>
<td>31.23</td>
<td>1.12</td>
<td>0.21</td>
<td>15</td>
<td>468.5</td>
</tr>
</tbody>
</table>

Standard protocol: miRNeasy serum/plasma kit per manufacturer instructions.

The poor 260/230 ratio of isolated RNA samples is indicative of contamination with organic compounds. To remove potential contaminants, we tested different post-extraction cleanup techniques, including ethanol (EtOH) precipitation and column-based techniques (Zymo, Amicon). However, we observed that the 260/230 ratio did not improve with any clean up protocol, ranging from 0.09-0.36 for no cleanup, 0.12-0.43 for EtOH precipitation, 0.1-0.19 for Amicon, and 0.11-0.35 for Zymo (Table 3.3).
Table 3.3 Summary of RNA extractions performed with or without a cleanup protocol

<table>
<thead>
<tr>
<th>Description</th>
<th>Average RNA recovered (ng)</th>
<th>260/230 range</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH precipitation</td>
<td>25.53</td>
<td>0.12-0.43</td>
</tr>
<tr>
<td>Amicon</td>
<td>101.11</td>
<td>0.10-0.19</td>
</tr>
<tr>
<td>Zymo</td>
<td>84.70</td>
<td>0.11-0.30</td>
</tr>
<tr>
<td>No cleanup</td>
<td>259.91</td>
<td>0.09-0.36</td>
</tr>
</tbody>
</table>

Isolated RNA samples from eight PCa patients were further purified with post-extraction cleanup protocols. Nanodrop was used to quantify RNA. Cleanup protocols did not improve 260/230 ratio. RNA yield of each individual post-extraction cleanup can be found in Supplementary Table 4.

Extractions with Amicon, Zymo, or no cleanup in five serum samples were also assessed with qRT-PCR for endogenous and spike-in RNAs (Figure 3.3). Generally, the cleanup protocols showed lower levels of RNAs (higher Ct), which indicates that RNAs were lost due to the cleanup protocols. Amplification of miRNAs was robust regardless of whether a cleanup protocol was employed.
Figure 3.3 qRT-PCR analysis of RNA extracted using miRNAeasy serum/plasma kit with various post-extraction cleanup protocols. Error bars represent standard deviation of Ct values. Extractions were performed for five samples with each type of cleanup. Taqman qRT-PCR assays were used to quantify target RNAs. RNA levels of individual extractions can be found in Supplementary Table 5.

3.1.1.2 Summary of RNA extraction optimizations

Due to the limited sample size of optimization experiments described in section 3.1.1, we did not perform statistical analysis to compare the RNA yield resulting from various methods and modification. However, we observed a consistent increase in RNA yield when miRNeasy serum/plasma kit was used compared to mirVana or Norgen, and addition of glycogen carrier molecules and phase-lock gel tubes also appeared to improve the extraction efficiency. Usage of post-extraction clean up protocols did not further improve sample purity and caused loss of RNA. Therefore, we found that an optimal approach to RNA isolation from serum is to use the miRNeasy serum plasma kit with the addition of 5μg glycogen carrier molecule during sample lysis and phase-lock gel tubes during phenol-chloroform separation.
3.1.2 Comparison of platforms for miRNA quantification

We performed preliminary experiments to assess three platforms for quantifying serum miRNAs: NanoString nCounter, Taqman microRNA assay, and miRCURY assay.

Twelve RNA samples were extracted with the optimized protocol described in section 3.1.3. NanoString profiling of these samples yielded very low miRNA counts (Table 3.4). Average count per miRNA ranged between 12.73-42.87, well below the detection threshold (100 counts). The number of miRNA that were above detection threshold ranged from 1 - 18.

In these 12 samples, we also quantified miR-26a and let-7a with Taqman assays. These two endogenous miRNAs were reliably detected by Taqman assays, but were below the detection threshold for NanoString nCounter (Table 3.4).
Table 3.4 Summary of profiling with NanoString and targeted quantification with Taqman microRNA assay

<table>
<thead>
<tr>
<th>Sample ID</th>
<th># miRNA with count &gt; 100</th>
<th>miR-26a qRT-PCR (Ct)</th>
<th>let-7f qRT-PCR (Ct)</th>
<th>miR-26a NanoString (counts)</th>
<th>let-7f NanoString (counts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>10</td>
<td>27.27</td>
<td>30.59</td>
<td>8</td>
<td>39</td>
</tr>
<tr>
<td>02</td>
<td>2</td>
<td>31.23</td>
<td>34.97</td>
<td>9</td>
<td>15</td>
</tr>
<tr>
<td>03</td>
<td>18</td>
<td>27.61</td>
<td>30.91</td>
<td>11</td>
<td>65</td>
</tr>
<tr>
<td>04</td>
<td>14</td>
<td>24.35</td>
<td>29.37</td>
<td>17</td>
<td>53</td>
</tr>
<tr>
<td>05</td>
<td>6</td>
<td>30.01</td>
<td>35.13</td>
<td>11</td>
<td>26</td>
</tr>
<tr>
<td>06</td>
<td>2</td>
<td>37.8</td>
<td>39.43</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>07</td>
<td>3</td>
<td>34.95</td>
<td>40.62</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>08</td>
<td>1</td>
<td>28.04</td>
<td>34.63</td>
<td>7</td>
<td>23</td>
</tr>
<tr>
<td>09</td>
<td>1</td>
<td>33.95</td>
<td>43.99</td>
<td>7</td>
<td>18</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>32.58</td>
<td>37.2</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td>11</td>
<td>6</td>
<td>28.76</td>
<td>32.46</td>
<td>10</td>
<td>26</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>32.64</td>
<td>37.68</td>
<td>7</td>
<td>6</td>
</tr>
</tbody>
</table>

We also assessed miRNA detection with miRCURY assays. Three endogenous miRNA (miR-103, -30e, -425) and a spike-in RNA (UniSp6) were reliably detected in all samples (Table 3.5). The spike-in RNA UniSp6 showed less variation than potential endogenous control miRNAs miR-30e and -425. Thus, we used a spike-in RNA (cel-miR-39) in subsequent experiments for normalization of endogenous miRNA.
### Table 3.5 Detection of endogenous and spike-in miRNAs with miRCURY assay

<table>
<thead>
<tr>
<th></th>
<th>miR-30e</th>
<th>miR-103</th>
<th>miR-425</th>
<th>UniSp6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Ct</td>
<td>29.7</td>
<td>28.4</td>
<td>28.8</td>
<td>25.5</td>
</tr>
<tr>
<td>Ct Range</td>
<td>26.1-34.1</td>
<td>24.1-32.0</td>
<td>25.2-33.4</td>
<td>24.318-26.622</td>
</tr>
<tr>
<td>Ct standard deviation</td>
<td>1.73</td>
<td>1.76</td>
<td>1.71</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Though both Taqman and miRCURY assays were both able to reliably detect targeted miRNAs, we moved forward using the miRCURY assay due to its higher sensitivity (0.08 µL vs 0.5µL equivalent of serum volume used per reaction).

### 3.2 Assessment of candidate miRNAs for predicting patient reclassification

#### 3.2.1 Cohort description

Clinicalopathological characteristics of the SB (training) and UHN (validation) cohorts are summarized in Table 3.6. In the SB cohort, the median age was 68.6 years (range 45.2-93.2), median PSA at sample collection was 5.21 ng/mL (range 0.01-38.68), and %core at diagnosis was 12.3% (range 1.3-83.3). Median follow-up after sample collection was 45.7 months (range 0.6-79.4). During follow-up, 47 (24.0%) of 196 patients have been reclassified due to GS upgrading (n = 35), PSA-DT < 3yrs (n = 10), or MRI progression (n = 2).

In the UHN cohort, the median age was 67.5 years (range 48.0-83.0), median PSA at sample collection was 5.60 ng/mL (range 1.21-18.47), and %core at diagnosis was 14.3% (range 5.0-75.0). Median follow-up after sample collection was 9.9 months (range 0.0-17.1). During follow-up, 21 (19.1%) of 110 patients have been reclassified due to GS upgrading (n = 15), PSA-DT<3yrs (n= 4), or MRI progression (n = 2).
Table 3.6 Summary of clinicopathological information of patient cohorts

<table>
<thead>
<tr>
<th>Clinicopathological characteristics</th>
<th>SB</th>
<th>UHN</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gleason Score at diagnosis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>196</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td><strong>Clinical Stage at diagnosis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>179 (91.3%)</td>
<td>69 (62.7%)</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>11 (5.6%)</td>
<td>9 (8.2%)</td>
<td></td>
</tr>
<tr>
<td>Not available</td>
<td>6 (3.1%)</td>
<td>32 (29.1%)</td>
<td></td>
</tr>
<tr>
<td><strong>PSA at sample collection (ng/mL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>5.21</td>
<td>5.6</td>
<td>0.639&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Range</td>
<td>0.01-38.68</td>
<td>1.21-18.47</td>
<td></td>
</tr>
<tr>
<td><strong>Cores positive at diagnosis (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>12.3</td>
<td>14.3</td>
<td>0.429&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Range</td>
<td>1.25-83.33</td>
<td>5.00-75.00</td>
<td></td>
</tr>
<tr>
<td>Not available</td>
<td>10 patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Age (Years)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>68.6</td>
<td>67.5</td>
<td>0.016&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Range</td>
<td>45.2-93.2</td>
<td>48.0-83.0</td>
<td></td>
</tr>
<tr>
<td><strong>Followup time (Months)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>45.73</td>
<td>9.9</td>
<td>&lt;0.001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Range</td>
<td>0.63-79.43</td>
<td>0.00-17.03</td>
<td></td>
</tr>
<tr>
<td><strong>Reclassification</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GS upgrading</td>
<td>35 (17.8%)</td>
<td>15 (13.6%)</td>
<td></td>
</tr>
<tr>
<td>PSA-DT&lt;3yrs</td>
<td>10 (5.1%)</td>
<td>4 (3.6%)</td>
<td></td>
</tr>
<tr>
<td>MRI progression</td>
<td>2 (1.0%)</td>
<td>2 (1.8%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>47 (24.0%)</td>
<td>21 (19.1%)</td>
<td>0.296&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>196</td>
<td>110</td>
<td></td>
</tr>
</tbody>
</table>

SB = Sunnybrook; UHN = University Health Network. <sup>a</sup> Mann-Whitney U test; <sup>b</sup> Student’s t-test; <sup>c</sup> Chi-squared test.
Statistical comparisons found that there were significant differences in the age (p=0.016) and followup time (p<0.001) between cohorts, but no significant differences were observed in PSA, %core, and rate of reclassification.

3.2.2 Prediction for patient reclassification in the training cohort

Univariable logistic regression analysis was performed to estimate the odds ratio (OR) and 95% confidence interval (CI) of individual miRNAs and clinical variables with respect to reclassification. Candidate miRNAs were not significant independent predictors with the exception of miR-375 (Table 3.7).

**Table 3.7 Logistic regression analysis of candidate miRNAs in the training cohort**

<table>
<thead>
<tr>
<th>Marker</th>
<th>OR</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Let-7a</td>
<td>1.011</td>
<td>0.916-1.115</td>
<td>0.830</td>
</tr>
<tr>
<td>miR-141</td>
<td>0.965</td>
<td>0.901-1.034</td>
<td>0.316</td>
</tr>
<tr>
<td>miR-145</td>
<td>1.063</td>
<td>0.986-1.147</td>
<td>0.113</td>
</tr>
<tr>
<td>miR-21</td>
<td>0.998</td>
<td>0.985-1.011</td>
<td>0.750</td>
</tr>
<tr>
<td>miR-223</td>
<td>0.989</td>
<td>0.968-1.010</td>
<td>0.292</td>
</tr>
<tr>
<td>miR-24</td>
<td>1.005</td>
<td>0.975-1.035</td>
<td>0.762</td>
</tr>
<tr>
<td>miR-26b</td>
<td>0.949</td>
<td>0.938-1.062</td>
<td>0.949</td>
</tr>
<tr>
<td>miR-30c</td>
<td>1.002</td>
<td>0.975-1.031</td>
<td>0.870</td>
</tr>
<tr>
<td>miR-30e</td>
<td>1.005</td>
<td>0.996-1.014</td>
<td>0.282</td>
</tr>
<tr>
<td>miR-375</td>
<td>0.969</td>
<td>0.940-0.998</td>
<td>0.034</td>
</tr>
</tbody>
</table>

Among clinical variables, PSA (OR = 1.071, 95% CI = 1.015-1.131), %core (OR = 1.033, 95% CI = 1.007-1.059) and prostate volume (OR = 0.973, 95% CI = 0.950-0.996) were statistically significant, but with very modest OR. Prostate volume data was only available for 103/196 patients, thus it was excluded from subsequent multivariable analysis. Backward stepwise logistic regression was performed to construct multivariable models of candidate miRNAs. A weighted combination of miR-223, -24, and -375 was found to be optimal (OR = 2.721, 95% CI = 1.499-4.939). This “3-miR”
score was a better predictor compared to any individual miRNA or clinical variable (Table 3.8). Similarly, we also constructed a panel of clinical variables for predicting reclassification, and the best combination consisted of PSA and %core (OR = 2.698, 95% CI = 1.458-4.994).

Table 3.8 Logistic regression analysis of 3-miR score and clinical variables in the training cohort

<table>
<thead>
<tr>
<th>Marker</th>
<th>OR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-miR*</td>
<td>2.721</td>
<td>1.499-4.939</td>
<td>0.001</td>
</tr>
<tr>
<td>% cores at diagnosis</td>
<td>1.033</td>
<td>1.007-1.059</td>
<td>0.012</td>
</tr>
<tr>
<td>PSA at collection</td>
<td>1.071</td>
<td>1.015-1.131</td>
<td>0.013</td>
</tr>
<tr>
<td>Age at collection</td>
<td>1.007</td>
<td>0.970-1.045</td>
<td>0.723</td>
</tr>
<tr>
<td>Prostate volume</td>
<td>0.973</td>
<td>0.950-0.996</td>
<td>0.020</td>
</tr>
<tr>
<td>Clinical panel**</td>
<td>2.698</td>
<td>1.461-4.993</td>
<td>0.002</td>
</tr>
</tbody>
</table>

* miR-223, -24, and -375; ** PSA at collection and %core at diagnosis.

Next, we assessed each type of reclassification (GS upgrading, PSA-DT < 3yrs, MRI progression) separately, and the 3-miR score was a significant predictor of GS upgrading (OR = 2.354, 95% CI = 1.232-4.498) and PSA-DT < 3yrs (OR = 4.339, 95% CI = 1.249-15.072). Analysis was not performed for prediction of MRI progression due to small number of events (n = 2).

In a multiple logistic regression model with the 3-miR score, PSA, %core, and age as covariates, the 3-miR score’s ability to predict reclassification was independent of clinical covariates and had a much higher OR in comparison (Table 3.9).
Table 3.9 Multivariable model of 3-miR score and clinical variables in the training cohort

<table>
<thead>
<tr>
<th>Marker</th>
<th>OR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-miR*</td>
<td>2.411</td>
<td>1.298-4.478</td>
<td>0.005</td>
</tr>
<tr>
<td>% cores at diagnosis</td>
<td>1.028</td>
<td>1.001-1.056</td>
<td>0.043</td>
</tr>
<tr>
<td>PSA at collection</td>
<td>1.049</td>
<td>0.983-1.120</td>
<td>0.147</td>
</tr>
<tr>
<td>Age at collection</td>
<td>0.997</td>
<td>0.952-1.038</td>
<td>0.791</td>
</tr>
</tbody>
</table>

*miR-223, -24, -375.

We then constructed ROC curves to assess the 3-miR score’s ability to differentiate between reclassified versus non-reclassified patients (Table 3.10). The 3-miR score (AUC = 0.690, 95% CI = 0.596-0.784) showed a higher AUC compared to PSA (AUC = 0.667, 95% CI = 0.580-0.754), %core (AUC = 0.632, 95% CI = 0.539-0.724), or clinical panel (AUC = 0.673, 95% CI = 1.458-4.994). Age did not yield a statistically significant AUC. A modest improvement was observed when incorporating both the 3-miR score and PSA value (AUC=0.700, 95% CI=0.682-0.884), which indicates that there is additive value in the 3-miR score to the conventional clinical biomarker, PSA, for predicting reclassification.

Table 3.10 ROC analysis of 3-miR score and clinical variables in the training cohort

<table>
<thead>
<tr>
<th>Marker</th>
<th>Area under the curve</th>
<th>P-value</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-miR*</td>
<td>0.690</td>
<td>&lt;0.001</td>
<td>0.596-0.784</td>
</tr>
<tr>
<td>%core at diagnosis</td>
<td>0.632</td>
<td>0.009</td>
<td>0.539-0.724</td>
</tr>
<tr>
<td>PSA at collection</td>
<td>0.667</td>
<td>&lt;0.001</td>
<td>0.580-0.754</td>
</tr>
<tr>
<td>Age at collection</td>
<td>0.530</td>
<td>0.546</td>
<td>0.440-0.621</td>
</tr>
<tr>
<td>3-miR + PSA</td>
<td>0.700</td>
<td>&lt;0.001</td>
<td>0.682-0.884</td>
</tr>
<tr>
<td>Clinical Panel**</td>
<td>0.673</td>
<td>0.01</td>
<td>0.578-0.768</td>
</tr>
</tbody>
</table>

* miR-223, -24, and -375; ** PSA at collection and %core at diagnosis.
Finally, we dichotomized patients based on 3-miR score + PSA values to assess how well this model can predict reclassification. We chose a cutoff value that yielded the highest sum of sensitivity + specificity from the previously constructed ROC curve. This “3-miR + PSA cutoff” yielded a modest positive predictive value (PPV = 39%), but an excellent negative predictive value (NPV = 89%), while correctly classifying 66% of non-reclassified patients (Table 3.11).

**Table 3.11 Accuracy of 3-miR + PSA cutoff for predicting active surveillance patient reclassification in the training cohort**

<table>
<thead>
<tr>
<th></th>
<th>Specificity</th>
<th>Sensitivity</th>
<th>PPV</th>
<th>NPV</th>
<th>$\chi^2$</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Training</td>
<td>66.4</td>
<td>72.1</td>
<td>39.2</td>
<td>88.8</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

3.2.3 Validation of markers in an independent cohort

Our findings from the training cohort were tested in the validation cohort. Univariable logistic regression analysis found that individual miRNAs were again not significant predictors for reclassification in the validation cohort with the exception of let-7a (Table 3.12).
Table 3.12 Logistic regression analysis of candidate miRNAs in the validation cohort

<table>
<thead>
<tr>
<th>Marker</th>
<th>OR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Let-7a</td>
<td>1.435</td>
<td>1.092-1.885</td>
<td>0.009</td>
</tr>
<tr>
<td>miR-141</td>
<td>0.850</td>
<td>0.692-1.043</td>
<td>0.120</td>
</tr>
<tr>
<td>miR-145</td>
<td>0.906</td>
<td>0.707-1.162</td>
<td>0.438</td>
</tr>
<tr>
<td>miR-21</td>
<td>0.813</td>
<td>0.535-1.237</td>
<td>0.334</td>
</tr>
<tr>
<td>miR-223</td>
<td>0.962</td>
<td>0.903-1.024</td>
<td>0.220</td>
</tr>
<tr>
<td>miR-24</td>
<td>1.035</td>
<td>0.951-1.127</td>
<td>0.430</td>
</tr>
<tr>
<td>miR-26b</td>
<td>0.949</td>
<td>0.830-1.086</td>
<td>0.449</td>
</tr>
<tr>
<td>miR-30c</td>
<td>1.191</td>
<td>0.995-1.426</td>
<td>0.057</td>
</tr>
<tr>
<td>miR-30e</td>
<td>0.667</td>
<td>0.399-1.116</td>
<td>0.123</td>
</tr>
<tr>
<td>miR-375</td>
<td>0.934</td>
<td>0.860-1.013</td>
<td>0.099</td>
</tr>
</tbody>
</table>

The 3-miR score (OR = 5.512, 95% CI = 1.502-20.229) was still a significant predictor of reclassification and outperformed PSA (OR = 1.256, 95% CI = 1.076). In the validation cohort, the clinical panel, age, %core, and prostate volume were not significant predictors for reclassification (Table 3.13).

Table 3.13 Logistic regression analysis of 3-miR score and clinical variables in the validation cohort

<table>
<thead>
<tr>
<th>Marker</th>
<th>OR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-miR*</td>
<td>5.512</td>
<td>1.502-20.229</td>
<td>0.010</td>
</tr>
<tr>
<td>% cores at diagnosis</td>
<td>1.023</td>
<td>0.991-1.056</td>
<td>0.155</td>
</tr>
<tr>
<td>PSA at collection</td>
<td>1.256</td>
<td>1.076-1.462</td>
<td>0.004</td>
</tr>
<tr>
<td>Age at collection</td>
<td>1.080</td>
<td>1.003-1.162</td>
<td>0.040</td>
</tr>
<tr>
<td>Prostate volume</td>
<td>1.014</td>
<td>0.991-1.036</td>
<td>0.232</td>
</tr>
<tr>
<td>Clinical Panel**</td>
<td>1.560</td>
<td>0.948-2.567</td>
<td>0.080</td>
</tr>
</tbody>
</table>

*miR-223,-24,-375; ** PSA at collection and %core at diagnosis.
Next, we assessed each type of reclassification triggers separately, and the 3-miR score was again a significant predictor of GS upgrading (OR = 7.495, 95% CI = 1.493-37.617). Analysis was not performed for prediction of PSA-DT<3yrs or MRI progression due to small number of events (PSA-DT < 3yrs n = 4, MRI progression n = 2).

Furthermore, in multiple logistic regression analysis of the 3-miR score, PSA, %core, and age as covariates, the 3-miR score’s ability to predict reclassification was significant independent of clinical variables (Table 3.14).

<table>
<thead>
<tr>
<th>Marker</th>
<th>OR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-miR*</td>
<td>10.876</td>
<td>2.123-55.712</td>
<td>0.004</td>
</tr>
<tr>
<td>% cores at diagnosis</td>
<td>1.040</td>
<td>1.000-1.082</td>
<td>0.048</td>
</tr>
<tr>
<td>PSA at collection</td>
<td>1.297</td>
<td>1.082-1.512</td>
<td>0.004</td>
</tr>
<tr>
<td>Age at collection</td>
<td>1.098</td>
<td>1.003-1.203</td>
<td>0.042</td>
</tr>
</tbody>
</table>

*miR-223, -24, -375.

We then constructed ROC curves to assess the 3-miR score and clinical variables in the validation cohort (Table 3.15). The 3-miR score (AUC = 0.708, 95% CI = 0.593-0.823), and PSA (AUC = 0.747, 95% CI = 0.643-0.851) were significant for predicting patient reclassification. Furthermore, when combining the 3-miR score and PSA, the AUC was again improved (AUC = 0.783, 95% CI = 0.682-0.884) and outperformed both the 3-miR score and PSA alone. The AUC’s of the clinical panel, age, %core, and prostate volume were not statistically significant.
Table 3.15 ROC analysis of 3-miR score and clinical variables in the validation cohort

<table>
<thead>
<tr>
<th>Test Result Variable(s)</th>
<th>Area under the curve</th>
<th>P-value</th>
<th>95% confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-miR*</td>
<td>0.708</td>
<td>0.003</td>
<td>0.593-0.823</td>
</tr>
<tr>
<td>%core at diagnosis</td>
<td>0.576</td>
<td>0.279</td>
<td>0.430-0.723</td>
</tr>
<tr>
<td>PSA at collection</td>
<td>0.747</td>
<td>&lt;0.001</td>
<td>0.643-0.851</td>
</tr>
<tr>
<td>Age at collection</td>
<td>0.635</td>
<td>0.055</td>
<td>0.519-0.752</td>
</tr>
<tr>
<td>3-miR + PSA</td>
<td>0.783</td>
<td>&lt;0.001</td>
<td>0.682-0.884</td>
</tr>
<tr>
<td>Clinical Panel**</td>
<td>0.621</td>
<td>0.085</td>
<td>0.487-0.755</td>
</tr>
</tbody>
</table>

*miR-223, -24, -375; **PSA at collection and %core at diagnosis.

Lastly, we applied the 3-miR + PSA cutoff to the validation cohort, and it again had a high NPV (89%) and correctly categorized 80.1% of non-reclassified patients (Table 3.16).

Table 3.16 Accuracy of 3-miR + PSA cutoff for predicting active surveillance patient reclassification

<table>
<thead>
<tr>
<th></th>
<th>Specificity (%)</th>
<th>Sensitivity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>$\chi^2$ P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Validation</td>
<td>80.7</td>
<td>57.1</td>
<td>41.4</td>
<td>88.8</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Chapter 4 Discussion

4.1 Optimization experiments and comparison of techniques

4.1.1 MS2 and glycogen as carrier molecules

A sample of pure RNA will have a 260/230 absorption ratio of 2, whereas ratios below 0.5 were typical in our isolated RNA samples. This is likely due to organic contaminants that were carried over from the extraction process. Organic compounds commonly found in nucleic acid extraction reagents, such as phenolate or thiocyanate, absorb at 230nm. With this in mind, the quantity estimates from Nanodrop were likely to be greatly inflated. However, we did observe a proportional increase in isolated RNA when using increased volumes of serum. Thus, the Nanodrop estimation of RNA levels may still be used as a relative comparison between extractions rather than absolute quantification.

We found that endogenous miRNA levels from glycogen-supplemented extraction replicates were highly similar. In contrast, there were large variances between replicates of extractions supplemented with MS2 bacteriophage RNA. Both carrier molecules have been reported to facilitate co-precipitation and binding of nucleic acids, which improves yield during extractions of low RNA quantities\textsuperscript{166,167}. MS2 RNA is characterized as miRNA-free. Its genome consists of a single stranded RNA 3569 nucleotides in length\textsuperscript{168}. However, due to the large variations between MS2-supplemented extraction replicates, we speculate that there was non-specific amplification due to binding of miRNA primers to MS2 RNA.

4.1.2 Quantification of miRNA

The NanoString platform could not reliably detect miRNAs isolated from 200 µL of serum. However, endogenous miRNAs that could not be detected by NanoString were reliably quantified with qRT-PCR. Thus, we believe a qPCR approach is more suitable for analysis of low RNA quantities than NanoString. At the time of study design, it was unclear if 200 µL of serum was enough for nCounter profiling. A search of the MEDLINE database in Dec 2015 identified only two studies
which analyzed circulating miRNAs with NanoString Counter, both of which used greater than 200µL of serum (500µL\textsuperscript{169} and 1000µL\textsuperscript{170} of plasma). More recently, a white paper was published by NanoString Inc., which suggested a minimum 400µL of biomaterials for profiling.

4.1.3 Normalization of circulating miRNAs

Due to lack of established endogenous controls available for circulating miRNAs, we performed a preliminary experiment to assess the variability of miR-30e, -425, and UniSp6 between samples. The two endogenous controls, miR-30e and -425, showed significant variation between samples, which suggest that they are not appropriate for normalization. In contrast, the spike-in RNA UniSp6 showed considerably lower variation compared to the two endogenous miRNAs. Other reports have also proposed that spike-in RNA is a superior choice for normalization of circulating miRNAs in non-high-throughput datasets due to lack of established endogenous controls\textsuperscript{171,172}.

We assessed miR-30e and -425 as potential endogenous controls because they were previously proposed to be stably expressed in serum or plasma of PCa patients\textsuperscript{151,153}. Though we did not find this to be the case in our serum samples, it was unsurprising, since most proposed endogenous controls for circulating miRNAs lack extensive validation and are likely to be disease or cohort-specific. In addition, Kelly\textit{et al.}, who used miR-425 as an endogenous control examined whole-blood, which contains cellular RNA\textsuperscript{151}. In their study, miR-425 was originally a candidate miRNA due to dysregulation in PCa tissue and was retrospectively selected as a normalization control because it was the most stably expressed miRNA among 12 candidate miRNAs analyzed. Huang\textit{et al.} reported that miR-30e was highly stable in a cohort of 123 PCa patients\textsuperscript{153}. However, RNA from serum exosomes were used, which may have a different expression profile compared to whole serum\textsuperscript{122,173}. These differences in sample types may also be a factor in the stability of miR-30e and miR-425 expression observed in these reports.

4.2 Prognostic miRNA panel for AS patient reclassification

To the best of our knowledge, this study is the first to investigate circulating miRNAs as non-invasive biomarkers for predicting AS patient reclassification. We identified and validated a miRNA
panel (miR-223, -24, -375) which can predict reclassification independently of clinical variables and confer additive predictive value to PSA. Our findings show that most patients below the 3-miR + PSA cutoff are unlikely to show disease progression. In our cohorts, the majority (66% - 81%) of indolent patients were below the 3-miR + PSA threshold, and only a small portion (11%) of patients in this subset would eventually reclassify.

Currently, AS patients suffer repeat biopsies every 1-4 years depending on the intensity of the program in which they have enrolled. It has been previously demonstrated that biopsies carry a significant risk of infection as well as contributing to patient anxiety. Furthermore, given the 30% rate of GS upgrading upon pathologic examination after RP, a significant portion of AS patients are likely undergraded. Using serum miRNA biomarkers may address these shortcomings of prostate biopsies. By identifying patients unlikely to experience disease progression using the 3-miR + PSA threshold, we can offer less rigorous monitoring with reduced frequency of biopsies, which will minimize the associated morbidities. Furthermore, surveillance with these non-invasive biomarkers overcomes the sampling bias of biopsies, which fails to account for multi-foci occurrences and heterogeneity of PCa. Patients with high 3-miR + PSA scores may also warrant more intensive surveillance. The PPV achieved is modest, but it may be tested with other biomarker tests, such as PCA3 to potential improve identification of high-risk AS patients.

4.3 Comparison to other biomarkers and tools in PCa monitoring

Patients on AS are subjected to repeat prostate biopsies. Non-invasive biomarkers which can reduce or replace biopsies is the next step in optimizing AS protocols. Currently, there are only a few FDA or CLIA certified biomarker tests which may benefit AS patients, specifically PHI, 4K score, and OncoType DX. In a recent multi-centered study, Heidegger and colleagues showed that PHI had an very modest OR of 1.039 (p = 0.004) for predicting GS upgrading of GS6 patients after surgery. In comparison, the 3-miR marker reported in our study has OR of 2.721-5.512 for predicting patient reclassification, including GS upgrading. Parekh et al. conducted a multi-centred study which showed the 4K score is able to discriminate between benign or low-grade PCa versus high-grade PCa. However, the comparison included a majority of benign patients and some GS ≥
8 patients, which are not representative of patients on AS or recently reclassified from AS. Finally, OncoType DX is a biopsy-based test and marketed for better selection of patients for AS at diagnosis rather than monitoring\textsuperscript{92}. Aside from aforementioned blood based biomarkers, numerous biopsy-based tests are also available. Confirm MDx is a biopsy-based test which predicts the presence of PCa upon repeat biopsy after at least one negative biopsy\textsuperscript{91}. Prolaris and Decipher are biopsy-based tests which predicts the likelihood of disease recurrence after radical prostatectomy\textsuperscript{97,174-176}. Finally, OncoType DX can estimate risk of occult, high-grade PCa when needle biopsy has only found GS6 or GS3 + 4 cancer\textsuperscript{92}. Among these biopsy-based tests, Oncotype DX may be helpful to AS patients, but it still requires invasive tissue biopsies. Furthermore, OncoType DX assays a large number of genes (17), which would be costlier than a smaller biomarker panel. As such, the 3-miR score better addresses the unmet need of non-invasive biomarkers for AS patients than any FDA or CLIA certified biomarker tests.

A new tool in contemporary AS protocols is MRI, which has demonstrated high NPV for ruling out clinically significant PCa\textsuperscript{57}. In our study, the 3-miR score + PSA cutoff also had excellent NPV (89%). Unfortunately, MRI was not consistently performed for patients in this study. However, comparison of our biomarkers to results from a recent clinical trial (PROMIS)\textsuperscript{57} suggests that the 3-miR + PSA cutoff may outperform MRI for identifying patients with insignificant PCa. Based on patient population breakdown obtained through correspondences with Dr. Hashim Ahmed of the PROMIS trial\textsuperscript{57}, we found that the NPV and specificity were both considerably lower at 77% and 32%, respectively, when MRI was used to distinguish between significant versus insignificant PCa (excluding non-PCa patients). It should be noted, however, that the population of PCa patients harbouring significant PCa was much higher (56%) compared to our cohorts (19-24%). Serum miRNAs are also potentially more economical and accessible, as MRI procedures are relatively costly and difficult to access in rural areas. Further testing of these non-invasive miRNA biomarkers is warranted, and assessment of their utility alongside MRI in the same AS population as well as a cost-effectiveness analysis are needed for better comparison.
4.4 Limitations

4.4.1 Normalization of miRNAs

There is currently no validated endogenous control for circulating miRNAs. Commonly used small RNA controls for tissue studies, such as U6, have been shown to have widely varying levels in circulation\textsuperscript{128}. Global mean normalization is unreliable when a small number of miRNAs is analyzed (candidate approach). Thus, we used a spike-in RNA to control for technical variability between samples during processing and analysis, however this method does not account for biological differences between patients.

4.4.2 Cohort composition

The followup period for the validation cohort (UHN) is quite short. However, the followup time was calculated starting from date of sample collection, and these patients may have been on AS for much longer. Beginning from the date of diagnosis, the median followup for this group becomes 49.7 months (range 0-183), thus we are confident most non-reclassified patients are harbouring clinically insignificant PCa. There are 10 patients, however, which had not received a confirmatory biopsy, with median followup from diagnosis of 2.5 months (range 0-14.5). We elected to include these patients in the analysis to keep the cohort clinical representative, as all AS populations will have a small proportion of newly enrolled members.

A notable proportion of men in both the training (18%) and validation cohort (11%) had PSA measurements greater than 10ng/mL, which does not conform to the conventional (NCCN) definition of low-risk patients. We decided to include these patients in the analysis because there is increasing evidence that GS6 tumours would not become significant regardless of the tumour volume or serum PSA level of the patient\textsuperscript{80-82}, which would make these men suitable candidates for AS. However, these patients may not be considered eligible for monitoring depending on the policy of specific AS programs.
4.4.3 Statistical modeling

During statistical analysis, we employed a stepwise model building method to construct the 3-miR score. This approach is commonly used in biomedical research but also criticized, because it is prone to overfitting\textsuperscript{177}. However, measures were taken to mitigate overfitting in our study design. Specifically, we incorporated only miRNAs that were previously demonstrated to be associated with PCa progression. We also kept a high event per variable (EPV = 16) during analysis of the training cohort. Finally, we performed external validation in an independent cohort, which is the gold standard for validation of multivariable logistic regression models\textsuperscript{178}.

4.5 MiRNAs in the statistical model

In univariable logistic regression analysis of candidate miRNAs, no individual miRNA was consistently significant in the two cohorts. The sample size in the study may not have been sufficiently powered to detect the minute differences in individual miRNAs which occurs during early stages of PCa progression. However, we constructed a 3-miR score which include three of nine candidate genes tested, miR-223, -24, and -375, that was significant for predicting patient reclassification. In the statistical model, lower levels of miR-223 and miR-375, and higher levels of miR-24 were associated with patient reclassification. This was surprising, as miR-375 and miR-24 were reported to upregulated and downregulated, respectively, in late stage PCa compared to low-grade PCa or non-PCa patients.

4.5.1 miR-375

Several groups reported increased levels of circulating miR-375 when comparing PCa to non-PCa patients or advanced-stage PCa (metastatic, high-grade, or CRPC) to localized PCa\textsuperscript{129,140,142}. Bryant \textit{et al.} reported that circulating miR-375 is upregulated among post-RP patients who experience recurrence compared to those who do not\textsuperscript{135}, and Huang \textit{et al.} found that circulating miR-375 is upregulated in patients with poor recurrence-free survival after RP\textsuperscript{179}. One study, however, did report a downregulation of circulating miR-375 in PCa versus BPH and healthy patients\textsuperscript{157}. In our statistical model, downregulation of serum miR-375 was associated with disease progression.
(lower miR-375 levels in the reclassified group). This difference in the direction of dysregulation may be largely due to the difference in the types of patients analyzed in our report versus earlier studies. The aforementioned studies compared advanced PCa to localized PCa or benign patients. In contrast, we aimed to detect changes at very early stages of PCa development, which may have been overshadowed by larger changes in late stage cancer developments. For example, a tumour suppressor miRNA may be downregulated in early progression of localized tumours, but apoptosis of circulating tumour cells in late-stage, metastatic cancer may increase the level of all miRNAs in circulation, resulting in a net increase of miRNAs.

MiR-375 is typically known as a tumour suppressor miRNA. Outside of PCa and breast cancer, miR-375 is almost universally downregulated and lower levels are associated with poor prognosis. This has been shown in gastric cancer\textsuperscript{180}, head and neck squamous cell cancer\textsuperscript{181}, cervical cancer\textsuperscript{182}, esophageal squamous cell carcinoma\textsuperscript{183}, lung cancer\textsuperscript{184}, hepatocellular carcinoma\textsuperscript{185}, and colorectal cancer\textsuperscript{186}. In breast cancer, there has been reports of both up- and downregulation of miR-375. The tumour suppressive effects of miR-375 are well established. In breast cancer, miR-375 has been found to inhibit CSC and resistance to tamoxifen, a treatment for hormone-sensitive breast cancer, by targeting and downregulating HOXB3. Interestingly, overexpression of HOXB3 has been found in PCa tissues compared to adjacent normal tissues and predicts poor outcome and GS $\geq 7$ after surgery\textsuperscript{187}. Furthermore, \textit{in vitro} and \textit{in vivo} experiments found that depletion of HOXB3 decreased cell proliferation\textsuperscript{187}.

4.5.2 miR-24

Circulating miR-24 was reported to be downregulated in intermediate- and high-risk compared to low-risk PCa patients (CAPRA score)\textsuperscript{130}, and high-grade versus low-grade PCa patients\textsuperscript{152}. MiR-24 was also found to be downregulated in PCa versus normal prostate tissue, though in a very limited sample size (10 PCa, 10 normal)\textsuperscript{188}. Interestingly, in the study by Mihelic et al., upregulation of miR-24 was observed from BPH to low-grade PCa patients\textsuperscript{152}. It may be that early progression of PCa involves increase of miR-24, while other changes in late-stage PCa cause a reversal of miR-24 levels.
There have been equally frequent reports of both tumour suppressive and promoting effects of miR-24. For example, miR-24 has been found to be upregulated in patients with metastatic hepatocellular carcinoma and increase metastasis in cell lines by targeting \( p53 \)\textsuperscript{189}, yet another study in adenoid cystic carcinoma cells reported that miR-24 targets \( PRKCH \), a negative regulator of \( p53 \), leading to upregulation of \( p53 \) and subsequently decreased migration and invasion\textsuperscript{190}. In tongue squamous cell carcinoma tissue, miR-24 is speculated to have a tumour promoting effect, as it was significantly and negatively correlated with \( PTEN \) expression\textsuperscript{191}. In gastric cancer cell lines, miR-24 has been found to target \( BCL2L11 \), leading to promotion of cell growth and migration, as well as inhibition of apoptosis\textsuperscript{192}. The opposite effect has been reported in experiments in cell lines of breast cancer and hepatoma by targeting \( BCAR1 \), an oncogene which promotes proliferation and migration\textsuperscript{193}.

### 4.5.3 miR-223

miR-223 was first reported by Moltzahn \textit{et al.} in 2011 to be downregulated in PCa versus healthy patient serum samples\textsuperscript{130}. Mihelic \textit{et al.} later reported that serum miR-223 was also downregulated in high-grade versus low-grade PCa patients\textsuperscript{152}. In tissue, miR-223 was found to be downregulated in PCa compared to healthy controls\textsuperscript{188,194}. Similar miR-223 dysregulation is also observed in other cancer types. Downregulation of circulating miR-223 has been associated with metastasis and disease progression in non-small cell lung cancer\textsuperscript{195}, esophageal squamous cell carcinoma\textsuperscript{196}, pancreatic cancer\textsuperscript{197}, and lymphocytic leukemia\textsuperscript{198}.

MiR-223 is characterized as a tumour suppressor miRNA. Several mechanistic studies have elucidated potential downstream effects of epigenetic regulation by this miRNA. Jia \textit{et al.} reported miR-223 inhibits proliferation and colony formation of HeLa cells through targeting \( IGH-1R \) and consequently the downstream \( Akt/mTOR/p70S6K \) pathway. Targeting \( IGF-1R \) by miR-223 was also reported in leukemia, hepatoma cells, oral carcinoma, and lung cancer\textsuperscript{199}. In prostate cancer, it has been shown that miR-223 directly targets \( ITGA3 \) and \( ITGB1 \) in PCa cell lines, leading to decreased cell proliferation, migration, and invasion\textsuperscript{194}.
4.5.4 Perspective

Due to the vast number of targets of individual miRNAs, it is difficult to discern the “big picture” effects resulting from alterations to a single miRNA. Mechanistic studies elucidate targets of commonly dysregulated miRNAs and give us insight on their role in cancer development. However, given that only a fraction of putative targets of even the most studied miRNA has been confirmed, it is likely that many other biological processes are simultaneously affected by any given miRNA. Furthermore, multiple miRNAs can co-regulate cancer pathways, which may resist or contribute to carcinogenesis depending upon their interactions. With this in mind, the 3-miR model proposed in our work implicate a specific balance of miR-223, -24, and -375, rather than changes in individual miRNAs. Furthermore, this may also be the reason why individual candidate miRNAs were not significant for predicting reclassification. Finally, there is a lack of data on miRNA profile changes during the very early stages of PCa progression, such as the progression from an AS to reclassification. The changes in these early disease stages may not reflect the more drastic overall miRNA alterations when considering progression from early to advanced stage PCa.
Summary and future directions

Summary and conclusion

This study identified and validated a combination of 3 miRNAs (miR-24, -223, -375) which could significantly predict reclassification of AS patients (training OR = 2.271, 95% CI = 1.499-4.939; validation OR = 5.512, 95% CI = 1.502-20.229). Multivariable analysis demonstrated that the predictive ability of this 3-miR score is independent of clinical variables (PSA, age, %core). Furthermore, we found that there was additive value in combining the 3-miR score and PSA. An optimal cutoff of the 3-miR + PSA panel was able to achieve high NPV (89% in training and validation cohort) and sensitivity (66% in training cohort and 81% in validation cohort). This non-invasive biomarker may be used to identify AS patients who are unlikely to experience disease progression and may be safely monitored with a less intensive protocol. Incorporation of non-invasive biomarkers can further personalize management of PCa patients and reduce morbidity associated with AS. Further independent validations need to be performed, but our findings thus far suggest that the 3-miR score has great potential as a non-invasive biomarker that would improve contemporary AS protocols.

Future directions

Sample collection from UHN is ongoing. Two hundred serum samples are expected to be accrued by the end of 2017. Patient clinicopathological information will be updated annually. As the UHN cohort follow-up time is relatively short, we expect the proportion of reclassification patients to increase. At each update, analysis will be performed to confirm the robustness of our biomarkers. Post-DRE urine cell pellet samples are also available for GUBiobank patients and a subset of Sunnybrook patients (141 out of 196). DNA was isolated from urinary cells and methylation of PCa related genes were analyzed with real-time, methylation-specific PCR. We plan to assess miRNAs and DNA methylation biomarkers in concert to produce a more robust biomarker panel which can predict AS patient reclassification.
References


## Appendix

### Supplementary Table 1 qRT-PCR analysis of RNA isolated with various extraction kits

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Supplementary Table 2 RNA extractions supplemented with various carrier molecules

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Extractions were performed for six serum samples. Nanodrop was used to quantified isolated RNA.

Supplementary Table 3 Protocol for filtering out MS2 RNA carrier molecules
Supplementary Table 3 RNA extraction with miRNeasy mini kit with small RNA enrichment step

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Unusually high RNA levels after extraction indicates that MS2 was not filtered out in the extraction process.
**Supplementary Table 4 Summary of RNA extractions performed with or without a cleanup protocol**

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RT-PCR analysis of RNA extracted using miRNAeasy serum/plasma kit with various post-extraction cleanup protocols.
Supplementary Table 5 qRT-PCR analysis of RNA extracted using miRNeasy serum/plasma kit with various post-extraction cleanup protocols

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