

# Energy restriction and the risk of spontaneous mammary tumors in mice: a meta-analysis

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## ENERGY RESTRICTION AND THE RISK OF SPONTANEOUS MAMMARY TUMORS IN MICE: A META-ANALYSIS

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**Our meta-analysis was aimed at providing a systematic review of the literature regarding the effect of energy restriction on spontaneous mammary tumors in mice and at providing a more precise pooled (summary) estimate of the risk of mammary tumors. A sensitivity analysis was conducted to obtain insight in potential heterogeneity between the animal studies. A literature search was conducted with the following terms to identify relevant articles: animal studies, mammary tumors, fat restricted, dietary carbohydrates, energy restriction and calorie restriction. A criteria list for the assessment of quality items (i.e., study characteristics) in animal experiments was developed that was intended to quantitatively assess potential factors that underlie heterogeneous results of different animal experiments. Incidence figures were used to calculate the risk difference. The pooled risk difference was calculated by random effects meta regression analysis. Fourteen animal experiments were included in this meta-analysis. Publication bias could not be identified. The pooled risk difference for the 14 studies was  $-0.55$  with a narrow 95% confidence interval ( $-0.69$ ;  $-0.41$ ), implying that the energy-restricted animal groups developed 55% less mammary tumors than the control groups. No heterogeneity could be detected between the studies based on study characteristics that included the age of mice at the start of intervention, duration of intervention, allocation of the mice, use of *ad libitum* control group, fertility of the mice and the type of energy-providing nutrient (fat, carbohydrate or protein). This meta-analysis confirms that energy restriction in itself consistently protects against the development of mammary tumor in mice, irrespective of the type of restricted nutrient or other study characteristics.**

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**Key words:** energy restriction; mammary tumors; mice; meta-analysis

Worldwide, breast cancer is the most frequent cancer in women and is one of the main causes of death in women today.<sup>1,2</sup> It is important to understand more about the etiology and possibilities for prevention of breast cancer. The hypothesis that high fat intake will increase the risk of breast cancer has often been examined in human studies. Although epidemiological studies in humans suggested that diet (energy and fat) is related to the incidence of breast cancer,<sup>3–5</sup> the results are not consistent. A pooled analysis of the results of several cohort studies yielded no evidence of a positive association between total dietary fat intake and the risk of breast cancer.<sup>6</sup>

To clarify the role of dietary factors in the etiology of breast cancer, much research has been conducted in rodents, especially regarding the effect of dietary fat-intake and energy (i.e., caloric) restriction on breast cancer risk. Energy restriction has been suggested to be a very effective way of reducing the incidence of mammary tumors in rodents.<sup>7,8</sup> The animal experimental data supports the hypothesis that, in mammary tumor development, there is a specific enhancing effect of dietary fat, as well as a general enhancing effect of calories. The tumor enhancing effect of excess intake of fat was also shown in the review of Freedman.<sup>8</sup>

In contrast with the human studies,<sup>3,6</sup> these results appeared to be consistent between different studies. Suggestions have been made that reduced calorie intake may lower carcinogenic risk by influencing hormones, cytokines, growth factors, rates of cellular proliferation and levels of immunological responsiveness.<sup>9,10</sup>

Thompson *et al.*<sup>10</sup> stated that energy restriction exerts its effect by altering one or more aspects of cell cycle regulation. Energy restriction inhibited cell proliferation and increased cell death due to apoptosis. This was observed in a model system of experimentally induced mammary carcinogenesis.<sup>10,11</sup> Others report that energy restriction enhances DNA repair and moderates oxidative damage to DNA.<sup>12,13</sup> The overall consequences of energy restriction appear to be the up-regulation of cellular and molecular defense systems and down-regulations of reproductive aging.<sup>14,15</sup> Nevertheless, the precise mechanism by which caloric restriction decreases the incidence of mammary tumors in mice is still unknown.

Several investigators have discussed the effect of different levels of total fat intake and different levels of total energy intake.<sup>16–19</sup> However, no clear conclusions are available on whether the effect of fat intake on tumor incidence is modified by the level of calorie intake. In a review described by Freedman *et al.*,<sup>8</sup> the effect of fat was 2/3 the magnitude of the calorie effect in both Sprague-Dawley rats and mice. The experimental animal models used in these kinds of studies can vary between an animal model with induced carcinogenesis or spontaneous development of tumors. We focused in this review on the spontaneous animal model because of the comparability with the human situation. To induce mammary tumors is unrealistic in comparison with the human situation.

Energy restriction in experimental animal studies can be achieved in several ways. Sources of energy are nutrients such as fat, carbohydrates and protein. Normally, the diet consists of 30% fat, 60% carbohydrates and 10% protein. In some animal studies energy restriction was achieved by reducing the amount of fat,<sup>20,21</sup> in other studies by reducing the amount of carbohydrates.<sup>16,17,22,23</sup> The effect of reducing the amount of fat in the diet was greater compared to the effect of reducing carbohydrates. In more recent studies, a combination of several energy providing nutrients was used to achieve energy restriction.<sup>24,25</sup>

The purpose of our study was to systematically review the animal literature on energy restriction and spontaneous mammary tumors and to provide precise quantitative pooled (summary) estimates of the risk of mammary tumors by means of a meta-analysis. The purpose of a meta-analysis is to combine results from different studies in an attempt to identify consistent patterns and sources of disagreement among those results.<sup>26</sup> The technique of

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meta-analysis is mostly used for human randomized clinical trials (RCTs). However, the concept of meta-analyses may also be applied to animal studies, allowing the evaluation of the influence of the type of nutrient restricted, the age of mice at the start of the experiment and the duration of the intervention period on the association between energy restriction and the development of mammary tumors. Our meta-analysis had an emphasis on type of energy restriction and duration of intervention period. Also, a sensitivity analysis can be performed in order to get insight in causes of possible heterogeneity in results between the studies. Furthermore, we evaluated changes in summary estimates according to study methodology. The age at which the energy restriction took place had our special interest because the hypothesis has been put forward that energy restriction early in life may affect breast cancer risk.<sup>27,28</sup>

## MATERIAL AND METHODS

### Literature search

A literature search was conducted using the computerized databases MEDLINE and Current Contents. We restricted this systematic review to experiments with mice and spontaneous mammary tumors because of the comparability of this animal model to the human situation and the comparability between the studies.

The following terms were used to identify relevant articles: animal studies, mammary tumors, fat restricted, dietary carbohydrates, energy restriction and calorie restriction. The computerized search covered the years 1966 to 1999. Furthermore, references cited in published original and review articles were examined. For inclusion in this analysis, studies had to meet the following criteria. The study had to be a randomized trial with mice and spontaneously occurring mammary tumors (*i.e.*, no induced tumors). Secondly, sufficient information had to be available in the report to determine the degree of energy restriction and to estimate risk differences for mammary breast tumors when comparing groups. The incidence of mammary tumors and the number of mice per dietary group had to be reported. Excluded were studies with animal groups who were fed an isocaloric diet. An isocaloric diet means that the control group gets the same amount of energy although the diet has a different composition than the diet of the experimental group. Our main interest of this meta-analysis was the impact of energy restriction. This review had no limitation on language.

### Data collection

A criteria list for the assessment of quality items (*i.e.*, study characteristics) in animal experiments was developed. This list was intended to quantitatively assess potential factors of heterogeneity in results between the animal experiments. The list calls for aspects of the studies such as general information on mouse strain, age at start of the study and virginal status. Intervention information (quantity and duration of energy restriction, type of diet and individual *vs.* group caging) and the number of mice in the experimental and control group was also assessed. The criteria list also assessed whether the effect measurement was blinded and if the studies made use of an *ad libitum* fed control group.

All publications eligible for reviewing were blinded regarding author(s), journal, publication year, acknowledgments and affiliations. They were distributed to 3 independent reviewers who assessed the studies using the criteria list (MJMD, MPAZ and PCD). In case of any discrepancies between the 3 reviewers, consensus was reached in subsequent meetings. If more than one animal experiment was described in one publication, they were considered as separate studies.<sup>16,17,20,21</sup>

Mammary tumors in the experiments were detected by palpation or autopsy. Incidence rates were based on the intervention period because none of the studies had a follow-up period after the end of the intervention. Based on the starting numbers of mice in each study, the number of incident cases in each group and the degree of energy restriction, 2-way contingency tables were constructed

for each study. The animal group with the highest degree of energy restriction was compared to the animal group that was fed *ad libitum* or with the group with the highest intake of calories. Not all the experiments used an *ad libitum* control group. In studies with more than 2 restricted animal groups, the 2 groups with the highest contrast were compared. Data were extracted from the experiments in order to construct the tables. The tables were used to calculate the risk difference [incidence in the experimental group minus the incidence in the control group, ( $I_e - I_o$ )] and its corresponding variance. The risk difference has a range from  $-1.00$  to  $+1.00$  and is dimensionless.<sup>26</sup> A risk difference of  $-0.3$  ( $-30\%$ ) means that in the energy restricted group 30% less spontaneous mammary tumors were developed compared to the control group.

In one study, the contingency table showed 2 cells with zero (no mice with incident tumors) that would yield a variance of zero. Since the inverse of the variance was used as weight,<sup>29</sup> the mean variance of the studies was utilized instead for this specific study.<sup>21</sup> Mortality rates were not available for each individual study and could therefore not be used in this meta-analysis.

### Determinant

The primary determinant studied in this review was the degree of energy restriction in the studies. Because of the small number of studies ( $n=14$ ), energy restriction was divided into 3 approximately equivalent categories ( $\leq 33\%$  energy restriction, 34–40% energy restriction or  $> 40\%$  energy restriction). However, the distribution of this variable is skewed because 5 studies had an energy restriction equal to the cut-off value of 40%. Furthermore, the type of restricted nutrient was also evaluated (carbohydrates, fat or protein restricted).

### Statistical analysis

To detect publication bias, we explored asymmetry in funnel plots, *i.e.*, plots of effect estimates against their estimated precision, and we measured the degree of asymmetry using Egger's unweighted regression asymmetry test.<sup>30,31</sup> The pooled (summary) risk difference was calculated by random effects meta regression analysis using the STATA statistical software package.<sup>29,32,33</sup> To account for both sources of variation (between and within studies), we used random effects meta regression analysis to combine the results from the primary studies. The random effect approach provides some allowance for heterogeneity in studies beyond sampling error. To explore possible reasons for the observed heterogeneity in results between different studies, sensitivity analyses were performed on the following study characteristics: age of mice at the start of the intervention ( $<1-5$  weeks old, 6–9.5 weeks old and  $>9.5$  weeks old), duration of intervention period ( $\leq 56.6$  weeks, 57–71.5 weeks and intervention period longer than 71.5 weeks). The cut-off points of these study characteristics were based on tertiles. Allocation of the mice (individually caged or 2 or more mice in one cage) and the state of fertility of the animals at the start of the experiment (virgin or not) were also tested in sensitivity analyses to explore their influence on the association between energy restriction and mammary cancer.

## RESULTS

Based on the literature search, 16 articles were identified reporting animal experiments with mice on energy restriction and development of spontaneous mammary tumors of which 6 studies reported the use of isocaloric diets. The latter studies were excluded. In 4 articles more than one animal experiment was described; these were considered as separate studies. Thus, a total number of 14 animal experiments was included in this meta-analysis.

In Table I characteristics of the different studies are summarized.<sup>16,17,20,24,34–36</sup> All studies were published between 1942 and 1994. The mean energy restriction in the 14 included studies was 37% (range 23–50%) and mean age of the mice at the start of the

TABLE I—CHARACTERISTICS OF MICE STUDIES ON CALORIE RESTRICTION AND SPONTANEOUS MAMMARY TUMORS, ORDERED BY PUBLICATION YEAR

| Author                 | Year <sup>1</sup>  | Mice strain  | Age of mice (in weeks) | Intervention period (in weeks) | Degree of restriction <sup>2</sup> (%), (source) | Total number of mice in study |
|------------------------|--------------------|--|------------------------|--------------------------------|--|-------------------------------|
| Visscher <i>et al.</i> | 1942               | C3H, virgin  | 4.5                    | 67                             | 33% (fat + CHO*)                                 | 95                            |
| Tannenbaum             | 1945a <sup>a</sup> | DBA, virgin  | 9.5                    | 126.5                          | 23% (CHO)  | 94                            |
| Tannenbaum             | 1945a <sup>b</sup> | C3H, virgin  | 10                     | 54                             | 33% (CHO + protein)                              | 58                            |
| Tannenbaum             | 1945b <sup>a</sup> | DBA, virgin  | 10                     | 90                             | 35% (CHO)  | 98                            |
| Tannenbaum             | 1945b <sup>b</sup> | DBA  | 23                     | 113                            | 33% (CHO)  | 50                            |
| Sarkar <i>et al.</i>   | 1982               | C3H, virgin  | 5                      | 107                            | 50% (protein)                                    | 144                           |
| Shao <i>et al.</i>     | 1990 <sup>a</sup>  | C3H/B1   | 20                     | 56.5                           | 40% (CHO)  | 25                            |
| Shao <i>et al.</i>     | 1990 <sup>b</sup>  | C3H/B1   | 20                     | 56.5                           | 40% (fat)  | 30                            |
| Koizumi <i>et al.</i>  | 1990               | (C3H/SHN) F1-hybrid (C3H/He male*SHN female), virgin | 3                      | 64                             | 50% (CHO)  | 65                            |
| Engelman <i>et al.</i> | 1990               | C3H/HeOu, virgin                                     | 4                      | 72                             | 32% (CHO)  | 48                            |
| Engelman <i>et al.</i> | 1991 <sup>a</sup>  | C3H/Ou, virgin                                       | 7                      | 54                             | 40% (CHO + protein)                              | 53                            |
| Engelman <i>et al.</i> | 1991 <sup>b</sup>  | C3H/Ou, virgin                                       | 7                      | 54                             | 40% (fat)  | 46                            |
| Engelman <i>et al.</i> | 1994               | C3H/Ou, virgin                                       | 6                      | 38                             | 40% (CHO)  | 20                            |
| Li <i>et al.</i>       | 1994               | RIII/Sa, virgin                                      | 5                      | 72                             | 37.5% (CHO)                                      | 103                           |

<sup>1</sup>a and <sup>b</sup>: one article describing 2 independent animal experiments—<sup>2</sup>CHO: carbohydrate. 30% restriction means that animals received 70% of amount eaten by *ad libitum* group.

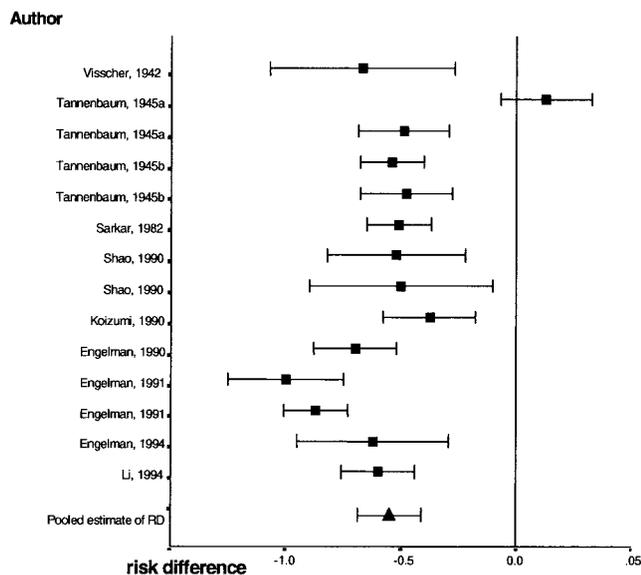


FIGURE 1—Difference in incidence of mammary tumors (risk difference) for energy restricted groups relative to *ad libitum* (or highest amount of calories) fed groups, and 95% confidence intervals for each study separately and pooled risk difference (pooled RD). Solid line indicates no effect, a risk difference of  $-0.55$  means that the energy-restricted group has 55% less mammary tumors developed compared to the control (nonrestricted) group.

experiments was 9.6 weeks (range 3–23 weeks). The mean duration of the experiments was 72.6 weeks (range 38–126.5 weeks). All experiments stopped after the intervention was stopped. The mean number of mice per study was 66 (range 20–144 mice). None of the reports mentioned whether the effect measurement was blinded. Most of the mice in the energy-restricted groups in the studies were individually caged. The incidence of spontaneous mammary tumors in the control groups ranged from 37% to 100%. Mice strains used in these experiments varied between DBA, C3H, C3H/Bi, C3H/Ou and RIII/Sa.

Risk differences were below zero in all individual studies except one [16], with values ranging from  $-1.0$  to  $0.13$ . The pooled risk difference (pooled RD) for the 14 studies was  $-0.55$  (95%CI  $-0.69$ ;  $-0.41$ ), implying that the energy-restricted animal groups developed 55% less spontaneous mammary tumors than the con-

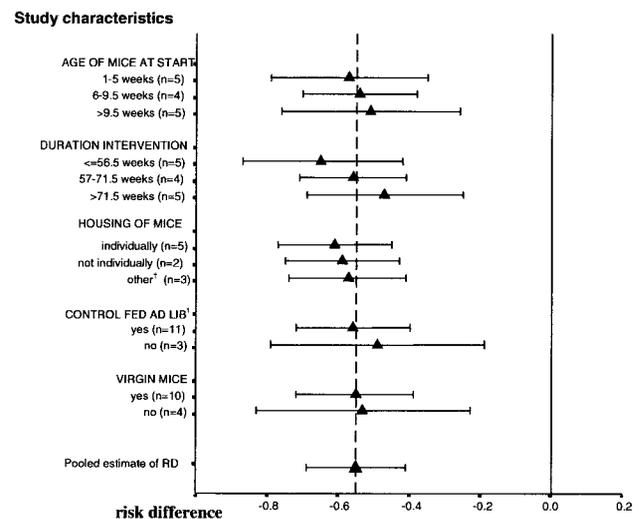


FIGURE 2—Summary risk differences for energy restricted groups vs. *ad libitum* (or highest amount of calories) and 95% confidence intervals of mammary tumors for pooled studies with different study characteristics. Solid line refers to null effect and dashed line refers to pooled estimate of risk difference for all studies. Other housing, the experimental (restricted) group is individually caged and the control group is not individually caged. *ad lib*, *ad libitum*

trol groups. Figure 1 shows the different risk differences for the individual studies and the pooled (summary) risk difference. We could not identify publication bias, either visually or in terms of statistical significance (Egger's test  $p > 0.8$ ).

To explore the influence of study characteristics on outcome estimates, heterogeneity between the different studies was evaluated. Figure 2 shows the relationship between energy restriction and spontaneous mammary tumors by age of the mice at start of intervention, duration of the intervention, housing (individual or not) of the mice, use of an *ad libitum* control group and fertility of the mice.

The pooled risk differences for energy restriction and spontaneous mammary tumors were  $-0.57$  (95%CI  $-0.79$ ;  $-0.35$ ) for mice aged 1–5 weeks,  $-0.54$  (95% CI  $-0.70$ ;  $-0.38$ ) for mice aged 6–10 weeks and  $-0.51$  (95% CI  $-0.76$ ;  $-0.26$ ) for mice aged over 10 weeks at the start of the study. In experiments with a short intervention period, the greatest reduction in development of spontaneous mammary tumors was seen. For studies with a

short duration of intervention ( $\leq 56.5$  weeks) the pooled risk difference was  $-0.65$  (95%CI  $-0.87; -0.42$ ) and  $-0.56$  (95%CI  $-0.71; -0.41$ ) for studies with a median duration of intervention period (57–71.5 weeks). For studies with a long intervention period ( $> 71.5$  weeks) the pooled risk difference was  $-0.47$  (95%CI  $-0.69; -0.25$ ). For studies using individual caging the pooled risk difference was  $-0.61$  (95%CI  $-0.77; -0.45$ ). For studies using an *ad libitum* control group, the pooled risk difference was (95%CI  $-0.72; -0.40$ ). For studies that did not use an *ad libitum* control group the pooled risk difference was  $-0.49$  (95%CI  $-0.79; -0.19$ ). No difference was seen in pooled RD between studies using virgin mice or not. Studies that used virgin mice the pooled risk difference was  $-0.55$  (95%CI  $-0.72; -0.39$ ) and for studies that used no virgin mice the pooled risk difference was  $-0.53$  (95%CI  $-0.83; -0.23$ ) (Fig. 2). None of the above mentioned results were significantly different between the subgroups ( $p$ -values of interaction  $> 0.20$ ).

In Figure 3, results according to different dietary exposure characteristics are presented. For studies with  $\leq 33\%$  energy restriction the pooled risk difference was  $-0.50$  (95%CI  $-0.72; -0.28$ ). For studies with an energy restriction between 34 and 40%, the pooled risk difference was  $-0.56$  (95%CI  $-0.7010; -0.41$ ). For studies with the highest degree of energy restriction ( $> 40\%$ ), the pooled risk difference was  $-0.62$  (95%CI  $-0.91; -0.33$ ). This means that in studies with more than 40% energy restriction, 62% fewer animals in the intervention groups developed spontaneous mammary tumors than in the control groups. For carbohydrate-restricted studies, the pooled risk difference was  $-0.52$  (95%CI  $-0.68; -0.36$ ), and for studies that did not restrict the carbohydrates the pooled risk difference was  $-0.64$  (95%CI  $-0.93; -0.35$ ). Fat-restricted studies showed a pooled risk difference of  $-0.71$  (95%CI  $-1.04; -0.39$ ) and non-fat restricted studies  $-0.51$  (95%CI  $-0.66; -0.36$ ). Studies with and without protein restriction showed a pooled risk difference of  $-0.57$  (95%CI  $-0.86; -0.27$ ) and  $-0.55$  (95%CI  $-0.75; -0.35$ ), respectively. None of the interactions were significant ( $p$ -values  $> 0.30$ ).

## DISCUSSION

We performed a meta-analysis of 14 animal experiments that reports on energy restriction and the incidence of spontaneous mammary tumors mice. Energy-restricted animal groups showed a

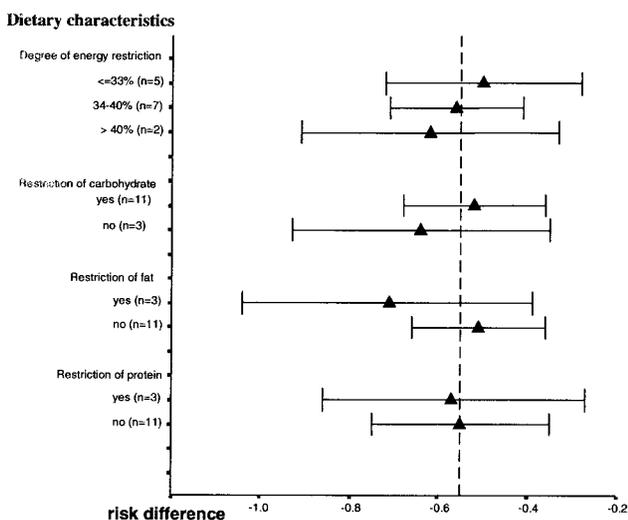
statistically significant lower incidence of spontaneous mammary tumors compared to the non-restricted groups (control groups), with a pooled risk difference of  $-0.55$ . All individual studies, except one,<sup>16</sup> showed a negative risk difference. The results of this systematic review show that energy restriction protects against the development of spontaneous mammary tumors in mice and support earlier studies. An energy restriction of more than 40% shows an apparently greater preventive effect (pooled RD  $-0.62$ ) than 33% energy restriction or less (pooled RD  $-0.50$ ), but the difference was not statistically significant. The type of nutrient which was restricted (fat, carbohydrate or protein) seemed not to have an important influence on the pooled risk difference, although fat-restricted studies showed a pooled RD of  $-0.71$  (again not statistically significant). These results are in accordance with the narrative review of Albanes.<sup>37</sup>

The only study that showed a positive risk difference of 0.13 was the study with the longest duration of intervention period, namely, 126.5 weeks (almost 2.5 years) and the lowest degree of energy restriction (23%).<sup>16</sup>

Because of heterogeneity of the studies with regard to animal strain, duration of the intervention period, age of mice and housing, we assumed that the true effect of dietary restriction varied between the studies in addition to the usual sampling variation in the estimates. In order to account for both sources of variation, we used random effects meta regression analysis to combine the results from the primary studies. The random effect approach provides some allowance for heterogeneity in studies beyond sampling error. Results showed that pooled risk differences were independent of the age of the mice, housing of mice, fertility of mice, the use or non-use of an *ad libitum* control group and the duration of intervention period. No heterogeneity could be detected between the studies based on study characteristics, although a longer duration of intervention period tended to be associated with a smaller risk difference. A possible explanation could be the fact that almost every mouse of the strains used in these experiments may finally develop a mammary tumor, provided the observation period is long enough. In experiments used in this meta-analysis, the incidence in the control groups varied between 37% and 100%.<sup>35</sup> In comparison, lifetime cumulative incidence of breast cancer among women varies between 8% and 15% worldwide.<sup>1</sup> Therefore, strains with lower incidence would be needed to achieve a situation that more closely resembles the human situation.

It has been suggested that the age at which animals are exposed to energy restriction may be an important factor in the development of mammary tumors. Early restriction will permanently stunt growth and reduce total cell numbers in all organs. Exposure to undernutrition in the prepubertal years leads to a decrease in cell division and a reduction in the total number of dividing cells (mitosis). It was hypothesized that when feeding the animals an energy-restricted diet from birth till weaning age and *ad libitum* after weaning, the deficiency in the number of cells will not completely be corrected, although the reduction in cell volume will generally recover.<sup>38,39</sup> When energy restriction occurs later in life, it will affect the rate of mitosis in the regenerating tissue.<sup>40</sup> Cell division can be interrupted by malnutrition at any time. When the rate of cell division is very high, the cellular DNA is subject to frequent damage. The effects of energy restriction might therefore depend on the timing of the restriction. In this meta-analysis, we could not detect an effect by the age of mice. Silverman<sup>41</sup> also concluded that the age of puberty in itself does not act as a risk factor for mammary carcinogenesis in animal experiments if a high fat/high calorie diet was fed throughout the period.

Another point of discussion is the duration of intervention period, which differs considerably between the various studies. In our meta-analysis, the duration of the intervention period did not significantly influence the association between energy restriction and spontaneous mammary tumor risk. However, the results sug-



**FIGURE 3** – Summary risk difference for energy restricted groups vs. *ad libitum* (or highest degree of calories) and 95% confidence interval of mammary tumors for studies by different exposure characteristics. Dashed line refers to pooled estimate of risk difference for all the studies.

gest that studies with a long intervention period reported a lower pooled risk difference. The shortest duration of intervention period was 38 weeks. It would be interesting to see whether shorter experiments would show the same results.

Another point of discussion is the fact that in our meta-analysis studies are included from 1942 onwards. The composition of the diets was different in the studies that were conducted before the 1980s. In these studies,<sup>16,17,34</sup> the diets were unrefined, which means that they could have contained a number of ingredients, particularly from plant, vegetable and cereal sources that may have contributed to some of the cancer protective effects observed. The other studies used purified<sup>20,22,35,36</sup> or semi-purified diets.<sup>21,23,24,42</sup>

Given the strong and consistent effect of energy restriction on cancer prevention in experimental animals, it is clearly possible that the balance between energy consumption, retention and expenditure, perhaps in combination with frequency and length of fasting periods between meals, represents an underlying and central link in the relationship between diet, nutrition and cancer in Western society.<sup>43</sup> Future dietary cohort studies and intervention trials are needed to get insight into the effects of energy and/or fat restriction on human breast cancer development.

In conclusion, this meta-analysis confirms that energy restriction in itself consistently protects against the development of spontaneous mammary tumor in mice, irrespective of the type of restricted nutrient and/or other study characteristics.

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