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ORIGINAL ARTICLE

# Comparison of Cumulus Cells Preparation among Diminished Ovarian Reserved and Normal Ovarian Reserved Women for RNA Extraction and Review of Literature

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***ABSTRAK***

*Kajian ini bertujuan untuk menentukan kaedah pengumpulan sel kumulus (CC) yang lebih sesuai dengan membandingkan pengendalian sampel yang diperoleh daripada wanita dengan rizab ovari yang berkurang (DOR) dan rizab ovari normal (NOR). Ini bertujuan untuk mendapatkan hasil ribonukleik asid (RNA) yang optimum bagi membolehkan analisis ekspresi gen yang lebih baik. Kami membandingkan pengumpulan sampel melalui kaedah mekanikal dengan enzimatik, "standard rotor-stator homogeniser" (RSH) dengan "disposable easy grind homogeniser" untuk teknik penyediaan sampel dan seterusnya proses penghasilan cDNA dan penilaian qPCR untuk keputusan terakhir. Kami juga turut menjalankan kajian sistematik untuk menyatukan penemuan kami dengan bukti terkini mengenai teknik pengekstrakan RNA. Hasilnya, strategi optimum adalah melalui denudasi mekanikal tanpa proses enzimatik, diikuti oleh penggunaan media bersifat penstabilan RNA sebelum pengekstrakan RNA. Selepas itu, penggunaan kawalan tekanan manual dengan penapis, adalah penting untuk penghasilan sampel tulen dan sekata serta setanding di antara sel kumulus wanita NOR dan DOR. Teknik penyediaan dan pengekstrakan sampel ini menghasilkan jumlah RNA yang optimum dan analisis ekspresi gen yang berjaya. Berdasarkan hasil kajian, ia*

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*dapat menambah nilai sebagai strategi semasa untuk mengoptimumkan pengekstrakan RNA dalam sel kumulus untuk kajian eksperimen. Secara kesimpulannya, penemuan kami menyumbang dalam merumuskan strategi yang lebih baik untuk mengoptimumkan pengekstrakan RNA dalam CC manusia untuk kajian transkriptik dan genomic asas.*
***Kata kunci:*** *Analisis gen; pengekstrakan RNA; penstabil RNA; rizab ovari berkurangan; sel cumulus*

## ABSTRACT

Our study aims to consolidate appropriate cumulus cells (CCs) collection methods by comparing sample handling obtained from diminished ovarian reserved (DOR) and normal ovarian reserved (NOR) women, aiming for a good yield of ribonucleic acid (RNA) for better gene expression analysis. We compared the sample collection by mechanical versus enzymatic method, standard rotor-stator homogeniser (RSH) versus disposable easy grind homogeniser for sample preparation technique and subsequently the cDNA synthesis and qPCR for final evaluation. We also conducted a systematic review to consolidate our findings with current evidence of RNA extraction technique. The optimal strategy was via mechanical denudation without an enzymatic process, followed by RNA stabiliser prior to RNA extraction. Subsequently, utilising a manual pressure control with filter, is paramount for a pure and homogenised sample and comparable for both NOR and DOR women CCs. These sample preparation and extraction techniques yielded an optimum RNA concentration and successful gene expression analysis. Our review also added value as a current strategy for optimising RNA extraction in CCs for experimental studies. Our findings contribute to formulating a better strategy for optimising RNA extraction in human CCs for transcriptomic studies.
**Keywords:** Cumulus cells; diminished ovarian reserved; gene analysis; RNA extraction; RNA stabiliser

## INTRODUCTION

In reproductive biology, the intricate interplay of various cell types orchestrates the complex oocyte maturation process, culminating in successful fertilisation and embryogenesis (Carvacho et al. 2018; He et al. 2021). Among these crucial players, cumulus cells (CCs) emerge as indispensable companions to oocytes, providing vital support and regulatory functions throughout the maturation process. The CCs, specialised somatic cells, reside near the oocyte within the ovarian follicles. Their intimate association and intricate communication with the oocyte are essential for guiding and facilitating its developmental journey (Turathum et al. 2021). The cumulus-oocyte complex forms a dynamic microenvironment, fostering bidirectional signalling that influences oocyte growth, meiotic progression, and eventual ovulation. This proximity enables CCs to exchange nutrients, growth factors, and metabolites with the oocyte, thereby ensuring optimal oocyte maturation (Carvacho et al. 2018; Huang & Wells 2010). At the molecular level, the CCs support the oocyte development closely by secreting hyaluronic acid (HA) while expanding the follicle-stimulating hormone (FSH) stimulation (Assidi et al. 2013). The mural granulosa cell (MGCs), which supports the growth of follicles, coordinates the endocrine function externally. The ovulation phase occurs due to increased

HA secretion by CCs once the rupture of the dominant follicles responds for luteinising hormone surge (van Gijn & Gijselhart 2011; Zhang et al. 2023a). Morphologically, CCs exhibit diverse shapes and sizes, ranging from spindle-like to cuboidal. These variations in morphology reflect the dynamic nature of their functions and underline their adaptability to the changing needs of oocyte maturation (Huang & Wells 2010; Salimov et al. 2023). The CC layer enveloping the oocyte forms a protective shield and a communication bridge between the oocyte and the surrounding ovarian microenvironment (Xie et al. 2023). The number of CCs enveloping an oocyte can differ depending on the follicular development stage. During the early stages of folliculogenesis, the number of CCs is relatively sparse. As the follicle progresses and matures, a corona radiata forms around the oocyte, comprising numerous layers of closely packed CCs. This protective cocoon of CCs serves as a conduit for nutrient exchange, signalling molecules, and regulatory factors that profoundly impact oocyte maturation (He et al. 2021; Huang & Wells 2010; Xie et al. 2023). The size and morphology of CCs exhibit remarkable diversity, reflecting the complex interplay of various intrinsic and extrinsic factors. Several factors contribute to the variability in CC size (Sfakianoudis et al. 2021). Apart from the follicular stage, the number, size, and morphology of the CCs are influenced by hormone levels, such as FSH and luteinising hormone (LH), metabolic status, ovarian microenvironment including oxygen tension and local growth factors, genetic variability, and aging (Coticchio et al. 2015). Minimal CCs are observed in cases of low oocyte quality, particularly in women with diminished ovarian reserved (DOR) (Nana et al. 2022) and in the elderly (Zhang et al. 2023b). Due to its minimal CCs, the challenges in obtaining a good concentration of ribonucleic acid (RNA) are even higher. The cumulative evidence has highlighted the pivotal role of CCs as invaluable indicators of oocyte quality.

As established, the molecular and biochemical composition of CCs can provide insightful information about the developmental competence of the oocyte and subsequent embryo quality (Gilchrist & Smitz 2023). CCs are endowed with the potential to harbour critical molecular biomarkers that offer predictive insights into the likelihood of successful fertilisation and embryo implantation. This burgeoning field holds promise for revolutionising assisted reproductive technologies and infertility treatments by allowing more informed decision-making regarding oocyte selection. Most evidence utilises the CC's RNA to study the critical mechanism of CC's in oocyte development and maturation. For these matters, the RNA extraction steps are crucial. It required a proper purification of RNA from the CC's tissue samples. Nevertheless, ribonuclease enzymes in the tissues can complicate it, leading to rapid degradation of RNA material upon extraction. To date, various methods are used in molecular biology to isolate RNA from CCs. The most commonly reported is the guanidinium thiocyanate-phenol-chloroform extraction. It consists of the filter paper-based lysis followed by the elution method, which features high throughput capacity. Despite all the reported RNA extraction methods in CCs, given their minute size and scarcity, they still pose significant challenges to effective RNA extraction. The ranges of RNA concentration is still below 10 ng/µl; ranging from 4-7 ng/µl as reported by previous study (Ferrari et al. 2010). Nevertheless, the limited number of CCs obtained from a single follicle necessitates meticulous optimisation of RNA extraction protocols to ensure sufficient and high-quality

RNA (Biase 2021; Maisarah et al. 2020). The significance of RNA extraction in CCs must be considered. Challenges also occur in DOR women as compared to normal ovarian reserved (NOR) women as their CCs mostly scanty. Thus, optimum strategy should be advocated to ensure purified RNA obtained following the extraction (Lu et al. 2022). However, despite its importance, limited published literature suggests the optimal method for achieving a pure final product. Therefore, it is imperative to conduct further research and experimentation to uncover the best approach for samples preparation during extracting RNA in CCs. While numerous original articles delve into the molecular insights gleaned from CCs, the intricacies of RNA extraction should be addressed. The present study addressed a significant gap in gene expression studies in CCs. We aimed to provide a comprehensive optimisation strategy that covered all aspects of sample collection, preparation, RNA extraction, and purification in both DOR and NOR women. By consolidating our findings with current literature, we offered valuable insights to help researchers prepare high-quality RNA for their studies. Our research paved the way for more accurate and reliable gene expression studies in the future.

## MATERIALS AND METHODS

### (A) Clinical Study

### Study design, recruitment and ethical approval

The prospective cohort study was conducted in the Advanced Reproductive Center, Hospital Canselor Tuanku Mukhriz (HCTM), Universiti Kebangsaan Malaysia (UKM) Cheras, Kuala Lumpur, from June to December 2022. This research was approved by the UKM Research Ethics Committee (JEP-2022-187), Faculty of Medicine, UKM Cheras, Kuala Lumpur, Malaysia. Consent was obtained prior to CC collection during the oocyte retrieval (OR) procedure for in-vitro fertilisation (IVF). Based on Bologna Criteria, the cut-off point of Anti Mullerian Hormone (AMH) level >8.5714 pmol/L is considered NOR. Women who are less than this level are regarded as DOR (Ferraretti & Gianaroli 2014). The CCs were discarded after separation from surrounding oocytes - "denudation" before the IVF procedure. Thus, obtaining these samples does not interfere with the IVF treatment. All women who underwent OR during the study period were included and group according to AMH level regardless of the causes of the infertility. A total of 40 women were included; 20 NOR women and 20 DOR women. A total of 40 samples of CCs for RNA extraction and optimise the housekeeping gene (HKG) hypoxanthine - guanine phosphoribosyl transferase (HPRT) amplification were collected and stored in an Eppendorf tube size 1.5 ml filled with 500 µl RNALater® solution (Thermo Fisher USA).

### RNA extraction method optimisation

For the purpose of our study, the RNA extraction method optimisation was divided into three stages:

(i) Sample collection optimisation

The oocyte denudation (OD) process was the initial step of CC sample collection. To date, most centers implement the enzymatic protocol for oocyte denudation (Tjahyadi et al. 2022). In brief, OD was conducted in two phases. Initially, the fresh retrieval oocytes were enzymolysed in hyaluronidase - hyase-10x TM (Vitrolife® Sweden) to dissolve the bonds between CCs within 40 seconds; then,

CCs were further removed from oocytes mechanically by repetitive pipette aspiration and deposition in a series of media without an enzyme. Finally, the "naked"-clean oocytes were examined for their maturity, and only meiosis II stage oocytes (MII) with the presence of polar body were used for IVF procedure either via intra-cytoplasmic sperm injection (ICSI) or conventional IVF. The comparison of cumulus-oocytes-complex (COCs) features with and without hyaluronidase and post-denudation of both techniques were showed in Figure 1. For this study, 20 CCs were collected via conventional method using enzymatic protocol, whereas 20 other CCs were collected without enzymatic protocol-purely mechanical using only manual force created via repetitive pipette aspiration and deposition in a series of media without an enzyme to obtain naked oocytes. The study flow was illustrated in Figure 2.

(ii) Sample preparation optimisation

For sample preparation, CCs were lysed to form smaller molecules to achieve excellent RNA extraction (Tan & Yiap 2009). This step required a mixer of RNA carrier solution (QIAamp® Circulating Nucleic Acid Kit) and diluted with buffer RLT, forming the RNA carrier dilution (RCD). The RLT buffer is a lysis buffer used to lyse CCs prior to RNA isolation. All the CC samples were prepared with RCD. In addition,

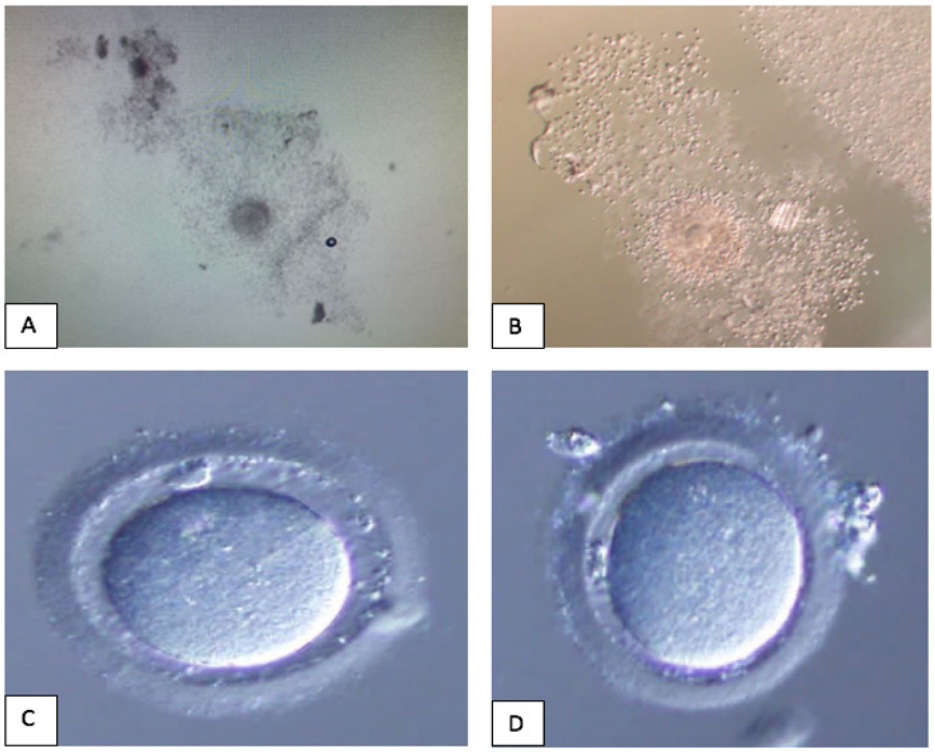


**FIGURE 1:** This showed oocytes denudation process. (A) Cumulus Oocytes Complex prepared for mechanical denudation without enzyme; (B) Cumulus Oocytes Complex enzymolysed in hyaluronidase; (C) "Naked" oocytes following enzymolysed in hyaluronidase – clean edge; (D) "Naked" oocytes following mechanical denudation – minimal cumulus cell at the edge

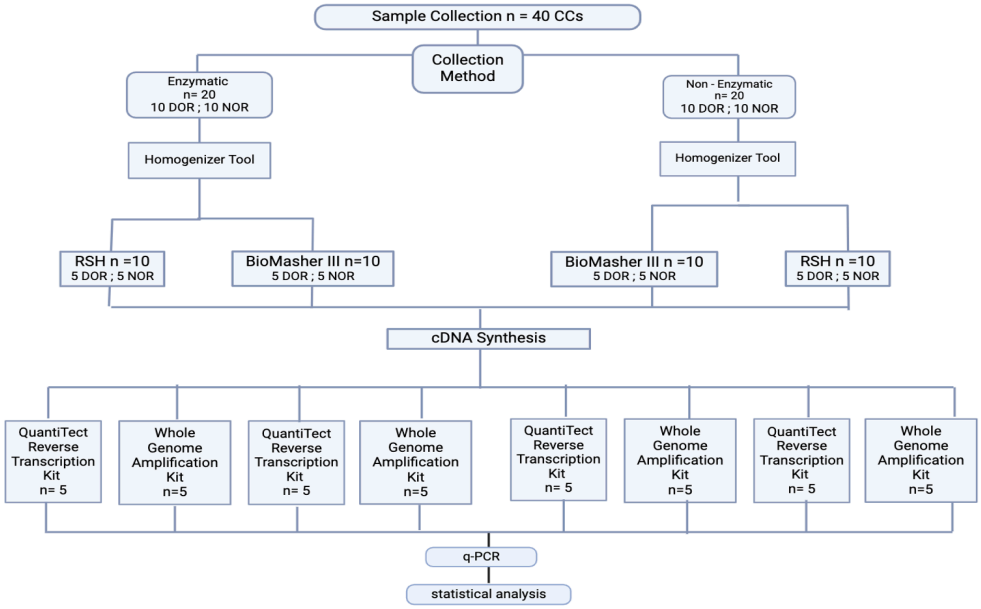


**FIGURE 2:** The study flow

the homogenisers were used as a mechanical disruption agent for CC preparation. This approach helps in forming an even mixture by forcing the CCs through the narrow space via multiple forces and turbulence to distribute the CC molecules evenly. In our study, we used two types of homogenisers, standard rotor-stator homogeniser (RSH) (Kaivo-oja et al. 2006) or disposable easy grind homogeniser, BioMasher III (Funakoshi®, Japan). In summary, the standard RSH consists of a fast-spinning inner rotor with a stationary outer sheath–stator. It homogenises CCs through mechanical tearing and shear fluid forces and mixing the CC samples in Eppendorf tubes (Maa & Hsu 1996). The BioMasher III (Funakoshi®, Japan) is formed by a filter tube with an abrasive surface inner wall and combined with a pestle with a textured surface. The pestle in the filtered tube layered on to centrifuge the tube is used for manual grinding (Iwai et al. 2022). Then, the CC-extracted samples were filtered and placed in the recovery tube. For these steps, every 10 CCs from NOR and 10 CCs from DOR women homogenised using a RSH, and the 10 other CCs from NOR and DOR women were manually ground via BioMasher III (Funakoshi®, Japan);

(a) RSH (Kaivo-oja et al. 2006) - Subsequently, the collected CCs were transferred into an empty 1.5 ml centrifuge tube using a sterile pipette tip. Then, 100 µl of RCD was added to the microcentrifuge. RSH was used to disrupt and homogenise the samples. Another 100 µl of RCD was added and centrifuged at 10,000 xg for 30 seconds. Thereafter, the "pass through" homogenised samples were collected for RNA purification.

(b) BioMasher III (Funakoshi®, Japan) - The collected CCs, together with their RNA stabiliser, were transferred into the BioMasher III (Funakoshi®, Japan) filter column. The CCs

were separated from RNA stabiliser through 2000 x g centrifugation for 10 seconds. The filtrate that contained RNA stabiliser was subsequently discarded. Then, 100 µl of RCD was added to the filter column. The CCs were grounded using the pestle. Another 100 µl of RCD was added, and the disrupted samples were homogenised through the filter by centrifugation at 10000 x g for 30 seconds. Subsequently, the "pass through" homogenised samples were collected for RNA purification.

(iii) RNA purification optimisation

The RNA from all samples was purified using an RNAeasy® micro kit (Qiagen, Venlo, Netherlands) with few modifications. In brief, post-homogenised CC samples were collected in a 1.5 ml tube and added with 200 µl of 70% ethanol. Subsequently, the solution was transferred into an RNAeasy column within a 2 ml tube and centrifuged at 8,000 xg for 15 seconds. The flowthrough was then discarded. The 350 ml buffer RW1 was added to the RNAeasy column and centrifuged at 8,000 xg for 15 seconds, and then the flowthrough was discarded. Thereafter, 80 µl of DNAse I dilution was added directly into the spin column RNAeasy. The solution was left to rest on a bench for 15 minutes at room temperature. Then, the 350 µl buffer RW1 was added to the RNAeasy column and centrifuged at 8,000 xg for 15 seconds, and the 2 ml collecting tube (Practice Committee of the American Society for Reproductive Medicine, Practice Committee of the American Society for Reproductive Medicine 2020) with flowthrough was discarded and replaced with a new 2 ml CT at the RNAeasy column. The 500 µl buffer RPE was added to the RNAeasy column and centrifuged at 8,000 xg for 2 minutes. Subsequently, the CT was discarded. The 500 µl of 80% ethanol was not added in the further modification. Subsequently, the new 2 ml CT was added to the RNAeasy column, with the lid open, centrifuged at full speed for 5 minutes to dry the membrane, and the flowthrough was discarded. Finally, the new 1.5 ml CT was placed at the RNAeasy column, and 14 µl RNAse free water was added at the direct center of the RNAeasy column and set for 5 minutes. The lid was closed gently, followed by centrifugation, which was performed at full speed for one minute to elute the final RNA.

**RNA concentration and purity measurement**

The concentration and purity of the purified RNAs were measured using a Nanodrop Thermo Fisher (Massachusetts, United States). The purity of the samples was evaluated by measuring the ratio of absorbance readings at 260 nm (specific for nucleic acids). Samples with A260/A280 ratios between 1.80 and 2.10 were considered to have no significant protein contamination, whereas the ratios of A260/A230 were used as a secondary measure of nucleic acid purity and was expected within the range of 2.0-2.2 (Olson & Morrow 2012).

**cDNA synthesis and qPCR**

Following RNA purification, the cDNA synthesis was conducted accordingly. We considered two types of cDNA synthesis kits, namely, the QuantiTect reverse transcription kit (Qiagen, Venlo, Netherlands)) and the whole genome amplification kit (Qiagen, Venlo, Netherlands)) given that the CC RNA purity was assumed to be smaller than extensive samples/tissues. The cDNA synthesis was performed according to the standard protocol of both kits. Then, the quality of each cDNA synthesised was validated by qPCR amplification. A housekeeping gene (HKG), the hypoxanthine-guanine phosphoribosyl transferase (HPRT),

was used to amplify the cDNA. The primer sequence used; Hs_HPRT1_1_SG. QuantiTect Primer Assay (Qiagen®, Venlo, Netherlands)) was used for the qPCR amplification according to the protocol. In brief, genomic DNA elimination was prepared on ice with total volume of 14 µl. Subsequently incubated for 2 minutes in 42˚C. The master mix solution also prepared according to protocol. In brief, the master mix final volume was 20 µl including 14 µl from template RNA. Subsequently was incubated for 15 minutes in 42˚C and then 3 minutes in 95˚C to inactivate the Quantiscript Reverse Transcriptase.

**(B) The Systematic Review**

The systematic review was done following the standard guideline protocol based on the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA)(Page et al. 2021) to identify the best RNA extraction method for CCs.

**Information source and search strategy**

A thorough search of the literature was conducted with EBSCOhost, PubMed, Science Direct and Scopus. Relevant research articles that were released up until 1st March 2024, were located. Keywords such as "cumulus cells" and "oocytes" were taken from the Medical Subject Heading (MeSH). MeSH terms from the Cochrane Library were used to generate synonyms for keywords. Through the evaluation of gathered review articles, further text terms were discovered. The following keywords were applied as part of the search strategy: ("cumulus cell" OR "granulosa cell," OR "oocytes" OR "cumulus oocyte complex") AND ("RNA extraction" OR "RNA purification" OR "RNA isolation").

**Inclusion and exclusion criteria**

Case-control and cross-sectional studies with abstracts investigating the methods used for RNA extraction from CCs, cumulus oocyte complex or oocytes. Only human and animal studies reporting on the RNA concentration and RNA purity of the extracted RNA were included to ensure the homogeneity of the data. In contrast, the editorials, case reports, conference proceedings, and narrative review articles were not included in this review since they had no primary data. Excluded studies included in silico studies, in vitro studies and any intervention studies. Studies that extracted DNA from cumulus or oocytes were excluded.

**Screening of articles for eligibility**

The articles that were collected from all databases underwent three stages of screening. In the first step, duplicates were removed and all articles with irrelevant titles were excluded. In the second phase, the abstracts of the remaining papers were reviewed and those that did not fit the inclusion criteria were eliminated. Lastly, a thorough review of the full text of remaining articles was conducted. In the stage, every article that did not adhere to the requirements for inclusion was eliminated. All authors involved in the phases of screening, choosing and extracting data. The PRISMA flow diagram that summarised the article assortment procedure and the grounds for item removal was displayed in Figure 3.

**Study selection, data extraction and risk of bias assessment**

Based on an initial search, five authors (A.M.F, A.M.A, M.H.I, M.J.N. and A.K.A.K.) screened all titles and abstracts of potential manuscripts. The selection criteria included

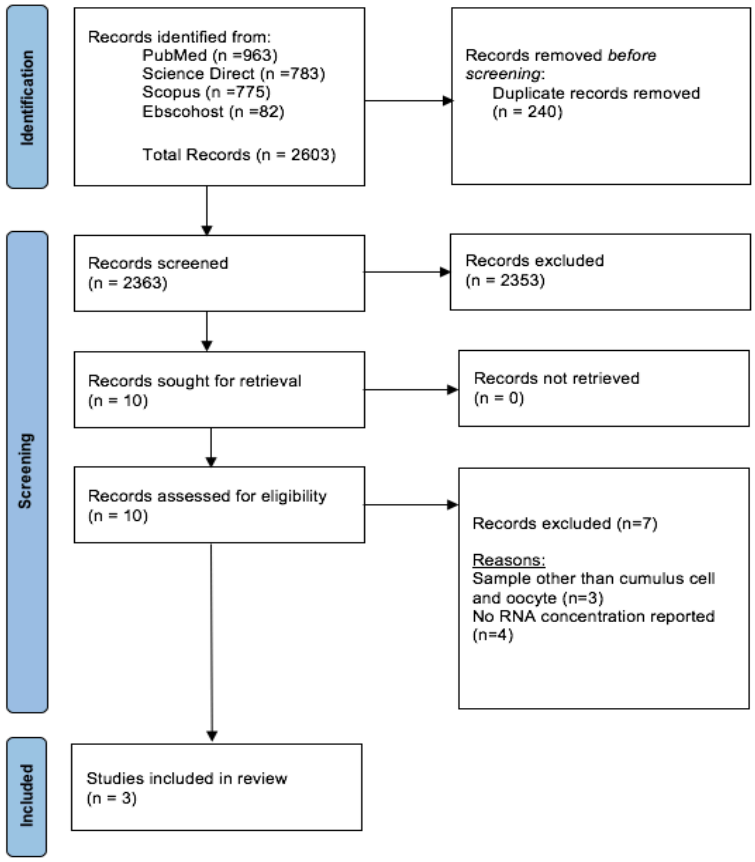


**FIGURE 3:** Prisma flow diagram for systematic review

manuscripts published in English from January 2012 to December 2022, evaluating the RNA extraction method for CCs and oocytes. Following the initial screening - title and abstract were screened, excluding manuscripts that used samples other than CCs and oocytes as experimental material, non-English language, case reports and review articles. The remaining potential manuscripts were then independently reviewed. The final selected manuscripts provided a detailed study design, focusing on RNA extraction methods with the final report of RNA concentration from each method. The conflicts in selection among authors were resolved through detailed discussions and opinions provided by the fifth, sixth and seventh authors (S.S.E, N.S. and A.A.Z). Additionally, the National Institutes of Health (N.I.H.) tool for observational studies was employed to assess the quality of the selected manuscripts. This evaluation was based on 14 variables, scoring 1 for 'yes,' 0 for 'no,' or 'non-applicable' for N.A. The manuscripts were then categorised as poor (0-5), fair (6-9) or good (10-14) based on their total scores (Table S1). Overall, the included studies in our review achieved a minimum fair to good score. Subsequently, the information gathered was as follows: (i) author name (year); (ii) article title; (iii) country; (iv) sample size; (v)

organism; (vi) RNA extraction methods; (vii) RNA concentration; and (viii) RNA purity. The summary of the collected data was listed in Table 1.

## RESULTS

### Clinical Study

We collected 40 samples of CCs from 40 follicles – 20 from each cohort (DOR and NOR) to extract RNA and optimise the HKG; the HPRT amplification. The samples were prepared and analysed according to the steps outlined in Figure 1. Our NOR women cohort had an average age of 36.40 ± 3.80 compared to 37.50 ± 4.51 in the DOR cohort. Besides that, their average AMH level was significantly higher: 17.22 ± 10.68 pmol/L compared to 4.32 ± 1.97 pmol/L (p<0.001) (Table 2). After the extraction, we measured RNA quantity and purity for all method combinations. Overall, the RNA concentration in non-enzymatic preparation was significantly 2.5-fold higher compared to enzymatic preparation: 9.25 (4.43-12.52) ng/µl vs 3.70 (2.75-4.43) ng/µl (p <0.001) (Table 2). In addition, using BioMasher III with RCD significantly yielded better RNA concentration than the RSH tool; 8.07 (3.93-12.50) ng/µl compared to 3.75 (2.70-4.80) ng/µl (p<0.001) (Table 3). Otherwise, the RNA purity is within an acceptable range (1.8-2.20) and comparable regardless of the preparation method or homogeniser type (Table 2; Figure 4). On the other hand, our study revealed that the RNA concentration yield was comparable

**TABLE 1:** The characteristics summary of the included studies

| Author, Year | Title | Country | Sample size (n) | Organism | Method for RNA extraction | RNA concentration (ng µl-1) | RNA Purity |
|---|---|---|---|---|---|---|---|
| Maisarah et al. (2020) | The challenge of getting a high quality of RNA from oocyte for gene expression study | Malaysia | COC (19) Oocyte (400) | Mouse | TRIzol | COC = 151.0 Oocyte = 126.7 | COC = 1.7 Oocyte = 1.68 |
| | | | | | RNeasy Mini Kit | COC = 3.8 Oocyte = 1.9 | COC = 1.68 Oocyte = 10.5 |
| Wiweko et al. (2017) | The quality of RNA isolation from frozen granulosa cells | Indonesia | Oocyte (28) | Human | QIAamp RNA Blood Mini Kit | 250 | 1.85 |
| Pavani et al. (2015) | Optimisation of total RNA extraction from bovine oocytes and embryos for gene expression studies and effects of cryoprotectants on total RNA extraction | Portugal | Oocyte (795) | Bovine | TRIzol | 152.8 | 1.5 |
| | | | | | Guanidinium thiocyanate | 47 | 1.18 |
| | | | | | Commercial kit | 31.2 | 2.06 |

COC: cumulus-oocytes-complex; RNA: ribonucleic acid

**TABLE 2 :** Demographic profile for both group

| Recruitment Profile | Normal Ovarian Reserved (NOR) Mean (±SD) | Diminished Ovarian Reserved (DOR) Mean (±SD) | p - value |
|---|---|---|---|
| Age | 36.40 (±3.80) years old | 37.50 (±4.51) years old | p = 0.410* |
| AMH Level | 17.22 (±10.68) pmol/L | 4.32 (±1.97) pmol/L | p < 0.001* |
| *independent t-test; signficant value p<0.05 | | | |

between NOR and DOR women; 4.35 (3.42-14.55) ng/µl versus 4.45 (3.35-16.24) ng/µl (p = 0.946) (Table 4). Our final cDNA and qPCR analysis showed a significant presence of HPRT gene amplification following the QuantiTect reverse transcriptase kit (Qiagen) for cDNA synthesis compared to no expression seen using Whole Genome AK kits (p = 0.005) (Table 5). This outcome was likely due to the RNA carrier use for CCs handling was incompatible with the whole genome amplification kit. The HPRT gene amplification graph and melt peak curve were shown in Figure 5. Unfortunately, the samples using whole genome amplification kits for cDNA synthesis with RCD and RSH tool with QuantiTect Reverse Transcriptase Kit (Qiagen) for cDNA showed no amplification for their qPCR (Table 5). The outcome likely due to sample degradation following RSH tool utilisation. Otherwise the comparison of RNA concentration for sample handling method (Hyase vs. non Hyase), type of homogeniser (RSH vs BioMasher III) and RNA purity was shown in Figure 5.

**TABLE 3:** Sample preparation and homogenizer method with RNA extraction outcome

| | RNA Concentration Median (IQR) | p - value | RNA Purification (A260/A280) Median (IQR) | p - value |
|---|---|---|---|---|
| Homogeniser Method | | | | |
| BioMasher III | 8.07 (3.39-12.50) ng/µl | p < 0.001# | 1.94 (1.88-2.04) | p = 0.596# |
| Rotor Stator Homogeniser (RSH) | 3.75 (2.70-4.80) ng/µl | | 1.95 (1.88-1.98) | |
| Sample Preparation Method | | | | |
| Non enzymatic ( Non-Hyase) | 9.25 (4.43-12.52) ng/µl | p < 0.001# | 1.97 (1.88-2.04) | p = 0.064# |
| Enzymatic (Hyase) | 3.70 (2.75-4.43) ng/µl | | 1.89 (1.88-1.98) | |
| #mann-whitney test | | | | |

**TABLE 4:** Comparison of RNA extraction outcome among DOR and NOR cohort

| RNA Concentration | Normal Ovarian Reserved (NOR) | Diminished Ovarian Reserved (DOR) | p - value |
|---|---|---|---|
| Median (IQR) | 4.35 (3.42-14.55) ng/µl | 4.45 (3.35-16.24) ng/µl | P=0.946# |
| #mann-whitney test; signficant value p<0.05 | | | |

**TABLE 5:** The HPRT gene amplification outcome

| cDNA synthesis kits | Amplification of HRPT Gene | | p - value |
|---|---|---|---|
| | Yes (n, %) | No (n, %) | |
| QuantiTect RT | 6 (42.8) | 14 (57.2) | p = 0.005^ |
| Whole Genome AK | 0 (0) | 20 (100) | |
| ^Fisher's Exact Test; signficant value p<0.05 | | | |

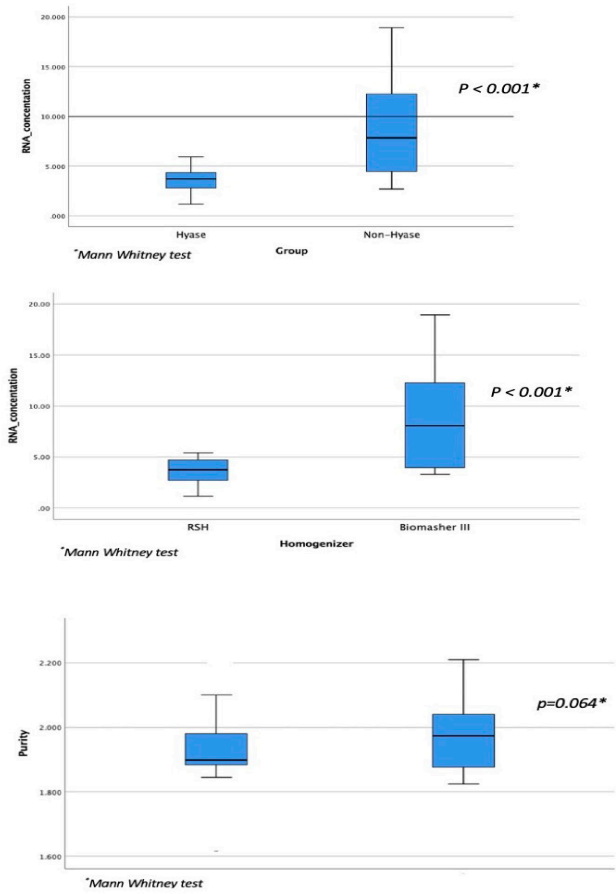


**FIGURE 4:** Sample preparation method, homogeniser type and RNA concentration outcome

## Systematic Review

To date, no available data had been reported that exclusively focuses on the RNA ex-traction method for CCs alone. Most RNA extraction methods have been briefly described in in-vitro studies, intervention, and silico studies, which were excluded from this review. Currently, most evidence for the technique combined CCs with oocytes or cumulus-oocytes complex (COCs). Hence, in our search strategy, initially, no paper yielded only the RNA method for CCs. As a result, we modified the search strategy to include CCs, oocytes and COCs.

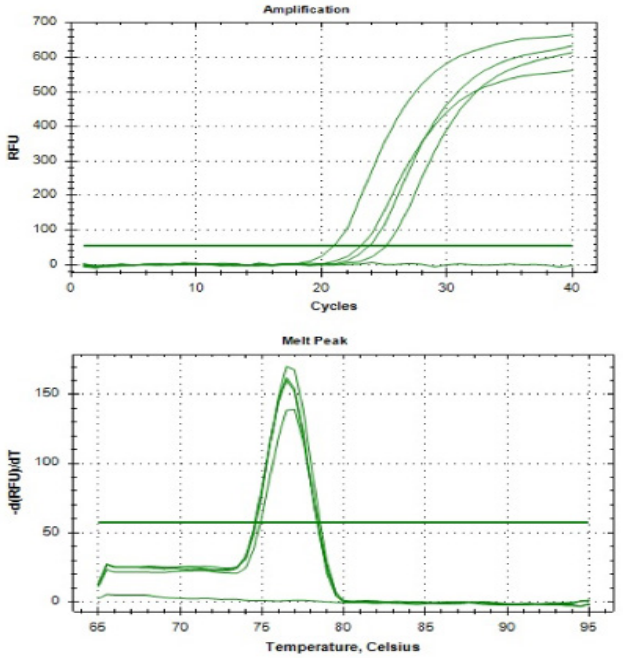


**FIGURE 5:** The HPRT gene amplification with melt peak curve

**Search Sequence and Quality Assessment**

A total of 2603 studies were retrieved during the primary search (Figure 3). After removing 240 duplicates, the remaining 2363 articles were thoroughly screened based on our inclusion criteria. Amongst them, 2,353 articles were excluded, leaving 10 for full-text evaluation. After a detailed evaluation, seven articles were discarded, 3 using samples other than CCs and oocytes, and four papers – no RNA final concentration was reported. Subsequently, three studies that focused on RNA extraction methods with available results of final RNA concentrations were selected for this review. All selected articles were evaluated using the National Institutes of Health (NIH) tool for observational studies to ensure quality and minimise bias. Notably, all four articles obtained a minimum fair to good score, indicating a low risk of bias (Table S1).

**Studies Characteristics**

A total of 1242 samples from three studies were included in this systematic review. All studies collected oocyte for RNA extraction optimisation study. Maisarah et al. (2020) included 19 samples of COC for RNA extraction (Maisarah et al. 2020). Wiweko et al. (2017) used human samples while Maisarah et al. (2020) and Pavani et al. (2015) were on murine model. The reported RNA extraction methods included for comparison TRIzol, Guanidium thiocyanate and few types of column based commercial kits. All the relevant information from the included studies had been summarised in Table 1.

**Main Outcome**

Most studies use pool of oocytes and COCs, ranging from 70 to 200 oocytes per pool, to yield the RNA and concluded that the RNA purity was good (1.0 to 2.0) using any preferred methods (Maisarah et al. 2020; Pavani et al. 2015; Wiweko et al. 2017). The two TRIzol protocol studies reported a higher RNA concentration with at least 150 ng/µl than commercial kits (Maisarah et al. 2020; Pavani et al. 2015). Similarly, these two studies compared Modified TRIzol Protocol (MTP) with commercial kits and revealed that MTP was considered the best option for RNA extraction in oocytes (Maisarah et al. 2020; Pavani et al. 2015). In contrast, one study concluded that the yield of RNA using commercial kits was comparable in fresh versus frozen oocytes (Wiweko et al. 2017). All the studies were summarised in Table 5. Unfortunately, none of these reported for RNA extraction for CCs alone.

## DISCUSSION

The current fertility field, the emergence of DOR women, is considered a challenging cohort to manage. Therefore, most of this cohort became interested in molecular studies to delineate a proper strategy for clinical implementation. Thus, we included DOR women in our study to propose an optimal strategy for harvesting the CCs and extracting the RNA for molecular study compared to NOR women. The minimal CCs obtained mainly in DOR women often lead to difficulty in harvesting RNA for transcriptome studies. Still, our study found that the RNA extraction was comparable between both cohorts with the current sample preparation method, thus providing good evidence for future references. Besides that, the high RNA concentration and purification are essential in molecular research, specifically in transcriptomic study. However, achieving excellent RNA concentration and purity could be challenging, especially for tiny samples, e.g. CCs (Iwai et al. 2022; Maisarah et al. 2020). Thus, evaluating an appropriate technique as an optimal strategy is crucial to improve the yield of RNA in these sample types. The comparison of sample preparation methods indicates that the combination of mechanical collection technique, BioMasher III (Funakoshi®, Japan), and RNA carrier improved the RNA concentration and purity obtained from the CCs. The current practice for oocyte denudation in most in-vitro fertilisation (IVF) centers worldwide is via the enzymatic technique, namely, hyaluronidase (Ishizuka et al. 2014; Moura et al. 2017). HYASE-10X™ (Vitrolife® Sweden) is often used to remove CCs from oocytes prior to intracytoplasmic sperm injection (ICSI) in our center. The maturation process in oocytes consists of the accumulation of HA in CCs as a protection mechanism. It is a high-molecular-weight glycosaminoglycan form with an alternate bond of D-glucuronic and N-acetylglucosamine (Nagyova 2018). The HA mainly accumulates within the CC oophorus to support the oocyte developing. These strong attachments of CCs and oocytes form a COC (Attanasio et al. 2020) facilitating the supply of nutrients and growth factors for further enhancement and maturation (Nagyova 2018; Salustri et al. 1995).

Therefore, separating the CCs from COC for ICSI preparation is technically challenging. To date, the denudation process is conducted in two phases (Esbert et al. 2013; Tjahyadi et al. 2022). The first step was that the COCs would be enzymolysed in hyaluronidase, such as HYASE-10X™ (Vitrolife® Sweden), to weaken the bond of HA within the CCs and oocytes. The process is performed rapidly because potential decremental effects can

occur if the process takes more than 40 seconds due to enzyme toxicity. Subsequently, the second phase is followed by mechanical denudation (Maldonado Rosas et al. 2022). It was conducted using microscope eyepieces and repetitive manipulation of COC in various media without the use of enzymes. This process involved the use of either mouth-controlled or hand-controlled pipettes to expose these oocytes. This process is technically challenging during manipulation because small oocytes within the separate CCs are difficult to identify, resulting in a laborious and demanding task. It is also often reported to vary reproducibility and inconsistency among the operators, and the yield rate and denudation efficiency vary significantly (Zhang et al. 2020). Thus, in collecting CCs for transcriptomic study, we found that the use of hyaluronidase impairs the end results of RNA yielding compared with mechanical denudation. The effect of these enzymes dissociated the CC composition by breaking the HA bonding in between CCs themselves, leading to over-destruction, thereby decreasing RNA concentration in our study. However, collecting the CCs using mechanical equipment is also laborious. Therefore, we opted to collect the CCs prior to hyaluronidase use via modification of the standard steps of denudation. Our study used the thinnest needle (22G) to enhance mechanical force in separating the CCs from COC to obtain the optimum size of CCs prior to performing the two standard steps of denudation. Hence, the CCs were not exposed to hyaluronidase. Nevertheless, the comparison with and without hyaluronidase significantly concurs with the potential outcome for future recommendations. Therefore, the non-enzymatic group offers higher RNA concentration yield. The RSH (Kaivo-oja et al. 2006) and BioMasher III (Funakoshi®, Japan) were compared to identify the best method for CC disruption and homogenisation. The comparative results show that the BioMasher III (Funakoshi®, Japan) significantly improved RNA concentration and purity of the CCs compared with the RSH. Incomplete disruption and homogenisation can stem from several factors and display cascading effects on downstream analyses and experimental outcomes, including gene expression profiling, given that specific RNA transcripts may be overrepresented or underrepresented due to incomplete disruption (Nouvel et al. 2021). Nevertheless, the partial disruption and homogenisation may result from cell and tissue types exhibiting varying degrees of resistance to these processes, influenced by differences in cellular structure and composition (Goldberg 2021). Stiffer tissues or samples with high connective tissue content, such as cartilage or fibrous tissues, are difficult to homogenise effectively (Choudhary & Choudhary 2018; Zhao et al. 2023). Thus, the selection of homogenisation methods, such as mechanical disruption, enzymatic digestion or bead-based methods, can impact the effectiveness of cell or tissue disruption. Complete homogenisation may occur if the selected method is optimised for the specific sample type (Ali et al. 2017).

The use of a homogeniser might raise the temperature of the RNA samples due to the high-frequency vibration, which could eventually lead to sample degradation. Inefficient homogenisation may cause the release of endogenous ribonucleases, leading to RNA degradation (Amiri Samani & Naji 2019). Subsequently, these enzymatic activities reduce RNA integrity and compromise downstream applications, such as gene expression analysis (Maisarah et al. 2020; Pagani et al. 2023). Thus, identifying the best homogenising method for different cell types is crucial. Apart from producing a complete homogenised sample, the use of BioMasher

III (Funakoshi®, Japan) has been reported to be efficient in processing a high number of samples by shortening the RNA extraction time through one-pot procedure, providing a DNase- and RNase-free condition, preventing cross-contamination and offering safety (Yamamoto et al. 2012). We found that the presence of filter column in BioMasher III improves the purity of the RNA by effectively removing RNA stabiliser and reducing CC loss during cell transfer for the disruption and homogenisation process in the RLT buffer. Therefore, despite obtaining RNA yield from the RSH group, potential degradation prevents the amplification of HKG even when using the QuantiTect Reverse Transcription Kit compared with the BioMasher III (Funakoshi®, Japan) with QuantiTect Reverse Transcription Kit for cDNA synthesis. Generally, various methods for RNA extraction from oocytes have been proposed for gene expression profiling studies in this context (Jones et al. 2008; Wells & Patrizio 2008). Most experiments employed the TRIzol method with some modifications and microRNA extraction kits as efficient methods to extract high-quality total RNA from oocytes (Duy et al. 2015; Trakunram et al. 2019). However, without the column-based system, the TRIzol method demands experience and skills in separating the RNA. Furthermore, the column-based method is more efficient for many samples. Several studies report on the low RNA concentration and quality of the RNeasy Mini Kit (Al-Adsani et al. 2022; Beltrame et al. 2015; Tavares et al. 2011). The RNeasy micro kit was used for the RNA extraction in this study. The RNA carrier effectively improved the RNA yield using the RNeasy micro kit protocol. Several studies have shown that adding RNA carriers increases yields and improves PCR amplification performance (Ramon-Nunez et al. 2017; Wright et al. 2020). When working with small sample sizes, limited cell populations, and precious clinical samples, such as CCs, using RNA carriers can reduce RNA loss during extraction. This approach is especially important for obtaining reliable and accurate downstream results. RNA carrier is also commonly used in viral RNA extraction kits to facilitate and aggregate viral RNA (Ogunbayo et al. 2023). However, we found that the RNA carrier is incompatible with the whole genome amplification kit given that no PCR amplification was detected in the downstream analysis. Nevertheless, specific amplification was observed for samples that utilised QuantiTect reverse transcription kit for the cDNA synthesis. Concerning the current RNA extraction techniques for micro-size cells, our review revealed that there is no doubt that the Modified TRIzol Protocol (MTP) is considered the best option for RNA yield from oocytes (Maisarah et al. 2020; Pavani et al. 2015). In contrast, commercial kits are considered acceptable for COCs RNA extraction (Maisarah et al. 2020). Our study was in concert with current findings as comparing two commercial kits revealed comparable overall RNA purity despite different handling method. Therefore, the sample preparation is the ultimate strategy to improve the overall RNA extraction outcome. Our study revealed that non-enzymatic handling with smaller manual grinding method is preferred. Thus, our clinical study adds value to the current RNA extraction particularly in sample handling strategy for human CCs for gene expression study, mainly via commercial kits.

Thus, our study delineated an excellent flow of harvesting the CCs to optimise RNA extraction in both NOR and DOR women. Based on various studies, we managed to evaluate the optimum strategy for producing a suitable protocol for RNA extraction in CCs for future reference. Utilising only mechanical denudation for CC collection,

BioMasher III as homogeniser, RNA carrier during RNA extraction and QuantiTect reverse transcription kit for the cDNA synthesis results in sound amplification in gene of interest (GOI). Meanwhile, our systematic review also consolidated the current strategy of RNA extraction method for micro cell RNA – mainly COCs and oocytes. However, our study only confined to RNA extraction and simple q-PCR of HPRT gene alone. Nevertheless, pooling several samples for high throughput analysis, such as NGS, can increase the required RNA concentration. Otherwise, a small number of papers could be added to our review due to our inclusion and exclusion criteria.

## CONCLUSION

Overall, our study revealed that sample handling of CCs should be done without standard enzymatic denudation. The manual pressure control homogeniser, RNeasy micro kit, and RNA carriers provide a better RNA yield regardless in NOR or DOR women. Our findings suggest a better strategy for optimising the RNA extraction in CCs for transcriptomic studies. Otherwise, our review also concluded that the use of MTP is the best choice for RNA extraction in oocyte sample.

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**TABLE S1:** NIH Quality Assessment Tool

| No | NIH Quality Assessment Tool | Maisarah et al. (2020) | Wiweko et al. (2017) | Pavani et al. (2015) |
|---|---|---|---|---|
| 1 | Was the research question or objective in this paper clearly stated? | Y | Y | Y |
| 2 | Was the study population clearly specified and defined? | Y | Y | Y |
| 3 | Was the participation rate of eligible persons at least 50%? | Y | Y | Y |
| 4 | Were all the subjects selected or recruited from the same or similar populations (including the same time period)? Were inclusion and exclusion criteria for being in the study prespecified and applied uniformly to all participants? | Y | Y | N |
| 5 | Was a sample size justification, power description, or variance and effect estimates provided? | N/A | N/A | N/A |
| 6 | For the analyses in this paper, were the exposure(s) of interest measured prior to the outcome(s) being measured? | Y | Y | Y |
| 7 | Was the timeframe sufficient so that one could reasonably expect to see an association between exposure and outcome if it existed? | Y | Y | Y |
| 8 | For exposures that can vary in amount or level, did the study examine different levels of the exposure as related to the outcome (e.g., categories of exposure, or exposure measured as continuous variable)? | Y | Y | Y |
| 9 | Were the exposure measures (independent variables) clearly defined, valid, reliable, and implemented consistently across all study participants? | Y | Y | Y |
| 10 | Was the exposure(s) assessed more than once over time? | N/A | N/A | N/A |
| 11 | Were the outcome measures (dependent variables) clearly defined, valid, reliable, and implemented consistently across all study participants? | Y | Y | Y |
| 12 | Were the outcome assessors blinded to the exposure status of participants? | N/A | N/A | N/A |
| 13 | Was loss to follow-up after baseline 20% or less? | Y | Y | Y |
| 14 | Were key potential confounding variables measured and adjusted statistically for their impact on the relationship between exposure(s) and outcome(s)? | Y | Y | Y |
| TOTAL (/14) (Y = Yes, N=No, NA=Non applicable) | | 11 | 11 | 10 |
| Quality Assessment Score Poor (0-5), fair (6-9), or good (10-14) | | Good | Good | Good |