Review of AREDS Report 38 publication and supporting Information

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Executive summary:. The data presented in AREDS 38 does not show a beneficial effect for any dietary supplement in the prevention or delay of AMD making the identification of subgroup effects difficult. Data from the AREDS Report 8 which is the only publication to show this "main effect" is presented as a departure point for a subgroup analysis, which is inappropriate. Reports 8 and 38 use very different statistical techniques to analyze progression events making them not directly comparable. AREDS Report 38 also has many internal data inconsistencies making it impossible to know what data was actually analyzed by the authors. The primary data can be used to test the hypothesis that there is an interaction between genetics and treatment. When the primary data is lifted from the manuscript and basic modeling statistics are performed, a strong interaction between CFH, ARMS2 and response to zinc use is observed.

1. Introduction: AREDS Report 8.

The 2001 Age Related Eye Diseases Study (AREDS) reported on the effect of 3 treatments (zinc, antioxidants and zinc+antioxidants) on the risk of progression from dry AMD to advanced AMD[1]. The study concluded that the combination of zinc and antioxidants (the "AREDS Formulation") was most efficacious in delaying or preventing the onset of advanced disease in the subset of patients with extensive drusen in at least one eye (AREDS category 3 or 4). These conclusions were based on the unplanned analysis of a subset of 2556 from the total study population of 4752 individuals.

Within this subset, 775 progressed (Table 4[1]). The report does not provide numbers of participants in each arm for Category 3 and 4 only, though data in Table 3[1] provides data for this group combined with Category 2 participants, allowing the following estimate:

Treatment	Count
Placebo	626
Antioxidants	674
Zinc	628
AREDS	635
Total (cat 3+4)	**2563**

This represents discrepancy from 2556 category 3/4 participants reported in Table 4 [1] (difference of 0.27%) but it is close to 2562 reported participants in these categories described in a table supplement to a latter publication from these authors (supplementary table 5)[2]. The data presented in Table 4 of the 2001 AREDS report for patients with category 3 and 4 disease at the time of enrollment will be considered further here[1]. The authors conclude that Zinc alone and the AREDS Formulation were both superior to placebo in lowering the risk of progression to advanced AMD ($p = 0.01$) with reduction of risk of 30% and 34%, respectively. Antioxidants were not superior to placebo after correcting the statistical significance threshold for multiple hypothesis testing ($p=0.03$), though the absolute reduction in progression rate was 24%. These results were analyzed using repeated-measures logistic regression. Repeated timed assessments of patients identified about 8% who spontaneously "recovered" from advanced disease, allowing them to be returned to the "at risk" pool of patients. For this reason survival modeling techniques, such as CoxPH regression were considered unsuitable. It is unclear from the reported data what the effect of dietary supplements would be if "reversion" from advanced disease was considered to be variation in retinal image interpretation rather than deviation from the established understanding of the pathophysiology of this disease.

2. AREDS Report 38.

The AREDS Report 38 study[3] analyzed a subset of the entire AREDS study set described above[1]. Out of 2562 AREDS participants with Category 3/4 AMD (supplementary Table 5[3]), SNP analysis was performed on 1237 of these selected for DNA availability. The authors don't explain how and additional 176 DNA samples were available for participants who had their secondary markers measured (CFH SNPs rs412852 and rs3766405, and ARMS2 deletion) to reach the reported sample size of 1413 for that analysis. Here we concern ourselves only with the results on primary CFH/AMRS genetic markers.

The main results of the AREDS 38 report are shown in Figure 1B, with results for secondary markers shown in Fig 1C. The analysis was performed using Cox PH regression model. Figure 1B shows the hazard ratios from repeated-measures analysis lifted from AREDS report 8[1](HR; are measures of relative risks and are similar, but not the same as ORs). In AREDS Report 38, data is presented for 3 treatment groups (zinc alone, antioxidants alone and the AREDS Formulation) stratified by 9 genetic risk groups (27 analyses). Only one combination of genetic risk group and treatment was superior to placebo with p-value = 0.001 (one risk allele for each of CFH and ARMS2 treated with the AREDS Formulation). The paper reports an overall test of interaction between genetic factors and treatment effects with p-value=0.06. It is worth noting that this omnibus test does not need to be corrected for multiple testing. Hence, if one takes a standard 0.05 confidence threshold, the test, while formally failing to detect a significant interaction between the whole set of 9 tested genotypes and 3 treatment arms does suggest the modification of supplement therapeutic effect by genetics.

There are 2 major problems with the AREDS 38 study as reported: A main effects analysis is not reported. Figure 1A reports main effects from the AREDS 8 study[1] not from the data in the AREDS 38 study. This is highly misleading, since that study used a much larger sample size (over twice as large) and a different modeling approach (repeated-measures logistic regression vs Cox PH regression used in AREDS Report 38). Without the demonstration of main effects one cannot properly interpret the absence of interaction. It would be interesting to see the comments of the peer reviewers on this point.

The analysis presented in AREDS 38 should not have been attempted before examining the cell-sizes. A simple examination of Fig 1B (or SupTable 6B) shows that many subgroups used in the analysis has extremely low cell sizes. For example, for 3 genotype groups "00/01/02" (nomenclature refers to the number of AMD risk alleles at the CFH and ARMS2 loci respectively), no count of events is larger than 5. Insufficient cell counts are observed for most other genotype groups except those with CFH=2. It is customary in cases such as this to combine groups in some biologically or clinically relevant way before any formal analysis and hypothesis testing is conducted (as has been described for a similar analysis[4]). Doing so will prevent interpretation of invalid confidence intervals and p-values but also limit the number of hypothesis tests and resulting multiple testing correction. If done in a sensible way it may lead to greater power for the analysis as a whole.

3. Repeat analysis of the AREDS 38 data from published data.

Due to the unavailability of primary survival data for the patients in AREDS 38, we employed a series of simple 2x2 table analysis and logistic regressions to revise this study's methodology. When follow-up periods have little variability this technique will approximate the results from Cox regression models. Figure 3 and 5 from AREDS Report 8[1] shows over 80% of participants were followed for at least 6 years and relatively small number of events occurred in year 7. Simple event count-based analysis should closely approximate results from time-to-event (e.g., Cox regression) analysis.

3.1 Data problems for event counts

AREDS Report 38, Fig 1B and SupTable 6b purport to show the number of events and totals in each combination of treatment and combined genotype group (27 total subgroups). While the numbers showing group sizes (third column in SupTable 6b and Total" columns in Fig 1B) generally agree, the counts of events (and, hence, non-events) show large discrepancies between these two, apparently equivalent, records of count data. For

example in Figure 1B group "00" subjects (no risk alleles at either CFH or ARMS2) who were treated with antioxidants 3 events for the corresponding placebo group is reported while SupTable 6b reports 2 events for this group. For genetic group, "01" (no CFH risk alleles and 1 ARMS2 risk allele) Figure 1B reports 7 events for placebo but 5 events are reported in SupTable 1B. In this genetic group 9 vs 5 events for antioxidant treated patients are reported. Some discrepancies are quite large. For group "21" (2 CHF risk alleles and 1 ARMS2 risk allele), Zinc, and AREDS Formulation treatment groups Figure 1 B reports 36, and 30 progression events respectively, while SupTable 6b reports 26, and 20 events. There are internal inconsistencies in Figure 1B as well. For the "21" genetic group the number of events in placebo-treated patients is quite different: '29' is reported in for the comparison to antioxidant and zinc-treated patients and '20' is reported for the comparison to AREDS Formulation-treated patients (these should obviously all be the same). Figure 1B and SuppTable 6b show totally discrepant data for genetic group "22" (2 risk alleles for both CFH and ARMS2) outcomes:
for patients treated with antioxidants, zinc and the AREDS Formulation, Fig 1b shows event counts: 19, 18 and 14, while the SuppTable 6b reports 15,16,12 events. Placebo counts are also different: 14 in Figure 1b and 10 in SuppTable 6b.

Given the discrepancy between the primary data tables/figures presented in the paper one source was selected (Figure 1b) by us to perform a repeat analysis of interaction between genetic makeup and treatment groups..

2.2 Marginal treatment analysis

Using **combined** counts from SupTable 5 (no DNA and DNA) representing all 2562 participants with AREDS category 3 and 4 disease one can attempt to replicate the results of Report 8, Table 4[1]. Using Odds Ratio estimates and ChiSquare tests for a series of 2x2 tables, the following
results are obtained:

Treatment	Odds Ratio	99% Confidence Interval	p-value
Antioxidants	0.797	(0.582-1.090)	0.058
Zinc	0.827	(0.602-1.135)	0.118
AREDS F	0.797	(0.579-1.095)	0.062

These should be compared with the results reported in (right-side of) Table 4 in the AREDS 8 publication[1]. AREDS Report 8 used repeated measures logistic regression which we approximate using a simple 2x2 table analysis of the AREDS Report 38 data. This is reasonable because of low transient event counts (8%, as reported in AREDS 8) and follow-up periods with low variability. We find significant discrepancies. While the OR for progression observed in antioxidant-treated patients is approximately the same in AREDS Report 8 and AREDS Report 38 (0.797 vs 0.76 using unadjusted estimates), the other treatments show markedly different ORs. This is a cause for serious concern about data integrity. None of the treatments in AREDS Report 38 show significance at the 0.01 level (threshold used in AREDS Report 8). This is in contrast to AREDS Report 8 which reported progression data from Zinc and antioxidant+zinc-treated patients in comparison to placebo-treated individuals to be different at p-values below 0.01 and that for antioxidant-treated individuals to be different from placebo-treated patients with a p-value of 0.03. In summary, except for antioxidant treated patients, we see large discrepancies between the marginal (ie: blinded to genetic data) results inferred from the numbers in AREDS Report 38 and those reported in AREDS Report 8.

A similar reanalysis was performed using data from **participants with available DNA samples** (ie, group used for most of the results in AREDS Report 38). It also shows largely insignificant treatment effects:

Treatment	Odds Ratio	99% Confidence Interval	p-value
Antioxidants	0.911	(0.574-1.446)	0.603
Zinc	0.882	(0.553-1.408)	0.483
AREDS Formulation	0.722	(0.449-1.158)	0.075

This shows that this subset of the AREDS data is markedly different than the full AREDS sample used in AREDS Report 8[1]. Combined with our failure to replicate the main results of AREDS Report 8 using counts from (non-DNA and DNA) datasets demonstrates a flaw in overall data integrity.

2.3 Treatment-by-genotype analysis

Given that the data used in AREDS Report 38 shows no overall treatment analysis any attempt to deduce interaction of genetic factors with treatment must be understood as a purely exploratory analysis. We cannot conclude that genetic make-up does not modify the treatment effect, since we do not observe a significant treatment effect. In this case, one would only expect to observe significant treatment differences among genetic subgroups if genetic modification was very large.

Data reanalysis: We have generated genetic subgroups in a sensible way to obtain larger cell sizes more suitable for analysis. First we attempt to replicate the AREDS Report 38 results shown in Fig1B using 2x2 table analysis or using an equivalently logistic regression since timed survival data is not available. For genetic group="00" (no risk alleles within CFH or ARMS2 genes) using data from Fig1B for determining risk for progression to advanced AMD we obtain OR= 0.74, 1.22, 0.33 for antioxidant, zinc, AREDS Formulation-treated individual compared to placebo-treated patients, respectively. This compares respectively to (hazard ratios) HRs=0.69, 1.34, 0.39, reported in the AREDS 38 report which used a Cox regression analysis. Since the event counts are extremely low ORs are accurate approximations of HRs. There also appears to be satisfactory agreement for larger genotype groups with more events, like "20" (2 CFH risk alleles and no ARMS2 risk alleles). We derive the OR (progression) within this genotype group to be 1.16, 2.41, 2.12 for antioxidant, zinc and AREDS formulation respectively compared to placebo-treated patients. This compares to the AREDS 38 published HR within this genotype group of 1.08,1.96,1.78 respectively. In this genotype group with larger event counts the OR and HR diverge slightly, though we do get the same order of effect among the treatment groups. We conclude that the Odds Ratios we derive adequately approximate HRs reported in AREDS 38 for groups with small and larger numbers of progression events. .

We studied interaction between genetic and treatment groups on progression events using logistic regression and data from Fig1B (35 degrees of freedom in total). The overall effect for interaction between genetic and treatment is significant with a p- value=0.0025, even if the

general p-value for treatment group is not significant (0.300). This should be compared with the interaction p-value=0.06 reported from the Cox regression model in AREDS Report 38. This provides strong support for genetic modification of treatment response. This analysis did not involve grouping of genetic risk groups.

Dichotomizing treatment arms into those containing Zinc (Zinc, and AREDS) and those without (Placebo and antioxidants alone) we examined the interaction between Zinc treatment and genetics. Using either allelic dosage (number of risk alleles – 0,1, or 2), or dominant/recessive coding for ARMS and CFH, respectively, we detect significant modification of Zinc treatment effect by genetics. We report results from allelic dosage model, noting that results from dominant/recessive model were even stronger. Across all genetic groups zinc does not reduce the risk of progression to advanced AMD (p-values 0.34, and 0.89 in allelic dosage and dominant/recessive models, respectively). Both CFH and ARMS2 were independently associated with AMD progression risk with OR of 1.33 (p=0.021) and 2.77 (p<0.00001)) respectively. Highly significant interactions between CFH and zinc or with ARMS2 and zinc treatment were observed in this model but with opposite effects. The coincidence of a risk allele at ARMS2 and zinc treatment produces an OR of 0.54 (p=0.0007) meaning that zinc attenuates the risk associated with ARMS2 risk alleles. In contrast, the coincidence of a risk allele at CFH with zinc has an OR of 1.56 (p=0.0152), meaning that the risk associated with CFH risk alleles is augmented by zinc treatment.

Similar models for antioxidant vs no antioxidant treatment dichotomy did not show significant interaction (or main treatment) effects .
Given that CFH and ARMS2 interact with zinc treatment individually and oppositely, one logical combination of genetic subgroups is worth highlighting. A subgroup analysis of genetic risk group “20" (2 high CFH risk and no ARMS2 risk alleles), gives the same pattern as reported by Awh *et al* [4]: Zinc and AREDS formulation have significantly deleterious effects.
Following are results using data from the Figure 1B from AREDS Report 38.

Treatment	Odds Ratio	Confidence Interval (99%)	p-value
Antioxidants	1.16	(0.352-3.81)	0.751
Zinc	2.43	(0.781-7.566)	0.041

AREDS Formulation	2.13	(0.703-6.506)	0.0752

In general the summation of these analysis point to the deleterious effect of CFH with zinc-containing treatments and enhanced beneficial effect of Zinc for people with at least one ARMS2 risk allele. All these results broadly support the conclusions of Awh et al. [4] though the significance levels are lower.

4. Discussion

The AREDS Report 38 by Chew *et al* was written as a direct response to Awh et al [5]. Carefully constructing genetic subgroups based on this approach would have reduced number of hypothesis and avoided very small counts. A fragmented interaction analysis, that was performed in AREDS Report 38 effectively subdivides the data into cells many of which contain event counts well below numbers suitable for analysis. The AREDS Report 38 contains a number of serious analytic and manuscript production errors. There is no description of the main effects of treatment on progression risk FROM THEIR DATA SET. The treatment effect reported in the AREDS 8 report was not replicated. Their use of results reproduced from the AREDS 8 Report in Fig1-A is quite misleading. It is also reproduced with error, since the confidence interval significance is 99%, not 95% as stated, which leads to interpretation problem of confidence intervals and p-values. The AREDS Report 98 data is based on a different, much larger dataset than that used in AREDS Report 38. Secondly, the data was derived using a different modeling approach (repeated measures logistic vs Cox regression used by Chew *et al*). Even using AREDS Report 38 data on all Category 3 and 4 patients at baseline (regardless of DNA availability) which is the full AREDS sample, we fail to observe a significant treatment effect. Without demonstrating a significant treatment effect in the data set under study, very little can be concluded by failing to detect significant genetic effect modifiers via interaction study. Even leaving apart a dogma of statistical inference - that one never accepts the null hypothesis, one can only fail to reject it - without demonstrating significant treatment effect in the first place, one cannot conclude anything by failing to detect significant effect modifier signal (here, putative genetic factor interaction with treatment).

Not withstanding this, a simple logistic regression using the counts obtained from Fig 1B, suggests that such effect modification may in fact be present (with a omnibus p-value for interaction (24 d.f.) equal 0.0025). With lack of significance for main treatment effect this suggests a very strong interaction between treatment and genetic factors.

A worrisome aspect of Chew *et al* report is the number of inconsistencies in data presentation. The numbers of patients and events reported in their main results figure (Fig 1B) differ significantly from the supposedly same data reported in the Supplementary table 6b. This is especially so in the division of participant outcomes between Events and Non-Events. There are also discrepancies between SuppTables 6a and 6b tables. SuppTable 6a is represented as a collapsed (marginalized) version of SuppTable 6b, without the ARMS2 subgrouping. An example of discrepancy is found for the CFH1-Zinc row in SuppTable 6a. There are 4 too many events (and 4 two few non-events) compared to that reported in SuppTable 6b. With these data issues and without the access to the full data, any re-analysis is very tentative. However using the reported data we find that the genetic modifier effect on AREDS treatment is significant and largely similar to that reported by Awh *et al* [4].

References

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