**RESEARCH ARTICLE**

N6-Methyladenosine (m6A) readers are dysregulated in renal cell carcinoma

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**Abstract**

N6-Methyladenosine (m6A) is the most common modification of messenger RNA (mRNA) in mammals. It critically influences RNA metabolism and plays an essential role in virtually all types of bioprocesses including gene expression, tissue development, self-renewal and differentiation of stem cells, stress response and circadian clock control. It plays a crucial role in carcinogenesis and could be used as a prognostic and a diagnostic tool and as a target for new anticancer therapies. m6A modification is dynamically and reversibly regulated by three types of proteins. Methyltransferases, so-called “writers” add a methyl group to the adenosine, which can be removed by demethylases, also called “erasers.” m6A-specific RNA-binding proteins, from here on referred to as “readers,” preferentially bind to the m6A site and mediate biological functions, such as translation, splicing or decay of RNA. In this study, we examined the expression of the six m6A readers HNRNPA2B1, HNRNPC, YTHDC1 and YTHDF1-3 in clear cell renal carcinoma (ccRCC). We show that on mRNA level the expression of all six m6A readers is significantly downregulated compared to normal renal tissue and on protein level five out of six readers are dysregulated. Lower levels of some m6A readers are correlated with advanced stage and grade as well as associated with a shorter overall, progression-free and cancer-specific survival. In summary, we could show that m6A readers are dysregulated in ccRCC and might therefore act as a tumor marker, could give further information on the individual prognosis and be a target of innovative cancer therapy.

**KEYWORDS**

N6-methyladenosine, renal cell carcinoma, YTHDC1, YTHDF1, YTHDF3

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1 | INTRODUCTION

Kidney cancer is one of the most abundant cancers in both men and women. In 2019, 73,820 new cases and 14,770 deaths were estimated by the American Cancer Society in the United States of America alone. Worldwide, about 270,000 new cases annually are estimated. Renal cell carcinoma (RCC) is by far the most common kidney cancer and furthermore the most lethal cancer in the genitourinary system. Approximately 80% of RCC are of the clear cell subtype (ccRCC). Partial or radical nephrectomy is still the gold-standard treatment with curative intent for patients with localized RCC. Patients with metastasis however are usually treated with a systemic treatment, which in these cases has a palliative intent. A significant therapeutic improvement was the introduction of immunotherapy and targeted drugs, but relative drug resistance and resistance to radiotherapy are still big problems in clinical practice and the median survival for patients with metastatic ccRCC still is under 3 years, which makes understanding the pathogenesis of ccRCC and finding new therapeutic targets such an important topic.

The methylation of N^6^-adenosine (m^6^A) is the most common messenger RNA (mRNA) modification in mammals as between 0.1% and 0.4% of total adenosine residues are methylated. It plays a very important role in cancer development and progression as well as in almost all crucial cellular processes and stages of mRNA metabolism. The field of “Epitranscriptomics” (posttranslational RNA modifications) is known to have diverse important roles in a wide range of biological and pathological processes, especially in cancer, which led to it being one of the hot spots in life science and resulted in a vast number of studies worldwide.

m^6^A was first discovered in the 1970s. The regulatory mechanisms and the functional characteristics, however, were mostly unknown until recent years. It was shown that the m^6^A modification is a reversible and dynamic progress that affects more than 7600 human genes with 12,000 m^6^A sites. m^6^A is evolutionary highly conserved, mainly occurs at the RM^6^ACH-sequences (R = A/G/U; R = A/G and H = A/C/U) and is especially highly concentrated in 3′-untranslated regions, around stop codons and in long internal exons. Furthermore, it appears in precursor mRNAs (pre-RNAs) and long noncoding RNAs (Inc-RNAs).

The m^6^A modification is dynamically regulated by three groups of proteins. The attachment of the methyl group is catalyzed by the m^6^A methyltransferase complex (MTC)—which is composed of METTL3, METTL14 and WATA—and by other proteins like METTL4 and -16, ZC3H13, KIAA1429, and RBM15, also being called “writers”. The removal on the other hand is facilitated by FTO and ALKBH5, two m^6^A demethylases, also called “erasers”.

In this study, we examined the six readers HNRNPA2B1, HNRNPC, YTHDC1, and YTHDF1, which recognize the methyl group and transform it into biological signals. In this study, we have examined the six readers HNRNPA2B1, HNRNPC, YTHDC1, and YTHDF1-3.

m^6^A has been proven to play a critical role in tumorigenesis of very many different types of cancer like breast cancer, hematologic malignancies, especially well examined in acute myeloid leukemia (AML), hepatocellular carcinoma (HCC), pancreatic cancer, colorectal cancer (CRC), and many more, but also to maybe serve as a therapeutic target in several types of cancers like glioblastoma and AML. It was found that m^6^A expression levels significantly correlate with the activity of many cancer hallmark-related pathways like BRD4, MYC, SOCS2, or EGFR by regulating the mRNA expression levels of both oncogenes and tumor suppressor genes, thus promoting or suppressing tumorigenesis.

In 2019, Zhou et al. for the first time showed that alterations of the m^6^A modifiers have an impact on the prognosis of patients with ccRCC. m^6^A levels in ccRCC were downregulated, just as in other tumor entities like breast cancer and glioblastoma. m^6^A-downregulation resulted in a poor prognosis and worse clinical characteristics. Zheng et al. and Wang et al. showed that many of the m^6^A-regulating proteins were dysregulated in ccRCC which indicated that the m^6^A-regulator proteins indeed play important roles in ccRCC carcinogenesis. Furthermore, they could establish a m^6^A-related risk signature serving as a prognostic tool for ccRCC, similar to Zhao et al., all further promoting the idea of future m^6^A-targeted treatments for ccRCC. Additionally, it was shown that m^6^A-regulator protein expression levels could serve as biomarkers to distinguish between RCC subtypes.

In this study, we examined the six readers HNRNPA2B1, HNRNPC, YTHDC1, and YTHDF1-3. The fates of m^6^A-modified mRNAs transcripts are diversely affected by the different readers.

2 | MATERIALS AND METHODS

2.1 | Patients

The tissue samples used were collected by the Biobank of the Center for Integrated Oncology Cologne-Bonn. All patients underwent radical or partial nephrectomy at the Department of Urology at the University Hospital Bonn. All patients gave written informed consent for the collection of biomaterials. The study was approved by the Ethics Committee of the University of Bonn (vote: 127/17).

Fresh-frozen tissue of both ccRCC and correspondent normal renal parenchyma were stored at −80°C and utilized for mRNA expression studies. For Immunohistochemistry, archived formalin-fixed and paraffin-embedded tissues were utilized. All used tissues were re-examined by an uro-pathologist and classified following the 2009 WHO classification. Clinicopathological parameters are provided in Table S1.

2.2 | Quantitative real-time polymerase chain reaction

RNA isolation was described in detail previously. Briefly, the mirVana miRNA Isolation Kit (Ambion) was used to isolate total RNA which was treated with DNase (Ambion). The RNA quantity was...
evaluated utilizing a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and the RNA integrity was measured by evaluating the 28S and 18S ribosomal RNA bands in gel electrophoresis.

The gene expression of HNRNPA2B1, HNRNPC, YTHDC1, and YTHDF1-3 was evaluated through quantitative real-time polymerase chain reaction (PCR). Complementary DNA (cDNA) was won from 1 µg total RNA utilizing the PrimeScript RT Reagent Kit with genomic DNA Eraser (Takara Bio). PCR experiments were performed using 2.5 ng/µl cDNA template, SYBR Premix Ex Taq II and Rox Plus and 10 pmol/µl forward/reverse primer on a QuantStudio (Applied Biosystems by Thermo Fisher Scientific). Relative expression levels were determined using the QuantStudio 3D AnalysisSuite Cloud Software (Applied Biosystems). Gene expression was normalized to beta-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase and peptidylprolyl isomerase A as described earlier. Primer sequences are provided in Table S2.

2.3 | Immunohistochemistry

The six readers’ expressions were further investigated in ccRCC, papillary RCC (pRCC), chromophobe RCC (chRCC), sarcomatoid RCC (sRCC), Oncocytoma and benign renal tissue utilizing a tissue microarray (TMA), described previously by Schrödter et al. Formalin-fixed, paraffin-embedded archival tissues were cut into 5 µm thick sections, placed inside a water bath at 45°C for ideal expansion, applied on slides and dried at 65°C for 60 min. Afterwards, the slides were proceeded using the Benchmark Ultra system (Ventana Medical Systems Inc.), which performed deparaffinization, pretreatment with cell conditioning buffer (CC1 buffer, pH 8), and incubation with the primary antibodies (HNRNPA2B1: 1:100; HNRNPC: 1:100; YTHDC1: 1:350; YTHDF1: 1:100; YTHDF2: 1:100; YTHDF3: 1:1000) at 37°C for 36 min. Signal detection was performed with the HRP Multimer technology of the UltraView DAB IHC Detection Kit (Ventana) and finally, the slides were counterstained using Mayer’s hemalaun and bluing reagent (Ventana). The staining intensities were measured quantitatively utilizing QuPath software. Clinicopathological parameters are provided in Table S3.

2.4 | Statistical analysis

Statistical analyses (t test, Mann–Whitney U test, Kruskal–Wallis test, univariate, and multivariate Cox regression analyses, Kaplan–Meier estimates, Spearman’s rank-order correlation test) were appropriately executed with the Statistical Package for the
Social Sciences (SPSS), version 26 (SPSS Inc., IBM Corp.). Statistical significance was approved at $p < .05$.

3 | RESULTS

3.1 | mRNA expression

Gene expression of the six readers was examined in 166 ccRCC and 102 normal renal tissues. Expression levels of all 6 genes were significantly reduced in ccRCC in comparison to normal tissue ($p$ all $< .001$) (Figure 1). Spearman’s rank-order correlation test also showed a significant correlation between all six readers (Table S4). Furthermore, downregulation of all six readers was correlated with clinical M stage in ccRCC patients ($p$ all $< .05$) (Figure S1). Also, YTHDC1-downregulation correlated with a worse AJCC-Staging ($p = .033$). Associations with other clinicopathological parameters were not observed ($p$ all $>.05$) (Table S3). The prognostic potential for all genes was studied via Kaplan–Meier estimates: Regarding YTHDC1 and YTHDF3, a correlation with shorter overall (OS), progression-free (PFS), and cancer-specific survival (CSS) was found (YTHDC1: OS: log-rank $p = .004$; PFS: log-rank $p = .031$; CSS: log-rank $p = .037$) (Figure 2) (YTHDF3: OS: log-rank $p = .008$; PFS: log-rank $p < .001$; CSS: log-rank $p = .007$) (Figure 2). YTHDF1 was correlated with a shorter OS ($p = .035$) (Figure 2). Univariate Cox regression analyses confirmed our findings from the Kaplan–Meier estimates ($p$ all $< .05$) and also demonstrated the prognostic value of m6A readers in patients with ccRCC. Also, advanced clinicopathological parameters like Grading (G1/2 vs. G3/4), pT-Stage (pT1/2 vs. pT3/4), pN-Stage (pNX/0 vs. pN+), and pM Stage (M0 vs. M1) were found to correlate with survival (OS, PFS, CSS) in univariate Cox regression analysis (except pN-Stage considering PFS). In the multivariate Cox regression analysis, this predictive information was not observed (see Table S6a–c).

For further validation, we also examined the genes via the kidney renal clear cell carcinoma-TCGA dataset. The results partly supported our findings: HNRNPA2B1 ($p < .01$), YTHDF2 ($p = .01$), and YTHDF3 ($p < .01$) are downregulated, just as in our study (Figure S2). The other genes, however, were not significantly dysregulated in the TCGA dataset. Considering survival, the prognostic value was examined with Kaplan–Meier estimates via the GEPIA-website. Most of the studied genes were correlated with a shorter survival: HNRNPC ($p = .02$), YTHDC1 ($p < .01$), YTHDF1 ($p = .03$), YTHDF2 ($p < .01$), and YTHDF3 ($p < .01$) were correlated with a shorter OS and YTHDC1 ($p < .01$), YTHDF1 ($p < .01$), YTHDF2 ($p = .01$), and YTHDF3 ($p = .03$) were correlated with a shorter PFS (see Figure S3).

FIGURE 2 The mRNA expression level of YTHDC1, YTHDF1, and YTHDF3 is predictive for a shortened survival in patients with ccRCC. ccRCC, clear cell renal carcinoma; mRNA, messenger RNA [Color figure can be viewed at wileyonlinelibrary.com]
3.2 | Protein expression

Immunohistochemical staining was executed using a TMA consisting of 154 ccRCC, 35 pRCC, 10 chRCC, 14 sRCC, 10 Oncocytoma, and 30 normal renal tissues. See Table S5 for clinicopathological parameters and Figure S4 for exemplary pictures. Protein expression partly supported our findings on the mRNA level, all genes except YTHDF3 were significantly dysregulated in ccRCC. HNRNPC and YTHDF2, surprisingly, were upregulated and the other genes downregulated in comparison to normal renal tissue (HNRNP2B1, HNRNPC, YTHDC1, and YTHDF2: \( p < .001 \); YTHDF1: \( p = .001 \)) (Figure 3).

For YTHDF2-downregulation, we found a correlation with a lower grading (\( p = .001 \)), which may be explained by the diverse roles YTHDF2 plays in several types of cancer.\(^{33}\) Furthermore, Kaplan–Meier estimates indicated a shorter PFS regarding YTHDC1 (log-rank \( p = .036 \)) (Figure 4). Univariate Cox regression analyses confirmed this finding, furthermore, a correlation between lower

![Figure 3](https://wileyonlinelibrary.com)

**FIGURE 3** The protein expression levels of m\(^6\)A readers are dysregulated in RCC compared to normal renal tissue. m\(^6\)A, N\(^6\)-Methyladenosine; RCC, renal cell carcinoma [Color figure can be viewed at wileyonlinelibrary.com]

![Figure 4](https://wileyonlinelibrary.com)

**FIGURE 4** Kaplan–Meier estimates demonstrate a shorter progression-free survival in patients with low YTHDC1 expression [Color figure can be viewed at wileyonlinelibrary.com]

YTHDC1 levels and a shorter CSS was found. Again, advanced clinicopathological parameters like Grading (G1/2 vs. G3/4), pT-sStage (pT1/2 vs. pT3/4), pN-Stage (pNX/0 vs. pN+) and pM Stage (pM+ vs. pM1) were found to partly correlate with survival (OS, PFS, CSS) in
univariate Cox regression analysis (Grading and pM-Stage correlated with OS, PFS and CSS; pT stage correlated with PFS and CSS, p all <.05). In the multivariate Cox regression analysis, however, this predictive information could not be observed (see Table S6d-f).

Among the other RCC subtypes, most of the genes correlated with a significant dysregulation (Table S7). In pRCC, all proteins were upregulated (p all <.01) (Figure 3). In chRCC, HNRNPC, and YTHDF1-3 were upregulated (p all <.01) (Figure 3). In sRCC, the dysregulation was more complex: HNRNPC, YTHDF2, and YTHDF3 were upregulated, YTHDC1 and YTHDF1 were downregulated compared to normal tissue (p all <.05), for HNRNPA2B1, no significant dysregulation was found (Figure 3). Finally, in Oncocytoma, all genes were upregulated (p all <.01) (Figure 3).

4 | DISCUSSION

In the present study, we showed that all six readers are downregulated in ccRCC compared to normal tissue on mRNA level and that five out of six readers are dysregulated on protein level. This goes accordingly to the common dysregulations of m^6^A levels in different types of cancer and especially the findings of Zhou et al. Considering ccRCC, who stated that decreased m^6^A levels in ccRCC resulted in worse clinical parameters and thus survival and that m^6^A downregulation plays a crucial role in progression of ccRCC. This is why the worse prognosis for ccRCC patients we could show for some of the readers is also in conformity with many studies like Zhou et al. Also the TCGA dataset partly confirms our findings and also suggests that m^6^A is downregulated in ccRCC and that this downregulation correlates with a worse prognosis. Because of all these hints at the m^6^A dysregulation in ccRCC and the worse prognosis, our findings were expected.

Particularly interesting are HNRPA2B1, YTHDC1, and -F1, since they are downregulated on both mRNA- and protein levels. From these genes, the best results in our study were achieved for YTHDC1. Not only could we show that it is consistently downregulated on both mRNA and protein levels in ccRCC, but also that high levels of YTHDC1 are linked with a better outcome (longer OS, PFS, and CSS), emphasizing the potential YTHDC1 may have as a tool for diagnosis and prognosis. The worse prognosis of YTHDC1 downregulation was expected since it was also described by Zhou et al. YTHDC1 downregulation on mRNA level, just like the downregulation of all other readers on mRNA level is correlated to the existence of distant metastases and therefore a higher AJCC-staging and a worse outcome.

The lack of consistency in the ways of dysregulation on mRNA and protein level for HNRNPC, YTHDF2, and -3 was very curious, especially considering HNRNPC and YTHDF2, since they were downregulated on mRNA level and upregulated on protein level. This might have been the case since these genes are known to have many splice variants. To further determine this, we examined both the primers of all our genes and the antibodies against HNRNPC and YTHDF2 considering which splice variants they detect and if that may be the cause for the different directions of dysregulation. To examine the primers, we used the Basic Local Alignment Search Tool of the U.S. National Library of Medicine. The HNRNPC- and YTHDF2-antibodies were studied with the use of the UniProt-Dataset. The primers were found to have been designed well since they all recognize all know important splice variants. The same could be said about the antibodies: For HNRNPC, there are four known isoforms by alternative splicing. The subunit structure is composed of a tetramer containing three copies of isoform C1 and one copy of isoform C2. Since the antibody used detects the C2-isofrom, it should detect the tetramers. For YTHDF2, there are two known isoforms, which are both recognized by the used antibody. This means that we haven’t found common splice variants that the primers resp. antibodies couldn’t detect and thus this is not the explanation for the lack of consistency in the dysregulation in said genes. It could be possible, that because of the tumor metabolism, mRNA stability is posttranscriptionally enhanced leading to the reduced mRNA being translated more and that this results in a reduction of the gene on mRNA, but not on protein level. However, since this wasn’t the scope of our study, we cannot examine this hypothesis in greater detail.

YTHDC1, just as the other members of the YT521-B-homology (YTH)-domain family is a cytoplasmic, direct m^6^A reader. Its main function is mRNA splicing, facilitating nuclear export of m^6^A-marked RNA and promoting transcriptional silencing from X chromosome genes mediated by XIST (X inactive specific transcript). It recruits serine and arginine-rich splicing factor 3 (SRFS3) to its mRNA-binding regions near m^6^A and promotes exon inclusion. SRFS10 on the other hand binds to its target mRNA regions and regulates exon skipping in the absence of YTHDC1 or m^6^A. Furthermore, it has been observed to affect alternative splicing patterns in a concentration-dependent manner, but the mechanism so far is still unclear. Alternative pre-mRNA splicing and thus aberrant alternative splicing variants are often found in cancer, creating another link between m^6^A and cancer and explaining, why YTHDC1 is such an important protein in the context of cancer. Unbalanced alternative splicing, which can occur because of YTHDC1 dysregulation has been implicated in several kinds of cancer and may facilitate tumor cell proliferation and invasion via certain protumorigenic and the reduction of anti-tumorigenic isoforms and it is linked to altering cancer-associated genes like BRCA2 and PGR.

SRFS3, which is recruited by YTHDC1, is a crucial promoting factor in tumorigenesis in several kinds of cancer, like breast cancer, CRC, ovarian cancer, osteosarcoma and glioblastoma. YTHDC1 is also described to interact with m^6^A-writers like METTL3. METTL3-dysregulation, for instance, results in lower m^6^A levels of SRFSs, via YTHDC1-dependent nonsense-mediated mRNA decay of SRFS transcripts, reducing expression of SRFS-proteins. This results in bigger changes in alternative splicing isoform-switches, which affect tumor cell growth and progression in glioblastoma.

It is important to note, that in different kinds of cancer, m^6^A-regulating proteins can play diverse roles. In prostate cancer (PCa), it could be proven that YTHDC1 acts as an oncogene and it is commonly upregulated in CRC while it is downregulated in ccRCC.
and an upregulation correlates with a better outcome. Furthermore, low YTHDC1 expression levels are directly linked to a poor clinical outcome of patients with endometrial cancer, similar to our findings considering ccRCC. This is not surprising, since different m^6^A-regulating proteins, writers, erasers and readers are each linked to distinct cancer pathway alterations which suggests that different m^6^A-regulators have diverse functional effects in different cancer types. Also, it is well known that certain m^6^A-regulator proteins can have opposite functions in different types of cancer and can either work as a facilitator or a barrier of tumorigenesis because they are context and tissue-specific and their downstream targets differ in different cancer types. Also, Liu et al. suggest, that these different dysregulations in different kinds of cancer lie within tumor heterogeneity.

YTHDF1, another member of the YTH-domain family is also a cytoplasmic direct m^6^A reader. It directly promotes the translation of methylated mRNAs. It is known that YTHDF1 plays an important role in the development of and is linked with worse outcome in many kinds of cancer like HCC, CRC, where its knockdown results in suppression of CRC cell proliferation and chemoresistance and non-small cell lung carcinoma, but also bladder cancer, endometrial cancer, and ovarian cancer.

Like the other readers of the YTH domain family, YTHDF2 is a cytoplasmic reader and directly binds to m^6^A-containing RNA. Its main function is to mediate mRNA decay. YTHDF2 depletion increases both the abundance and the half-lives of cellular mRNAs. After YTHDF2 knockdown a 21% increase of the total mRNA’s m^6^A ratio was observed. Furthermore, YTHDF2 has been linked to several types of cancer like HCC, lung cancer, AML, PCa, endometrial cancer, breast cancer, or pancreatic cancer.

Just as the other cytoplasmic readers of the YTH domain family, YTHDF3 is a direct m^6^A reader. It was observed to interact with both YTHDF1 and YTHDF2 to facilitate either mRNA translation or decay. All in all, it works as a fine-tuning for the RNA accessibility of both YTHDF1 and YTHDF2. Studies showed that YTHDF3 is involved in tumorigenesis of several types of cancer including CRC, lung cancer, and PCa.

HNRNP2B1 is a nuclear RNA-binding protein. It indirectly binds to m^6^A sites on RNAs and was identified as a regulator in microRNA (miRNA) processing by promoting and accelerating pri-miRNA (primary miRNA) processing and maturation. Additionally, it regulates alternative splicing of miRNA. It was shown that HNRNP2B1 plays a role in pancreatic cancer and breast cancer. Together with METTL3 and -14, HNRNP2B1 could be used by Zhao et al. to build a risk score for patients with ccRCC.

HNRNPC is an indirect nuclear m^6^A reader, meaning just as HNRNP2B1 it alters the mRNA secondary structure so that m^6^A binding is facilitated. HNRNPC is mainly involved in pre-mRNA (precursor-mRNA) processing and mRNA splicing. Summarizing, we could show that on mRNA level and partly confirmed on protein level, the six readers HNRNP2B1, HNRNPC, YTHDC1, and YTHDF1-3 are dysregulated in ccRCC and that this dysregulation can result in worse prognosis and worse clinicopathological parameters like a higher risk of metastatic ccRCC and therefore higher AJCC-Stage. Furthermore, we also found dysregulation of m^6^A in the other studied subtypes of RCC: pRCC, chRCC, and sRCC as well as in Oncocytoma. Despite the small number of tissues, this indicates a dysregulation not only in ccRCC but also other kinds of renal carcinomas and supports the theory that m^6^A could be used to distinguish between different kinds of RCCs.

All these findings are another hint at what great promises the understanding of m^6^A holds for RCC and cancer in general. The dysregulation might in the future be used as a diagnostic tool and the worse prognosis that was partly shown may be able to be used to develop better prognostic tools and individualized risk scores for ccRCC patients like Zheng et al. and Wang et al. did. Also, like Zheng et al. proposed, m^6^A might be used for differentiating different RCC subtypes. Especially YTHDC1 seems to be a very promising m^6^A-regulating protein.

## 5 CONCLUSION

The readers HNRNP2B1, HNRNPC, YTHDC1, and YTHDF1-3 are dysregulated in ccRCC and other subtypes of renal cell carcinoma. In ccRCC, they partly correlate with worse clinicopathological parameters and shorter survival of the patients. Further studies are needed to establish the potential use of m^6^A as a biomarker, a diagnostic tool and a target for future targeted anticancer therapy.

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The authors declare that there are no conflict of interests.

## DATA AVAILABILITY STATEMENT

Raw data are available on request from the authors.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the supporting information tab for this article.

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