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# **DNA ADDUCTS AS BIOMARKERS OF EXPOSURE TO SOME DIETARY CARCINOGENS**

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Cover illustration: Ethanol molecule, as interpreted by Viktor Jurén

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*I dedicate this thesis to my husband Michael, and our children, Vendela, Viktor, Liv and “Knyttet” who have always stood by me and believed that I would do it.*



*All substances are poisons; there are none which are not poison.  
The right dose differentiates poison and remedy.*

*Paracelsus (1493-1541)*



## ABSTRACT

Humans are exposed to many chemicals via intake of food and drink and there are strong associations between dietary factors and cancer. Exposure to the dietary carcinogens acrylamide and acetaldehyde are potential health risks that have made the headlines. High levels of acrylamide are found in various food items, such as potato chips. Acetaldehyde exposure in association with alcoholic beverages is clearly linked to increased cancer risk. In addition, acetaldehyde is a contaminant and present or produced in various foods, including certain dairy products.

Dietary exposure is normally assessed from levels in food together with consumption patterns. However, with such assessments it is difficult to take into account the metabolic changes to the various chemicals, as the interindividual variations are great. Efforts have therefore been made to improve measurements of internal exposure by use of biomarkers, such as DNA adducts.

The aim of this thesis was to develop and apply biomarkers for human exposure to acrylamide and acetaldehyde. This was done by characterizing the DNA adduct N1-(2-carboxy-2-hydroxyethyl)deoxyadenosine (N1-GA-dA), formed by glycidamide) and the adducts N<sup>2</sup>-ethyl-2'-deoxyguanosine (N<sup>2</sup>-ethyl-dG) and N<sup>6</sup>-ethyl-2'-deoxyadenosine (N<sup>6</sup>-ethyl-dG), both formed by acetaldehyde. In addition, LC-MS/MS and <sup>32</sup>P-postlabeling methods for the analysis of these DNA adducts were developed and used to analyze animal and human DNA and tissue samples.

The glycidamide-derived DNA adduct N1-GA-dA was for the first time shown to be formed when mammalian cells were treated with glycidamide. However, the adduct was not detected in liver DNA of mice exposed to acrylamide.

The adduct N<sup>2</sup>-ethyl-dG was detected in DNA treated in vitro with acetaldehyde, in human lung DNA from smokers and non-smokers and for the first time in DNA exposed in vitro to cannabis smoke. N<sup>2</sup>-Ethyl-dG was also analyzed in leukocyte DNA from a group of healthy men who had consumed a moderate amount of alcohol under controlled circumstances. Adduct levels were not significantly increased. The chemical stability of N<sup>2</sup>-ethyl-dG was studied and the findings imply that the rate of loss is more rapid than previously thought.

With the <sup>32</sup>P-postlabeling assay developed for the analysis of acetaldehyde adducts, it was shown for the first time that N<sup>6</sup>-ethyl-dA is formed in DNA in vitro exposed to acetaldehyde.

Sensitive biomonitoring methods were developed and several novel findings were made. The methods used could be applied in future animal and human studies of exposure to glycidamide and acetaldehyde. In order to make these biomarkers useful for epidemiological studies, they must be fully validated and future biomarker studies should aim for analysis of multiple endpoints in a large number of samples.

## LIST OF PUBLICATIONS

- I. Natalia Kotova, **Tina Jurén**, Kirsi Myöhänen, Michael Cornelius, Lilianne Abrahamsson-Zetterberg, Josefin Backman, Ulrike Menzel, Per Rydberg, Leif Kronberg, Kirsi Vähäkangas and Dan Segerbäck. (2011).  $^{32}\text{P}$ -HPLC analysis of N1-(2-carboxy-2-hydroxyethyl)deoxyadenosine: a DNA adduct of the acrylamide-derived epoxide glycidamide. Toxicology Letters. 207, 18-24.
- II. Rajinder Singh, Jatinderpal Sandhu, Balvinder Kaur, **Tina Jurén**, William P. Steward, Dan Segerbäck and Peter B. Farmer. (2009) Evaluation of the DNA damaging potential of cannabis cigarette smoke by the determination of acetaldehyde derived N<sup>2</sup>-ethyl-2'-deoxyguanosine adducts. Chem. Res Toxicol. 22, 1181-1188.
- III. Rajinder Singh, Jolanta Gromadzinska, Yogita Mistry, Rebecca Cordell, **Tina Jurén**, Dan Segerbäck and Peter B. Farmer. (2012). Detection of acetaldehyde derived N<sup>2</sup>-ethyl-2'-deoxyguanosine in human leukocyte DNA following alcohol consumption. 737, 8-11.
- IV. **Tina Jurén**, Daniel Håkansson, Josefin Backman, Rajinder Singh, Peter B. Farmer, Leif Kronberg and Dan Segerbäck. Development of sensitive  $^{32}\text{P}$ -postlabelling methods for the analysis of the acetaldehyde derived DNA adducts N<sup>6</sup>-ethyl-2'-deoxyadenosine and N<sup>2</sup>-ethyl-2'-deoxyguanosine (Manuscript).



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## LIST OF ABBREVIATIONS

ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
ATP	Adenosine-5'-triphosphate
dA	2'-Deoxyadenosine
dAMP	2'-Deoxyadenosine monophosphate
dG	2'-Deoxyguanosine
DNA	Deoxyribonucleic acid
GC	Gas chromatography
HPLC	High-performance liquid chromatography
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography -tandem mass spectrometry
MS	Mass spectrometry
N <sup>2</sup> -ethyl-dG	N <sup>2</sup> -ethyl-2'-deoxyguanosine
N <sup>1</sup> -GA-dA	N <sup>1</sup> -(2-carboxy-2-hydroxyethyl)deoxyadenosine
N <sup>6</sup> -GA-dA	N <sup>6</sup> -(2-carboxy-2-hydroxyethyl)deoxyadenosine
PAH	Poly aromatic hydrocarbons

# 1 INTRODUCTION

What we eat very much depends on our place of residence, history, culture and religion. Eating food is the way we survive — the way we obtain our energy and nutrition. Food is also one of the main sources of pleasure in life. But, what is a balanced diet that will not make us sick? Nature is full of edible substances, but some also contain substances harmful to health. There are no easy, foolproof ways for us to determine, with our sensory organs, what is edible, nutritious and nontoxic.

It is estimated that approximately one third of cancer mortality in the US is associated with dietary factors. For example, a higher incidence of cancer has been noted for diets high in meat (especially red, grilled and preserved meat), high in fat, high in sodium and high in alcohol consumption (Mosby, Cosgrove, Sarkardei, Platt, & Kaina, 2012). Other risk factors include cigarette smoking (Hecht, 2003; IARC, 2012), physical inactivity (Booth, Laye, Lees, Rector, & Thyfault, 2008) and being overweight (De Pergola & Silvestris, 2013), infections (Carbone & De Paoli, 2012) and exposure to environmental carcinogens (Belpomme et al., 2007) and radiation (Dolphin, 1980). Cancer is often called a disease of the genome, as the induction of changes in the genetic material in cells is a hallmark of cancer (Hanahan & Weinberg, 2011). Such changes can be induced by certain chemicals present in the diet; some of these chemical carcinogens or their metabolites are reactive species that can bind to DNA (form a DNA adduct) and thereby cause mutation, which is a critical step on the path to cancer (Poirier, 2012).

Assessing exposure to carcinogens in the diet or in the environment is important when estimating cancer risks. Levels of DNA adducts can be used to assess the dose that has reached the tissue DNA, and these levels may be indicative of the risk associated with an exposure (La & Swenberg, 1996). However, the fact that an adduct is present does not tell us anything about the consequences in the DNA, i.e., if the adduct will be removed or whether a mutation will result. Thus, studies on DNA adducts may simplify risk assessment and prompt preventive actions to reduce exposure to carcinogens.

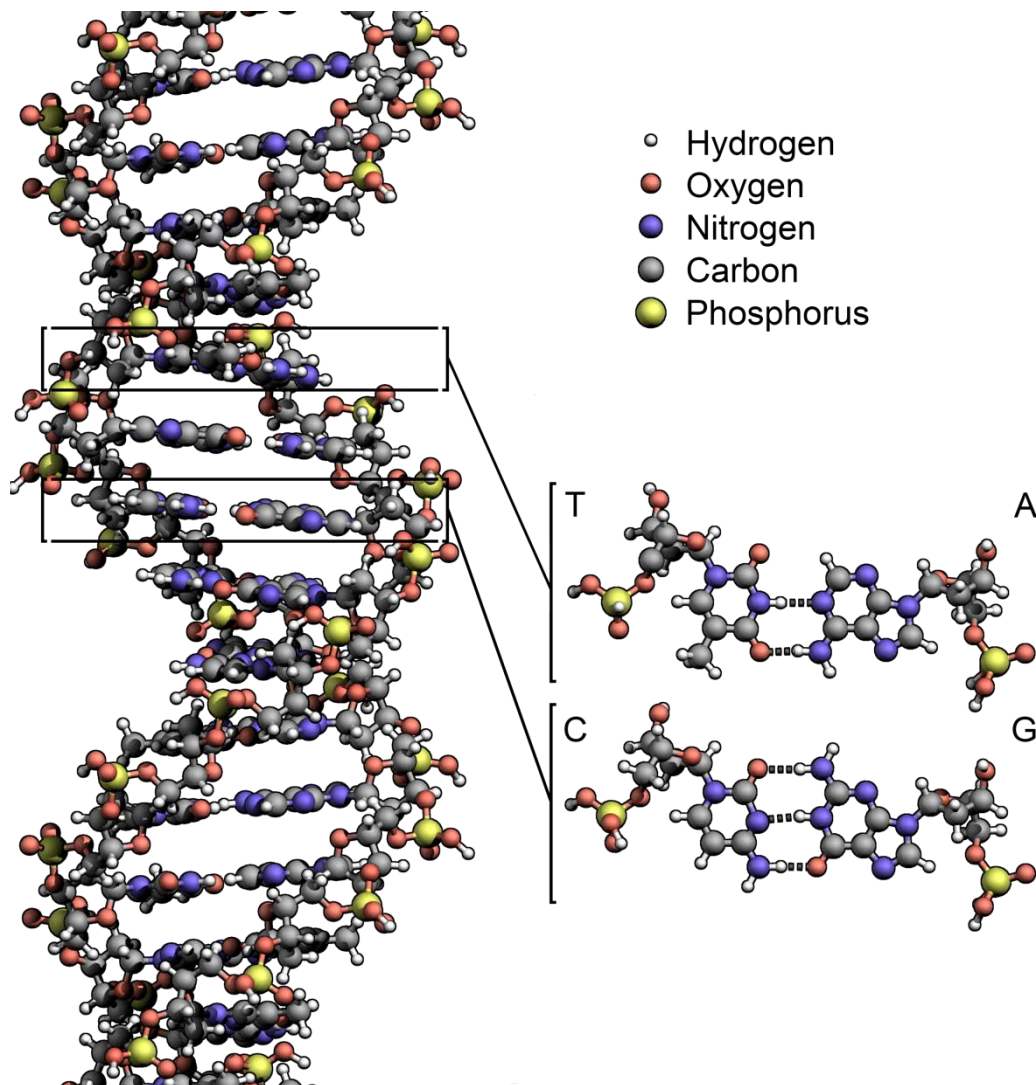
Food can also have a preventive effect on cancer incidence, and there is ample evidence concerning the benefits of some food groups. Plant-based foods, including fruit, vegetables and whole grains, a favorable omega-6/omega-3 polyunsaturated fatty acid ratio and fish consumption seem to have protective effects against cancer (Mosby, et al., 2012). Even if no single dietary factor can prevent or delay the occurrence of cancer, healthy eating may prevent the development of diet-associated cancers.

The cancers most preventable by appropriate food, nutrition, physical activity and body composition include those of the esophagus, mouth, pharynx, larynx, colon and rectum together with those of the breast. In efforts to reduce the risk of cancer, a healthy diet and lifestyle factors are second in importance only to abstinence from tobacco smoking.

In this thesis, studies on DNA adducts as a result of exposure to the two carcinogens acrylamide and acetaldehyde are presented.

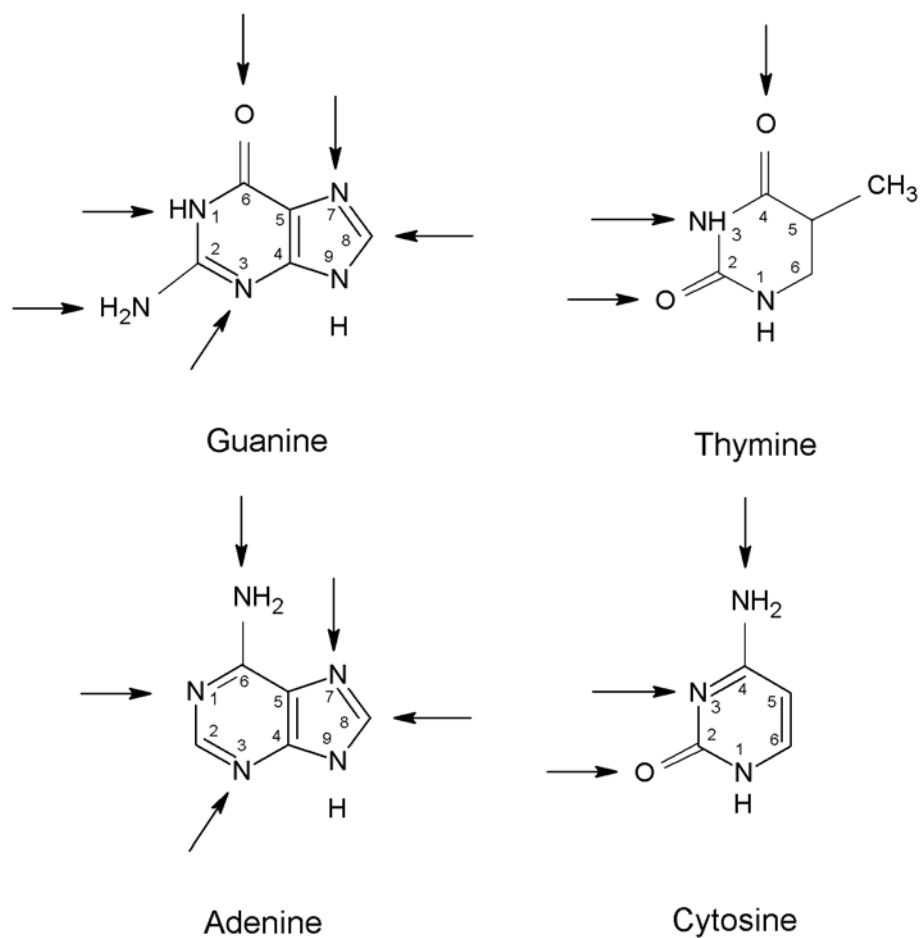
## 2 WHAT IS A DNA ADDUCT?

DNA is a biological macromolecule that carries our genetic information — our genome. The DNA molecule consists of the same building blocks in most organisms, including bacteria, plants, animals and humans. DNA is built of billions of small units called nucleotides, and every nucleotide consists of one of four bases: A (adenine), T (thymine), G (guanine) or C (cytosine) (**Figure 1**). These four bases form a letter sequence — the genetic code — with information encoding the sequence of amino acids in proteins. DNA is therefore often referred to as a recipe or a design drawing, containing the information for how to build the proteins contained in our cells. In order for the cell to function properly, it is crucial that the genetic code is copied without error to the next generation of cells.



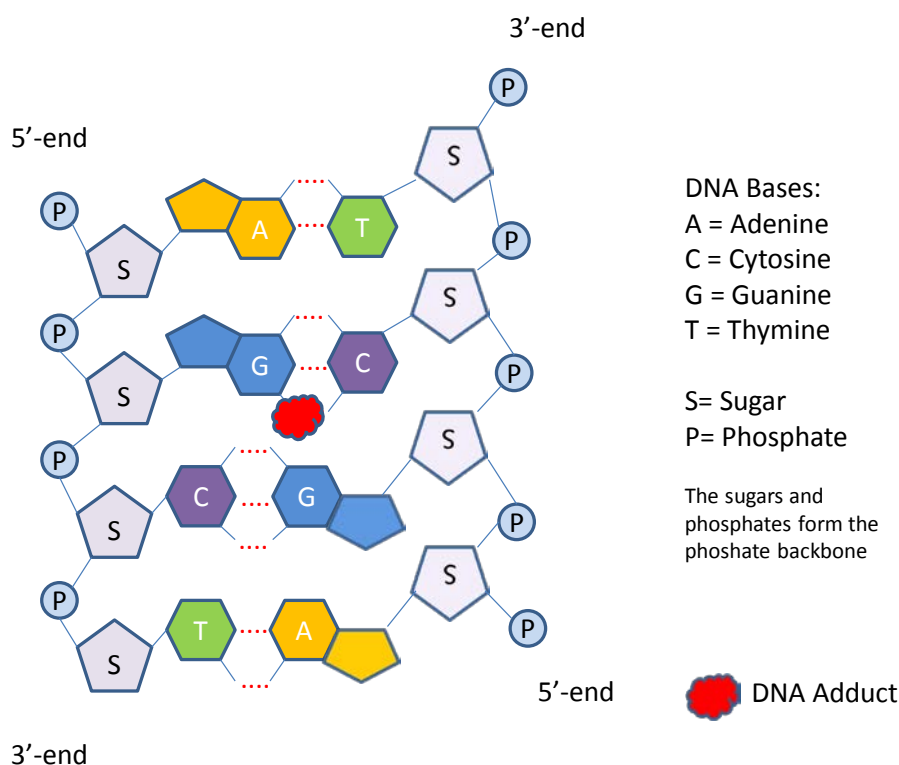
**Figure 1.** Schematic picture of a short section of DNA with the four DNA bases, A = Adenine, C = Cytosine, G = Guanine and T = Thymine and the sugars and phosphates forming the phosphate backbone  
(Illustration from <http://en.wikipedia.org/wiki/DNA>)

DNA adducts are examples of DNA damage that forms on one of the four nucleotides in DNA (**Figure 2**). Most chemical compounds that cause mutations are able to react and bind covalently (bind strongly by means of sharing electrons) to DNA. These addition products are called DNA adducts and are primarily formed with oxygen and nitrogen on the DNA bases (G>A>>C>T) and more rarely with oxygen on the phosphate groups (Dipple, 1995).



**Figure 2.** The DNA bases with common adduct forming sites indicated with arrows

The formation of a DNA adduct is preceded by a sequence of events. First, a carcinogen needs to be absorbed by the body. This can happen via the food we eat, the air we breathe or through contact or exposure to the skin. Once inside the body, the carcinogen is distributed by the blood stream to tissues and organs; thereafter, in some cases, the carcinogen is metabolized to a more reactive product, a metabolite. The carcinogen and/or metabolite penetrates the cell membrane and reacts with DNA, forming a DNA adduct somewhere along the DNA sequence (**Figure 3**).



**Figure 3.** Schematic picture of a snippet of DNA with an adduct attached to the DNA base guanine.

The adduct level is determined by the dose and the reactivity of the compound that binds to DNA, and the dose is determined by the exposure, absorption, distribution, activation and deactivation of the compound (Slikker et al., 2004). During chronic exposure, a steady-state level of adducts is built up as a consequence of formation and removal (Tornqvist & Landin, 1995). The efficiency of many cellular processes varies between individuals, and as a result, large interindividual differences in adduct levels can be found in response to similar exposures (Eder, 1999) .

Even if DNA adducts are formed every day, in each cell, only a tiny fraction of these lesions lead to permanent damage to the DNA. DNA adducts can be eliminated as a consequence of cell death or chemical instability. In addition, cells are equipped with sophisticated enzymatic repair systems that can remove DNA adducts (Sharova, 2005).

If the DNA adduct is not repaired or is repaired incorrectly, it may result in a mutation, which in turn may lead to altered metabolism, abnormal protein production and chromosomal instability. If the mutation is present in genes that are critical drivers in carcinogenesis, such as oncogenes and tumor suppressor genes, this in turn may lead to tumor induction (Ferguson, 2010).

Examples of chemical compounds that form DNA adducts include the following: simple mono- and bifunctional alkylating agents (e.g., dietary-formed carcinogenic nitrosamines found in cured meats); aromatic amines (e.g., heterocyclic amines formed when meat and fish are grilled); polycyclic aromatic hydrocarbons (PAH; found in foods cooked at high temperatures) and aldehydes (acetaldehyde) (Dipple, 1995; K. Hemminki, 1994).

### 3 DNA ADDUCTS AS BIOMARKERS OF EXPOSURE

The concept of biological markers (nowadays commonly denoted “biomarkers”) for the estimation of exposure and effects of chemical carcinogens was introduced 1987 (Perera, 1987). By measuring the levels of chemicals and complementing them with the effects of said chemicals in human cells or bodily fluids, this analysis can determine the exposure to the human body after metabolic processing. This would in turn result in improved measurement of the effects on various cellular targets, such as the amount of DNA damage. There are three types of biomarkers that are often discussed: “biomarkers of exposure” (chemicals, metabolites or adducts), “biomarkers of (early) effects” (a commonly used term for mutations and cytogenetic effects as a result of the chemical, metabolite) and “biomarkers of susceptibility” (meaning the genetic factors, often different polymorphism of relevant genes that will influence the pathway from exposure to disease) (Kyrtopoulos, Sarraf, Elliott, Schoket, & Demopoulos, 2006).

Biomarkers of exposure, for instance DNA adducts, can be measured in urine, blood, saliva and tissues. These markers are used to monitor the presence of a chemical in the body and its interactions with cellular components, including glutathione conjugates (van Welie, van Dijck, Vermeulen, & van Sittert, 1992). Biomarkers of exposure provide information on chemical exposure in individuals, changes in levels over time and variability among different populations. They may also provide information on the relative importance of different exposure pathways and associated risks. There are also different categories of biomarkers of exposure, including the chemical itself (e.g., in this project, acetaldehyde or acrylamide), its metabolite (conjugation with glutathione) or interactions between chemicals or metabolites and target molecules that are measurable in the human body (e.g., DNA- or protein adducts) (Swenberg et al., 2008).

DNA adducts are sometimes studied in experimental animals to determine if and which type of adducts are formed, to visualize the tissue distribution (in relation to the tissue specificities of tumor formation) and to investigate the shape of the dose-response curve for adduct formation. In addition, one can investigate the repair of adducts and the association to genotoxic endpoints, such as mutations or micronuclei (Beland & Poirier, 1993). One particular interesting aspect is the investigation of the effects of genes involved in metabolism or DNA repair using genetically modified rodents. For example, one such study established the importance of O<sup>6</sup>-methyl-dG for tumor formation (Allay, Veigl, & Gerson, 1999), and another study found that the consequences of modifying genes may not be as simple as previously believed and that the relationship between genes for metabolism and adduct formation is complicated (Arlt et al., 2012; Uno et al., 2001).



## 4 CANCER – A MULTISTEP PROCESS

The development of cancer involves the progressive transformation of normal human cells into highly malignant derivatives, i.e., cancer cells. During this process, cells undergo genetic changes that result in a number of effective ways to survive and propagate (Hanahan & Weinberg, 2011). To undergo active proliferation, cancer cells produce their own growth signals and at the same time become insensitive to antigrowth signals (Hynes & MacDonald, 2009; Lemmon & Schlessinger, 2010; Perona, 2006; Witsch, Sela, & Yarden, 2010). In addition, cancer cells can induce surrounding normal cells to supply them with growth factors (Bhowmick, Neilson, & Moses, 2004; N. Cheng, Chytil, Shyr, Joly, & Moses, 2008). Furthermore, resistance against programmed cell death (apoptosis) enables cancer cells to survive even if they are genetically damaged.

For the cancer tumor to grow to a macroscopic size, the cells require more than just a disruption in cell-to-cell signaling and evasion of apoptosis; cancer cells also need limitless replicative potential. Cancer cells are able to manipulate the number of times they can divide, which in turn permits unlimited multiplication of descendant cells (Eissenberg, 2013; Gunes & Rudolph, 2013; Lopez-Otin, Blasco, Partridge, Serrano, & Kroemer, 2013).

To survive, propagating cells require angiogenesis for the delivery of nutrients and oxygen via the blood supply. Sustained angiogenesis is therefore another important feature of cancer cells, similar to that required for wound healing, the menstrual cycle, inflammatory processes and fetal development (Carmeliet & Jain, 2000; Folkman, 1995). In fact, angiogenesis is a feature that is shared by most, and perhaps all, types of human tumors (Hanahan & Folkman, 1996; Raica, Cimpean, & Ribatti, 2009; Yoo & Kwon, 2013). As the tumor grows, nutrients and space become limited, but their capacity for invasion and metastasis enables cancer cells to escape the primary tumor mass and colonize new terrain in the body where, at least initially, nutrients and space are not limited.

Nevertheless, not all individuals exposed to carcinogens develop cancer, as there are differences between individuals in how they will respond to a given exposure or toxic agent. Some individuals are more susceptible and will experience an adverse health effect to one or more chemicals at lower or shorter exposures than the general population. Therefore, individual variations in genetic susceptibility contribute to the development of cancer in some people but not in others, despite exposure to the same levels of the same carcinogen (Fletcher & Houlston, 2010).

Most of the available carcinogenicity data on genotoxic compounds do not provide precise information regarding the specific stage or stages of the process of carcinogenesis at which the compounds act (Ferguson, 2010). The human diet is a complex mixture, and interactions between compounds and the role of anticarcinogens remains largely unknown.

## 5 EXPOSURE TO DIETARY CARCINOGENS

Exposure to dietary carcinogens is a function of the carcinogen content in food products and the amounts consumed. Even if a food product contains low levels of a specific carcinogen, it can still be a major contributor when consumed frequently or in large amounts.

Carcinogens have different levels of cancer-causing potential. One system for classifying those comes from the International Agency for Research on Cancer (IARC), which is part of the World Health Organization. In **Table 1**, some dietary carcinogens (classified by IARC) are listed, including acrylamide and acetaldehyde, which are discussed in this thesis. The fact that a substance (or exposure) is not classified as a carcinogen does not necessarily mean that it is safe. For instance, there are many more naturally occurring compounds than synthetic, and it should be noted that synthetic chemicals are highly regulated while natural chemicals are not.

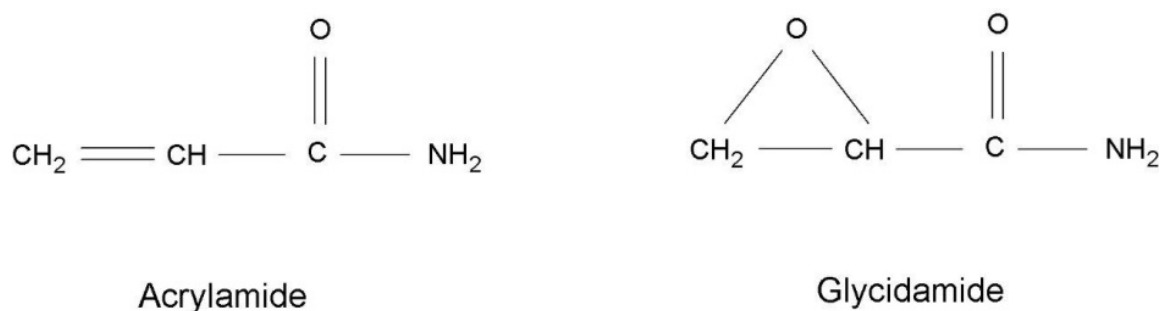
CARCINOGEN	GRO UP	CLASSIFICATION	FOOD SOURCE
Acrylamide	2A	Probably carcinogenic to humans	Potato chips and cereals
Acetaldehyde	2B	Possibly carcinogenic to humans	Alcoholic and non-alcoholic beverages, fruits, vegetables and yogurt.
Acetaldehyde in association with alcoholic beverages	1	Carcinogenic to humans	Alcoholic beverages
Ethanol in association with alcoholic beverages	1	Carcinogenic to humans	Alcoholic beverages
Aflatoxin	1	Carcinogenic to humans	Spices and dried foods like nuts, maize, rice and figs
2-Amino-3-methylimidazol[4,5-f]quinolone (heterocyclic amines)	2A	Probably carcinogenic to humans	Meat and fish cooked at high temperature
N-Nitroso-N-methylurea (N-Nitroso compounds)	2A	Probably carcinogenic to humans	Cured meat
Benzo[a]pyrene (polycyclic aromatic hydrocarbons)	1	Carcinogenic to humans	Meat and fish cooked at high temperature

**Table 1.** Classification of some dietary carcinogens (IARC Monographs).

Examples of dietary carcinogens include aflatoxins produced by fungi (Roze, Hong, & Linz, 2013), heterocyclic amines and PAHs formed when meat and fish are cooked at high temperatures (Layton et al., 1995; Sugimura, 1997), N-nitroso compounds found in cured meat, acrylamide present in potato chips and cereals and acetaldehyde in alcoholic beverages and food items.

Contamination of food can happen at any stage in the food chain, including in the naturally occurring product, as a result of farming methods, during industrial food processing, during storage and distribution, or during cooking (Abnet, 2007). In the complex mixture of our diet, the effects of these compounds may be additive, synergistic or inhibitory to one another. Naturally occurring carcinogens include, for example, acetaldehyde produced during the natural fermentation process of yogurt (H. Cheng, 2010). Deliberate additions include a variety of natural and synthetic additives and artificial substitutes for naturally grown food components, such as sweeteners, color and texture additives, fat substitutes and preservatives. Acetaldehyde is a deliberately added dietary carcinogen when it is used as a flavoring agent. Accidental contaminants are synthetic or natural environmental contaminants in the food chain, such as polychlorinated biphenyls and methyl mercury found in fish, microbial toxins such as those produced by *E. coli* in contaminated food and fungal toxins that may contaminate grain. Acrylamide in potato chips, as the result of the production process would be an example of an accidental contaminant.

## 5.1 EXPOSURE TO ACRYLAMIDE AND ITS CONSEQUENCES



**Figure 4.** Structures of acrylamide and glycidamide

In the year 2000, a Swedish study showed that when rats were fed a diet consisting of fried food, levels of hemoglobin adducts increased (Tareke, Rydberg, Karlsson, Eriksson, & Tornqvist, 2000). Two years later, the finding that acrylamide (figure 4) could be formed during cooking at high temperature (Tareke, Rydberg, Karlsson, Eriksson, & Tornqvist, 2002) led to big headlines in the press worldwide, and shortly thereafter, acrylamide was shown to be formed in the Maillard reaction via the heating of primarily the amino acid asparagine in the presence of glucose or fructose to above 120°C (Motttram, Wedzicha, & Dodson, 2002; Stadler et al., 2002). Acrylamide was

also reported in starchy foods, notably in potato chips and cereal-based foods processed at high temperatures (Lineback, Coughlin, & Stadler, 2012; Tornqvist, 2005). Daily human dietary exposures to acrylamide in the US and EU range from an average of 1 µg/kg bodyweight (bw) up to 4 µg/kg bw (JECFA, 2005). Indicative levels for the acrylamide contents of cereal- and potato-based foods range from 80 µg/kg for baby foods up to 1,000 µg/kg for potato chips (US chips) (EuropeanCommission, 2011).

Acrylamide is also a widely used industrial chemical, used in thickening compounds for wastewater treatment and in certain construction chemicals, such as the infamous sealant Rhoca Gil. During studies of occupational exposure, it was discovered that the control group inexplicably had high levels of adducts (Bergmark, 1997), which in turn led to the aforementioned studies of acrylamide as a dietary carcinogen that were published in 2000

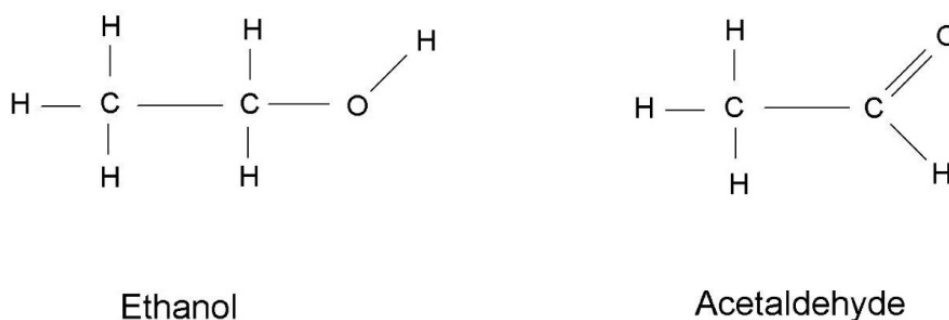
Many studies on acrylamide have been carried out since the findings published in 2000. As acrylamide is known to be a mutagen and has been shown to be carcinogenic in animals, exposure of acrylamide to humans has caused concern (Klaunig, 2008). Nevertheless, epidemiological studies thus far have shown no or only weak associations between exposure to acrylamide and cancer risk in humans (Marsh, Lucas, Youk, & Schall, 1999; Mucci, Adami, & Wolk, 2006; Mucci, Dickman, Steineck, Adami, & Augustsson, 2003; Mucci, Lindblad, Steineck, & Adami, 2004; Mucci et al., 2005; Obon-Santacana et al., 2013; Pelucchi et al., 2006). In a review from 2012 (Lipworth, Sonderman, Tarone, & McLaughlin, 2012), it was concluded that there is no convincing evidence of any carcinogenic effect of dietary acrylamide in humans. However, food questionnaires are difficult to use for exposure assessment because the levels of acrylamide vary not only between different foodstuffs but also between brands (and in some cases, between different production batches, e.g., potato chips), and this could have resulted in incorrect estimation of exposure and thus contributed to the negative findings.

In humans, acrylamide is metabolized to glycidamide (figure 4) by cytochrome P450 2E1 (Sumner et al., 1999). Like acrylamide, glycidamide is a small, uncharged water-soluble molecule, with a relatively short life span in vivo (approximately 2 h). Glycidamide does not seem to accumulate and is readily distributed to all tissues in the body (Doerge, Twaddle, Boettcher, McDaniel, & Angerer, 2007; Doerge, Young, McDaniel, Twaddle, & Churchwell, 2005; Shipp et al., 2006). It has also been shown that both acrylamide and glycidamide readily pass through the human placenta (Annola et al., 2008). Both acrylamide and glycidamide are mainly detoxified by conjugation with glutathione, a reaction carried out by glutathione-S-transferase (Gargas, Kirman, Sweeney, & Tardiff, 2009).

In a two-year cancer study, acrylamide administered in the drinking water induced tumors in the lungs and the Harderian glands in mice of both sexes (Beland et al., 2013). In addition, acrylamide given to male mice gave rise to tumors in the forestomach, whereas in female mice, this compound induced mammary gland, ovary and skin tumor formation. In male and female rats, thyroid tumors were increased, and male rats also demonstrated tumors in the testes, heart and pancreas. Female rats also developed an increased number of clitoral gland, mammary gland, oral cavity and skin

tumors. In the neonatal mouse tumor model, both acrylamide and glycidamide have been shown to be carcinogenic to the liver and to induce primarily A to G and A to T mutations (Von Tungeln et al., 2012). Compared to rats, humans may have a lower risk of developing cancer following acrylamide exposure because bioactivation of acrylamide to glycidamide is lower in humans compared to rats (Kopp & Dekant, 2009). Moreover, glycidamide is more reactive than acrylamide and is therefore considered to be responsible for many of the genotoxic effects of acrylamide (Gamboa da Costa et al., 2003) (Pruser & Flynn, 2011). Acrylamide in higher concentrations has been shown to act as a neurotoxin (IARC, 1994; Kuperman, 1958).

## 5.2 EXPOSURE TO ALCOHOL AND ACETALDEHYDE AND THEIR CONSEQUENCES



**Figure 5.** Structures of ethanol and acetaldehyde

For thousands of years, fermented grain, berries and honey have been used to make alcohol (ethyl alcohol or ethanol, figure 5). In many parts of the world, drinking alcoholic beverages is a common feature of social gatherings. Nevertheless, alcohol consumption increases the risk of acute health conditions, such as injuries incurred as a result of inebriation, and long-term risks such as negative social consequences, alcohol dependency and an increased risk of developing cancer.

The main human exposure to acetaldehyde (figure 5) is through the consumption of alcohol, either as a content of alcoholic beverages or as the main metabolite of ethanol (Lachenmeier, Kanteres, & Rehm, 2009).

Acetaldehyde is also found in smoke (notably tobacco smoke, cannabis smoke, automobile exhaust and wood smoke), various fruits and vegetables and yogurt and other fermented dairy products (Feron et al., 1991; IARC, 1985). In addition, acetaldehyde is produced by bacteria living in the gastrointestinal tract (e.g., in the mouth and colon) (Seitz & Becker, 2007). Pure acetaldehyde has a pungent irritating odor, but in lower concentrations, it has a pleasant fruity aroma. It is therefore also used as a flavoring substance in various foods, such as margarine. It is produced by bacteria

in yogurt and is an important taste-enhancer in these products (H. Cheng, 2010); acetaldehyde levels of at least 8-10 mg/kg have been considered to result in good-flavored yogurts (H. Cheng, 2010) The estimated daily intake of acetaldehyde is 100 µg per kg bw which is considerably lower than the exposure from alcohol consumption and tobacco smoking (Uebelacker & Lachenmeier, 2011). Currently, there is no consensus regarding the safe levels of exposure to acetaldehyde via dietary products. At the same time, acetaldehyde enjoys generally recognized as safe (GRAS) status as a flavoring agent, which has prompted discussions, as there are no defined safe limits for acetaldehyde (Salaspuro, 2009). Human exposure to acetaldehyde is therefore substantial, and the relative contribution of different sources is unknown, although a high level of alcohol consumption likely represents a significant contribution.

A number of epidemiological studies have identified chronic alcohol consumption as an important risk factor for various types of cancer (IARC, 2010, 2012). Even if the IARC has classified ethanol in alcoholic beverages as a carcinogen, there is evidence that acetaldehyde is the causative agent in alcohol-related cancers. The IARC has classified acetaldehyde as potentially carcinogenic to humans (group 2B), but in association with alcoholic beverages, acetaldehyde is classified as carcinogenic to humans (group 1) (IARC, 2012). Alcohol-associated tumors are generally located in the oral cavity, pharynx, esophagus, liver, colon, rectum and breast (in women).

### **5.2.1 Alcohol and acetaldehyde metabolism**

If two individuals consume the same amount of alcohol, they will most likely have very different levels of ethanol and acetaldehyde in their tissues. Blood alcohol concentration is influenced by several parameters, including the type of alcoholic beverage, the rate of consumption, the presence or absence of food in the stomach, chronic alcohol consumption, the elimination rate and genetic factors (Kopun & Propping, 1977). When ethanol enters the body, it is metabolized to acetaldehyde by the enzymes alcohol dehydrogenase (ADH), cytochrome P4502E1 (CYP2E1) and catalase. Several isoenzymes (variants of the same enzyme that catalyze identical chemical reactions) of ADH are present in different tissues and the rate at which ethanol is oxidized to acetaldehyde depends on the enzymatic activity of ADH (Seitz & Becker, 2007). Among people of Chinese, Korean and especially Japanese descent, a version of the ADH gene (allele *ADH1B\*2*) that encodes for a much more active enzyme is common (Druesne-Pecollo et al., 2009); this *ADH1B\*2* allele encodes an enzyme that is approximately 40 times more active than the enzyme encoded by the *ADH1B\*1* allele (Bosron, Ehrig, & Li, 1993; Ehrig, Muhoberac, Brems, & Bosron, 1993). Studies on Japanese populations with the more active ADH variant show that these individuals have a higher risk of pancreatic cancer than those with the more common form of ADH (Kanda et al., 2009).

The main pathway for the detoxification of acetaldehyde to acetate proceeds via mitochondrial aldehyde dehydrogenase (ALDH). There are multiple forms of ALDH with different activities, but ALDH2 is responsible for the majority of acetaldehyde metabolism (Seitz & Becker, 2007). People of Asian ethnic origin who have low or no

ALDH2 activity can develop facial flushing, nausea and hypotension due to acetaldehyde accumulation from drinking (Goedde et al., 1992).

In short, if ADH activity is low, low levels of acetaldehyde are produced from alcohol, and high ALDH activity indicates that acetaldehyde is eliminated effectively. In this case, the alcohol remains in the body for a longer time when the acetaldehyde levels are low, leaving the individual inebriated for a longer period of time. In the opposite case, high ADH activity metabolizes the alcohol to acetaldehyde effectively, and low ALDH activity eliminates the produced acetaldehyde slowly, leaving the individual feeling sick.

## 6 BIOMARKERS RELATED TO FOOD

Dietary contaminants, such as persistent halogenated chemicals and heavy metals, are often analyzed as such or as metabolites in human biological samples, and a large number of investigations of such contaminants have been carried out (Hedrick et al., 2012). Urinary aflatoxins have been studied, and for the urinary excreted DNA adduct aflatoxin-B1-N-7-guanine, it was shown that the amounts of dietary intake of the mycotoxin correlated to the DNA adduct level (Crews et al., 2001; Groopman, Wogan, Roebuck, & Kensler, 1994; Walton et al., 2001). PAH-DNA adducts in leukocytes are considered to be good biomarkers of exposure to PAHs (Schoket, 1999), and it has been shown that dietary intake of PAHs leads to increased adduct levels (Godschalk et al., 2000; Poirier et al., 1998; Rothman et al., 1993). For aflatoxin and PAHs, it has also been demonstrated that increased adduct levels are associated with a higher risk of developing cancer later in life (Qian et al., 1994; Veglia et al., 2008; Vineis & Perera, 2000). It would therefore be very interesting to perform more of these types of investigations. However, due to a lack of high-throughput techniques for DNA adduct analysis, it is not possible to carry out large-scale prospective studies involving mass screening of DNA adducts. Currently, it is feasible to analyze several hundred samples in a reasonable time frame, but not the tens of thousands of samples that would be required for large-scale human studies.

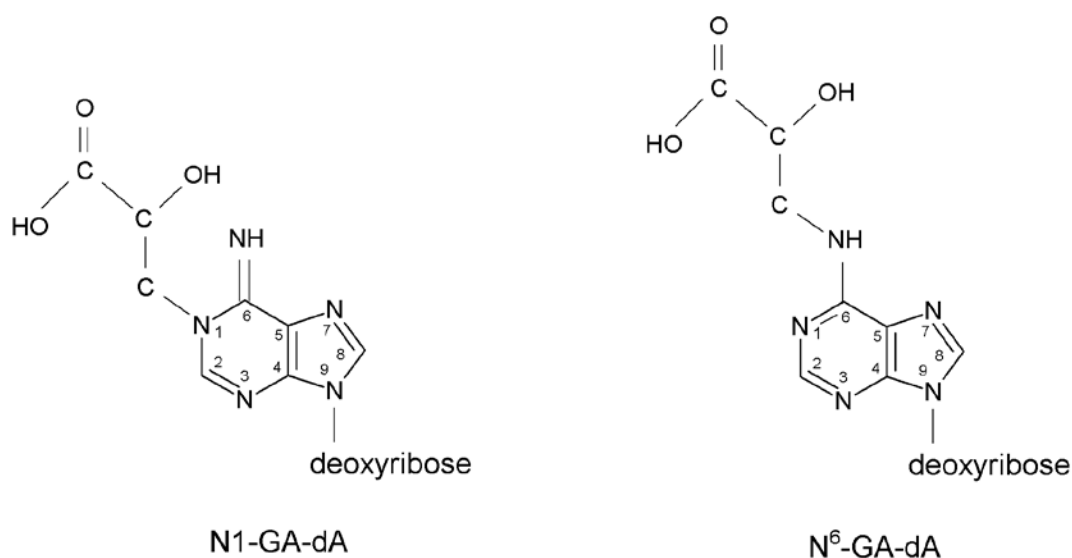
Protein (e.g., hemoglobin) adducts and urinary metabolites have been used to assess human exposure to acrylamide (Bergmark, Calleman, He, & Costa, 1993; Urban, Kavvadias, Riedel, Scherer, & Tricker, 2006; Vesper, Sternberg, Frame, & Pfeiffer, 2013). For these studies, the methods used were gas chromatography (GC)- or liquid chromatography (LC)-MS/MS-based, which represent sensitive approaches, and plenty of hemoglobin can be isolated from a single normal-sized blood sample (Tornqvist et al., 2002). These investigations have demonstrated the difficulty in assessing human exposure to acrylamide from register data on food concentrations, as mentioned above (Hagmar, Wirfalt, Paulsson, & Tornqvist, 2005; Wilson, Balter, et al., 2009; Wilson, Vesper, et al., 2009). Even in a very large study of 7,000 persons, the correlation between levels in food and hemoglobin-adduct levels was poor (Tran, Barraj, Bi, Schuda, & Moya, 2013). In other studies on acrylamide-derived hemoglobin adducts, it was observed that high adduct levels were associated with reduced fetal growth (Duarte-Salles et al., 2013) and with reduced birth weight and head circumference (Pedersen et al., 2013).

Acetaldehyde protein adduct formation has been presented in several studies (Nicholls, de Jersey, Worrall, & Wilce, 1992; Torrente, Freeman, & Vrana, 2012). The focus of these studies has been to develop biomarkers for research on alcohol abuse. To date, these biomarkers lack the sensitivity and accuracy to be able to differentiate between different drinking behaviors, such as abstinence, moderate drinking, heavy drinking or binge drinking.



## 6.1 DNA ADDUCTS OF GLYCIDAMIDE

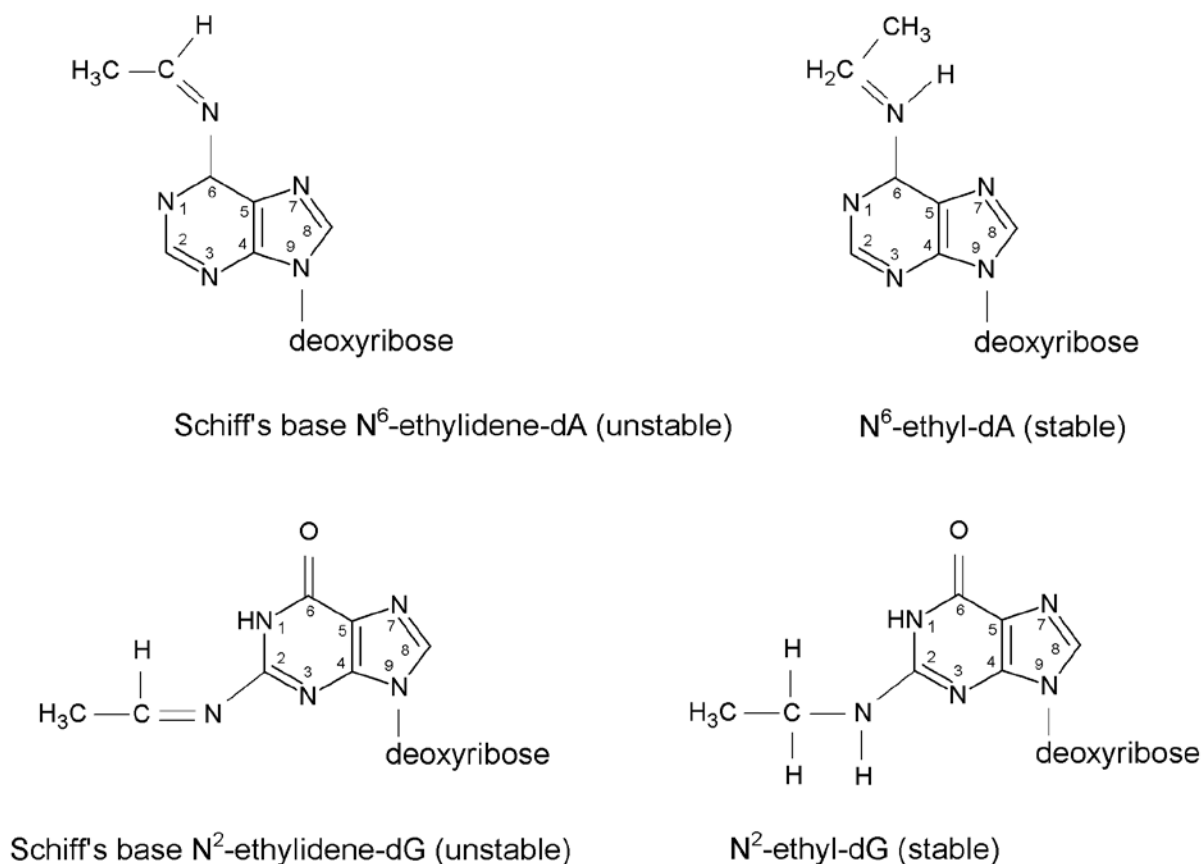
The most abundant DNA adduct of glycidamide is N7-(2-carbamoyl-2-hydroxyethyl)guanine (Segerback, Calleman, Schroeder, Costa, & Faustman, 1995). Other glycidamide-derived DNA adducts include N1-(2-carboxy-2-hydroxyethyl)deoxyadenosine (N1-GA-dA) (**Figure 6**) and its rearrangement product N<sup>6</sup>-(2-carboxy-2-hydroxyethyl)deoxy- adenosine (N<sup>6</sup>-GA-dA) (**Figure 6**), which was studied in this thesis (Gamboa da Costa, et al., 2003). The least abundant of the known DNA adducts of glycidamide is N3-(2-carbamoyl-2-hydroxyethyl)adenine. N1-GA-dA has to date not been detected in animals exposed to acrylamide, but both N7-(2-carbamoyl-2-hydroxyethyl)guanine and N3-(2-carbamoyl-2-hydroxyethyl)adenine have been analyzed in many studies following acrylamide and glycidamide exposure (Doerge et al., 2005; Gamboa da Costa, et al., 2003).



**Figure 6.** Adduct of glycidamide

DNA adducts from acrylamide itself have not been detected, possibly since the reactivity towards DNA of this compound is much less than that of glycidamide (Segerback, et al., 1995). In investigations of DNA adducts from glycidamide in rodents it has been shown that there is a linear dose-response relationship for adduct formation, at least at low doses (Tareke et al., 2006; Zeiger et al., 2009), and that the levels are similar in different tissues (Doerge, da Costa, et al., 2005). These results imply that the tissue specificity of cancers resulting from acrylamide exposure in mice and rats (Beland, et al., 2013) is not primarily driven by adduct formation.

## 6.2 DNA ADDUCTS OF ACETALDEHYDE



**Figure 7.** DNA adducts of acetaldehyde

The DNA adduct N<sup>2</sup>-ethylidene-dG (**Figure 7**) studied in this thesis is the one of the major DNA adducts resulting from the reaction of acetaldehyde (Vaca, Fang, & Schweda, 1995). Although N<sup>2</sup>-ethylidene-dG is unstable and difficult to measure directly, it can be measured after conversion to the stable N<sup>2</sup>-ethyl-dG (**Figure 7**) by treatment of DNA with a reducing agent, such as cyanoborohydride, NaBH<sub>3</sub>CN.

Acetaldehyde can also form 1,N<sup>2</sup>-propano-dG (Garcia et al., 2011; Wang et al., 2000) and interstrand DNA cross-links, which is a mutagenic lesion (Stein, Lao, Yang, Hecht, & Moriya, 2006). The mechanism for this has been suggested to occur via the propano adduct (Murakami, Esaka, & Uno, 2009; Theruvathu, Jaruga, Nath, Dizdaroglu, & Brooks, 2005). It has been shown that the propano adduct can be formed from the ethylidene adduct via participation of certain amino acids, histones or polyamines (Inagaki, Esaka, Deyashiki, Sako, & Goto, 2003; Sako, Yaekura, & Deyashiki, 2002; Theruvathu, et al., 2005).

It has also been suggested that ethanol can form the adduct 1-hydroxyethyl at the C8 position of guanine (Nakao, Fonseca, & Augusto, 2002). In the latter investigation, it

was found that control animals had high levels of this adduct in liver DNA. The levels were only slightly increased if the animals were treated with ethanol, and it was therefore suggested that lipid peroxidation was the source for adduct formation.

Almost 20 years ago, Fang and Vaca found increased levels of N<sup>2</sup>-ethyl-dG adducts in lymphocytes of alcohol consumers and in liver DNA from mice exposed to 10% alcohol in their drinking water, without treating DNA with a reducing agent (Fang & Vaca, 1995, 1997). The authors suggested that glutathione or cellular ascorbic acid could carry out this reduction *in vivo*. However, other researchers have not been able to detect N<sup>2</sup>-ethyl-dG without using a reducing agent; therefore, the efficiency of the potential endogenous reduction of N<sup>2</sup>-ethylidene-dG to N<sup>2</sup>-ethyl-dG remains uncertain. When using a reducing agent during the DNA isolation step, much higher levels of N<sup>2</sup>-ethyl-dG have been detected (Nagayoshi et al., 2009; Wang et al., 2006).

Formation of N<sup>2</sup>-ethylidene-dG (analyzed as N<sup>2</sup>-ethyl-dG after reduction) has been reported in both animal and human studies (Balbo et al., 2012a, 2012b; Nagayoshi, et al., 2009; Yu et al., 2010; Yukawa et al., 2012). In addition to demonstrating an increased adduct level following ethanol intake, these investigations have shown that there are effects (although rather weak) of alcohol consumption that can be measured as an increased level of N<sup>2</sup>-ethyl-dG. Furthermore, it has been shown that there are significant effects of genetic polymorphisms on adduct levels (Matsuda, Yabushita, Kanaly, Shibutani, & Yokoyama, 2006; Yukawa, et al., 2012) and that ALDH2 knockout mice have, in several tissues, higher adduct levels than wild-type animals (after alcohol consumption) (Nagayoshi, et al., 2009; Yu, et al., 2010).

The formation of N<sup>2</sup>-ethylidene-dA (**Figure 7**), one of the DNA adducts investigated in this thesis, has previously not been demonstrated. Therefore, no *in vivo* data have been reported.

N<sup>2</sup>-Ethyl-dG has been shown to induce G to C transversions during DNA synthesis via the actions of *E. coli* Pol I (Terashima et al., 2001). Human DNA synthesis seems to be strongly blocked by N<sup>2</sup>-ethyl-dG or able to bypass the adduct in an error-free manner, depending on the type of DNA polymerase studied (Choi & Guengerich, 2005, 2006; Matsuda et al., 1999; Perrino et al., 2003; Upton et al., 2006). Thus, it has been suggested that because N<sup>2</sup>-ethyl-dG can be efficiently bypassed by one of the main replicative DNA polymerases, this adduct may not be a pre-mutagenic source in humans.

## 7 METHODS FOR ANALYSIS OF DNA ADDUCTS

The levels of different types of DNA adducts detected in human blood or tissue samples range from  $1/10^6$ - $1/10^9$  adducts per normal nucleotide (Bartsch, 1996; Phillips et al., 2000). Sensitive analytical methods are therefore needed when analyzing human samples. The main quantitative methods employed consist of fluorescence, immune assays,  $^{32}\text{P}$ -postlabeling, mass spectrometry (MS) and accelerator MS (Phillips, et al., 2000). These methods have different advantages and disadvantages; for example, expensive equipment is required for certain methods (MS and accelerator MS). These methods also differ also in sensitivity and thus require different amounts of DNA (which is often a limiting factor for their application).  $^{32}\text{P}$ -Postlabeling may be the most sensitive method, but it is often applied in a semi-quantitative way (standards for correcting of recovery are not always used).

Two methods for analysis of DNA adducts of glycidamide and acetaldehyde were developed and tested in this thesis, LC-MS/MS and  $^{32}\text{P}$ -postlabeling.

### 7.1 LC-MS/MS

The use of MS for the detection of DNA adducts has increased with the development of this method. One example is the development of LC coupled to MS to help separate the adduct of interest from unmodified nucleosides. Prior to analysis, the DNA is digested to generate a mixture of normal and unmodified nucleosides or nucleotides. The DNA sample is then enriched, commonly by solid-phase extraction or the use of immunoaffinity columns or on-line column-switching (Koc & Swenberg, 2002).

The main advantage of MS, compared to other assays, is its chemical specificity. Limitations of the assay are the relatively large quantities of DNA needed, typically 10-100  $\mu\text{g}$  for a single analysis, and its sensitivity. However, the sensitivity is constantly improving, and a limit of detection in the order of 1 adduct / $10^8$  normal nucleotides is normally attained (Farmer et al., 2005).

### 7.2 $^{32}\text{P}$ -POSTLABELING

$^{32}\text{P}$ -postlabeling is a method for the sensitive detection of DNA damage produced by reactive chemicals and genotoxins. Postlabeling denotes that the sample is labeled (in this case, with a radioactive isotope) after the sample has been obtained.

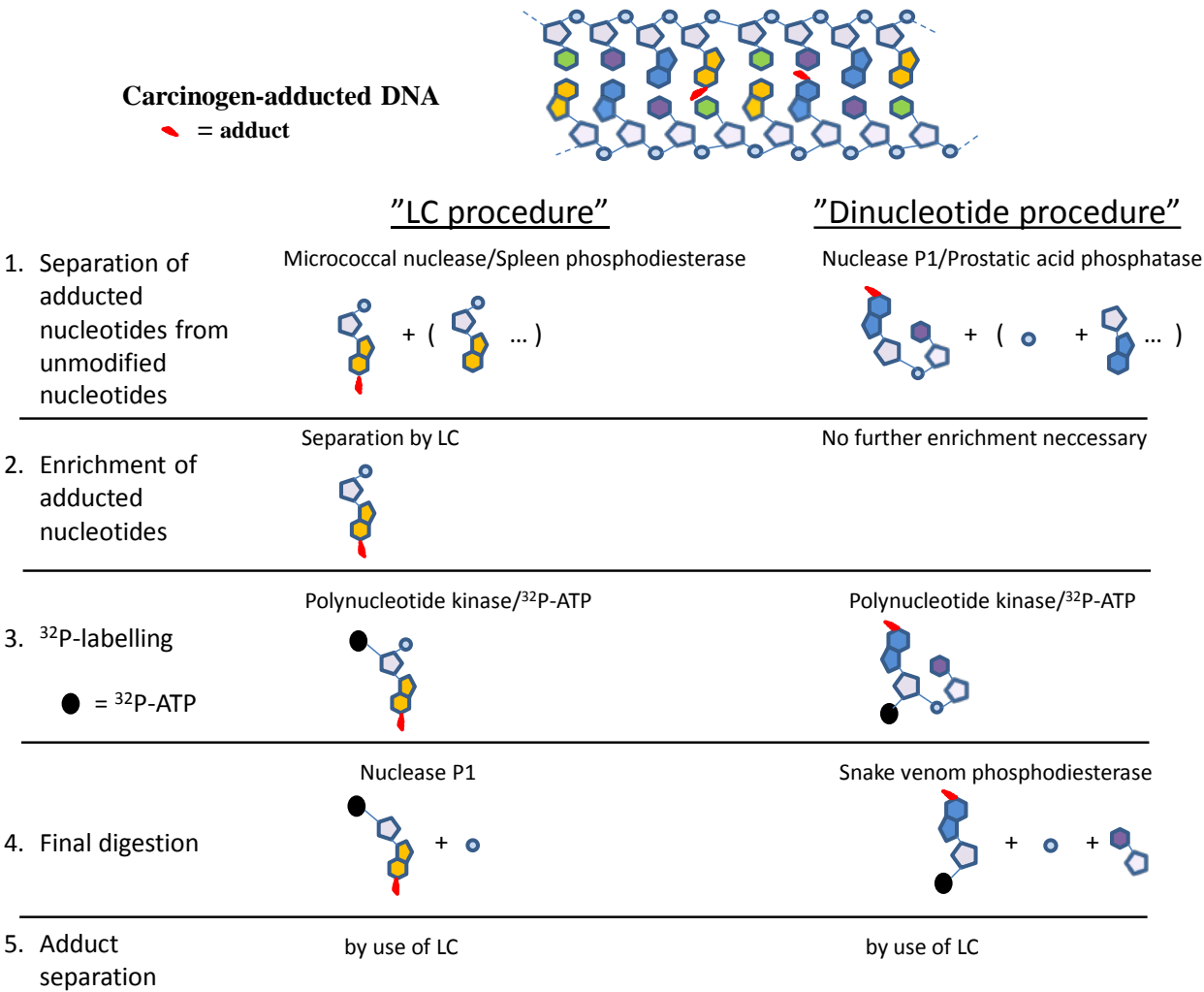
This technique began in the 1980s with the development of a  $^{32}\text{P}$ -labeling method for the analysis of simple alkyl-modified DNA adducts from reactions with, for example, formaldehyde. (Randerath, Reddy, & Gupta, 1981; Reddy, Gupta, Randerath, & Randerath, 1984). Since then, the method has been improved to enable the detection of a wide variety of genotoxins, including bulky aromatic and/or hydrophobic adducts

formed by, for example, benzo[a]pyrene or 4-aminobiphenyl, mycotoxins and complex mixtures such as tobacco smoke. Today, this method is widely used to monitor exposure to carcinogens in humans and to carry out DNA adduct analyses in animal experiments and in vitro studies. The assay is extremely sensitive and can detect a single adduct in  $10^7$  to  $10^{10}$  normal nucleotides (Beach & Gupta, 1992); in comparison, this sensitivity would be equivalent to being able to find a single individual amongst the entire world population. Other advantages of this method include the ability to detect structurally diverse DNA adducts without the need for prior characterization and its applicability to a wide range of sample types. In addition, the assay requires very low amounts of DNA (5-10  $\mu$ g compared to 50  $\mu$ g or more generally used for other techniques).

The limitations of the  $^{32}\text{P}$ -postlabeling method include the need to handle a specific high-activity radioisotope and the rather labor-intensive and low-throughput protocols. In addition, no structural information of the adduct is obtained, and the adducts are instead mainly characterized by their co-chromatography with synthetic adduct standards. The adducted nucleotides also need to be chemically stable, and the labeling efficiencies vary between adducts. Over the years, the assay has been improved by introducing new adduct-enrichment procedures, including enrichment with nuclease P1 digestion (and a variant of that called the dinucleotide monophosphate version), extraction with organic solvent (for hydrophobic adducts), LC-based separations and immune affinity purification. The use of such enrichment procedures (such as nuclease P1) may increase the sensitivity of the assay to a single adduct in  $10^{10}$  normal nucleotides (Jones, 2012).

In the original procedure, the DNA is digested to deoxyribonucleoside-3'-phosphates using micrococcal nuclease and phosphodiesterase (from calf spleen). The obtained 3'-nucleotides (adducted and normal nucleotides) are labeled on the 5'-side by transfer of a  $^{32}\text{P}$ -phosphate from  $[\gamma^{32}\text{P}]\text{-ATP}$  with the aid of T4 polynucleotide kinase. The mixture is then separated by thin-layer chromatography. An X-ray film is exposed on the plate on which the adducts (and normal nucleotides) appear as black spots. In the final step, the amount of radioactivity in the adduct spots is measured (after scintillation counting of the cut-out adduct spots), and adduct levels are calculated. This assay is not very sensitive, as the thin-layer chromatography step leaves a large excess of normal nucleotides. However, the process was improved by orders of magnitude after the introduction of an enrichment procedure following DNA digestion. If nuclease P1 is used for enrichment, the enzyme dephosphorylates normal nucleotides (which then cannot be labeled in the following step), but many adducted nucleotides (particularly bulky ones) are resistant towards the enzyme and will remain as 3'-phosphates. Using this procedure, more than 99.99% of normal nucleotides are dephosphorylated, thus improving the sensitivity. Some small hydrophilic adducts, e.g., alkyl groups bound to N7 of dG, are not resistant towards nuclease P1, and some LC-based methods (like solid phase extraction) can be used to separate adducted and normal nucleotides (Kumar & Hemminki, 1996).

The LC and dinucleotide procedures are outlined in the steps 1-5 below:



**Figure 8.** The <sup>32</sup>P-postlabeling assays used in this thesis, step by step

1. In the first step of the LC procedure (**Figure 8**), the DNA is digested by micrococcal nuclease and spleen phosphodiesterase to obtain a mixture of normal (unmodified) 3'-nucleotides and adducted 3'-nucleotides. In the dinucleotide procedure, the DNA is digested by nuclease P1 and prostatic acid phosphatase to obtain a mixture of normal 3'-nucleotides and adducted dinucleotides containing the adduct and a normal nucleotide on the 3'-end.
2. When using the LC procedure, this extra step removes the vast bulk of the normal nucleotides, leaving only adducted nucleotides for the following labeling step carried out on a HPLC system. For the dinucleotide procedure, an extra enrichment step is not needed because nuclease P1 enzymatically digests normal nucleotides so that they are no longer substrates for T4 polynucleotide kinase.
3. The obtained 3'- nucleotides or dinucleotides are labeled on the 5'-side by transfer of a  $^{32}\text{P}$ -phosphate from  $[\gamma^{32}\text{P}]\text{-ATP}$  with the aid of T4 polynucleotide kinase.
4. The bisphosphonates of the labeled mononucleotides are dephosphorylated on the 5'-side by treatment with nuclease P1. When adducts are labeled as dinucleotides, further digestion by snake venom phosphodiesterase after the labeling reaction generates a labeled 5'-monophosphate.
5. After addition of a UV marker to the adducted nucleotide being analyzed, the labeled 5'-monophosphates are separated using LC with online radioactive detection.

The main difference between how postlabeling is carried out in this laboratory compared to most other laboratories is that much smaller amounts of  $^{32}\text{P}\text{-ATP}$  are used, although there are recent reports of similar attempts (Kovacs, Anna, Rudnai, & Schoket, 2011). In addition, the assay has been made quantitative by introducing an external standard based on DNA samples with known adduct levels or by the use of a known amount of an adducted nucleotide. In this thesis, the size of the radioactive peak was quantified by parallel labeling of a DNA sample with a known level of N1-GA-dA or known amounts of the standards of N<sup>6</sup>-ethyl-dA or N<sup>2</sup>-ethyl-dG.

## **8 AIM**

- The aim of this thesis was to develop and apply biomarkers for human exposure to some dietary chemical carcinogens

### **8.1 OBJECTIVES**

- To characterize DNA adducts formed by glycidamide and acetaldehyde
- To develop methods for the analysis of DNA adducts from glycidamide and acetaldehyde
- To analyze such DNA adducts in animal and human tissue samples



## **9 BRIEF DESCRIPTION OF METHODS**

### **9.1 SYNTHESIS AND CHARACTERIZATION OF ADDUCT STANDARDS (PAPERS I-IV)**

Glycidamide and acetaldehyde were reacted with 2'-deoxyribonucleosides to produce the desired adducts. The adducted nucleosides prepared from glycidamide were N1-GA-dA and N<sup>6</sup>-GA-dA (the latter made from Dimroth rearrangement of the former). The adducted nucleosides prepared from acetaldehyde were N<sup>6</sup>-ethyl-dA and N<sup>2</sup>-ethyl-dG. After the initial reaction with acetaldehyde, an unstable Schiff base was obtained that was converted to the stable ethyl adduct by treatment with the reducing agent NaBH<sub>3</sub>CN (Wang, et al., 2000). The adducted nucleosides were purified by LC and sent to either Leicester University (N<sup>2</sup>-ethyl-dG) or Åbo Academy (N1-GA-dA and N<sup>6</sup>-ethyl-dA) for structure determination by MS. The adducted nucleosides were identified by their UV spectra. In addition, the corresponding adducts with 2'-deoxyribonucleoside-3'- (and 5') monophosphates were prepared. The adducted 3'-nucleotides were also used as standards in the postlabeling assay, and the corresponding adducted 5'-nucleotides were used as UV markers in the LC separation after postlabeling. The adducted nucleotides were identified by their UV spectra and co-chromatography (after dephosphorylation) with the corresponding adducted deoxyribonucleoside obtained.

The [<sup>15</sup>N<sub>5</sub>]N<sup>2</sup>-ethyl-dG stable isotope internal standard was synthesized by incubating [<sup>15</sup>N<sub>5</sub>]-labeled dG with acetaldehyde in the presence of NaBH<sub>3</sub>CN. This standard was used for MS analysis of the acetaldehyde-derived adducts obtained after exposure to cannabis smoke (Paper II) or alcohol consumption (Paper III).

### **9.2 ISOLATION OF DNA**

DNA from livers of mice treated with acrylamide was isolated by two different methods: a phenol/chloroform extraction protocol for tissues (Lagerqvist et al., 2008) and a slightly modified protocol for a commercial kit from Qiagen (Paper I). For analysis of acetaldehyde-derived adducts, the same commercial kit from Qiagen was used to isolate DNA from human lung (Paper II) and human leukocytes (Paper III). To isolate DNA from acetaldehyde-treated and untreated cells, a phenol/chloroform extraction protocol was used (Gupta, 1984) (Paper IV).

### **9.3 TREATMENT OF DNA, CELLS OR MICE WITH GLYCIDAMIDE**

To compare adduct levels of N1-GA-dA, both intact and lysed leukocytes (isolated from the buffy coat of a non-smoking blood donor), as well as double- and single stranded DNA, were treated with glycidamide.

Seven-week-old male CBA mice were given a single oral dose of acrylamide dissolved in phosphate-buffered saline (40 or 60 mg of acrylamide per kg bw) or a single oral dose of the same buffer as a control. The animals were sacrificed after 43 h, and livers were removed and stored at -80°C until further analysis with the postlabeling assay.

#### **9.4 TREATMENT OF DNA WITH CANNABIS AND CIGARETTE SMOKE (PAPER II)**

Calf thymus DNA was exposed to smoke from 10 tobacco or 1, 5 or 10 cannabis cigarettes, which was bubbled through the DNA solution in buffer using an aspirator, at room temperature (Singh, Kaur, & Farmer, 2005). The control DNA was incubated with the same buffer. These DNA samples were used for analysis of N<sup>2</sup>-ethyl-dG by LC-MS/MS at Leicester University.

#### **9.5 HUMAN INTERVENTION STUDY (PAPER III)**

Thirty healthy male non-smoking individuals (ranging from 21 to 44 years) participated in the study, which was carried out in Lodz, Poland. Venous blood samples were collected before consumption of 150 mL of vodka (containing 42% pure ethanol) and at 0 h, 3-5 h, 24 h and 48 h post-alcohol consumption. The blood samples were frozen and shipped to Leicester University for isolation of DNA and analysis of N<sup>2</sup>-ethyl-dG by LC-MS/MS.

#### **9.6 STABILITY OF N<sup>2</sup>-ETHYLIDENE-DG IN DNA AND CELLS (PAPER IV)**

Both DNA and Chinese hamster ovary cells were treated with 1.6 mM acetaldehyde at 37°C. The DNA used was obtained from human buffy coats because it had previously been noted that commercially available DNA, such as calf thymus or salmon sperm DNA, resulted in many radioactive peaks of unknown origin in the postlabeling assay.

After 24 h, DNA was precipitated, and cells were washed to remove the acetaldehyde. DNA and cells were further incubated for 0, 4, 8, 24, 48, 72 or 96 h at 37°C. In a separate experiment, the cells were lysed and their activity inhibited after treatment with acetaldehyde, but before the post incubation. Lysis was done by adding 1% sodium dodecyl sulfate and 1 mM ethylenediaminetetraacetic acid. To convert N<sup>2</sup>-ethylidene-dG to N<sup>2</sup>-ethyl-dG, DNA and cells were treated with NaBH<sub>3</sub>CN for 3 h and subsequently dialyzed to remove the NaBH<sub>3</sub>CN.

## **9.7 ADDUCT ENRICHMENT**

### **9.7.1 Nuclease P1 enrichment (Papers I and IV)**

Both glycidamide- and acetaldehyde-modified DNA was enzymatically digested by nuclease P1. This enzyme is a zinc-dependent endonuclease that yields nucleoside 5'-phosphates and 5'-phosphooligonucleotides, which are no longer substrates for the phosphorylation reaction with [ $\gamma$ - $^{32}\text{P}$ ]ATP and can thus not be labeled with  $^{32}\text{P}$ . Some DNA adducts are, however, resistant towards nuclease P1 (which cannot cleave the phosphodiester bond between the adducted and the normal nucleotide). To obtain dinucleotides that could be labeled with  $^{32}\text{P}$ , the phosphate groups on the adducted nucleotides were removed by incubation with prostatic acid phosphatase.

In this project, it was found that N<sup>6</sup>-ethyl-dA was not resistant to nuclease P1, although other adducts at N<sup>6</sup> dA had been shown to be resistant, for example, the N<sup>6</sup>-dA adduct of propylene oxide (Plna, Nilsson, Koskinen, & Segerback, 1999). Therefore, N<sup>6</sup>-ethyl-dA was enriched using LC (Paper IV).

### **9.7.2 LC enrichment of N<sup>6</sup>-ethyl-dA (Paper IV)**

DNA samples were digested with micrococcal nuclease and spleen phosphodiesterase. The resulting mixture of adducted and normal 3'-phosphomononucleotides was separated using LC, and the fraction containing the adduct was collected and used for postlabeling. The retention time of the adduct had previously been determined by repeated injections of sufficient amounts of N<sup>6</sup>-ethyl-3'-dAMP to allow the detection of the adduct by UV analysis.

### **9.7.3 DNA digestion for LC/MS-MS analysis (Papers II and III)**

Dried DNA with an added internal standard [ $^{15}\text{N}_5$ ]N<sup>2</sup>-ethyl-dG was dissolved in Tris-HCl buffer containing  $\text{MgCl}_2$  and  $\text{NaBH}_3\text{CN}$ . The DNA samples were then digested to 2'-deoxyribonucleosides using snake venom phosphodiesterase I, deoxyribonuclease I and shrimp alkaline phosphatase.

## **9.8 DNA ADDUCT DETECTION**

### **9.8.1 $^{32}\text{P}$ -postlabeling (Papers I and IV)**

The dried samples with adducted, enriched mono- or dinucleotides were enzymatically labeled through incubation with radioactive [ $\gamma$ - $^{32}\text{P}$ ]ATP in the presence of the catalyst enzyme T4 polynucleotide kinase. After labeling, samples were further digested using nuclease P1 or snake venom phosphodiesterase to generate adducted 5'-

monophosphates before separation by LC with online radioisotope detection. Recovery was optimized using nucleotide standards and adducted DNA.

### **9.8.2 LC-MS/MS (Papers II and III)**

The samples were loaded in the trap column, on which the unmodified nucleotides were eluted as waste. The purified adduct N<sup>2</sup>-ethyl-dG was trapped on the column and back-flushed from the trap column onto the analytical column. Finally, the adducts were eluted into the mass spectrometer for analysis. Using online-column switching valve technology, the enrichment step was carried out with the trap column, and there was no need for time-consuming off-line pre-purification steps of the adduct using, for example, solid-phase extraction.

For calibration, different amounts of N<sup>2</sup>-ethyl-dG and a fixed amount of the internal standard [<sup>15</sup>N<sub>5</sub>]N<sup>2</sup>-ethyl-dG were added to calf thymus DNA, followed by enzymatic hydrolysis in the absence of NaBH<sub>3</sub>CN.

## **9.9 STATISTICAL DATA ANALYSIS (PAPERS II AND III)**

Statistical evaluation of the change in levels of N<sup>2</sup>-ethyl-dG following alcohol intake (Paper III) was performed using the Wilcoxon signed-rank test with Statistica version 10 (StatSoft, Inc., Uppsala, Sweden). The level of statistical significance was set at  $p < 0.05$ . Spearman rank correlation was utilized to assess the association between N<sup>2</sup>-ethyl-dG and age or body mass index.

Statistical evaluation of the human lung data (Paper II) was performed using the Mann-Whitney U-test (StataCorp, TX). The level of statistical significance was set at  $p < 0.05$ . Least squares linear regression analysis was performed using the Stata statistical software package.

## **9.10 METHODOLOGICAL ISSUES**

The <sup>32</sup>P-postlabeling assay developed for the analysis of glycidamide (Paper I) demonstrated a high level of sensitivity, as this method could detect a single adduct per 10<sup>8</sup> normal nucleotides. N1-GA-dA (analyzed as N<sup>6</sup>-GA-5'-dAMP) was easy to detect in DNA in vitro and in cells exposed to glycidamide, which is here demonstrated for the first time with the <sup>32</sup>P-postlabeling method. The two assays used for the analysis of N<sup>2</sup>-ethyl-dG, LC-MS/MS and <sup>32</sup>P-postlabeling, demonstrated similar sensitivities, approximately a single adduct per 10<sup>8</sup> normal nucleotides. Similar LC-MS/MS methods have been used in two other laboratories, with reports of similar sensitivities (Matsuda, et al., 2006; Wang, et al., 2006).

Development of the new LC-MS/MS method in conjunction with online-column switching valve technology led to the successful detection of N<sup>2</sup>-ethyl-dG adducts in DNA (Paper II). This method allows for analysis without prior off-line pre-purification of the sample from the vast excess of unmodified nucleosides that would otherwise interfere with the MS analysis (Wang, et al., 2006). In our study (Paper II) it was for the first time shown that cannabis smoke could generate acetaldehyde-derived DNA adducts.

With the development of the postlabeling method described in paper IV, we were also able to show for the first time that N<sup>6</sup>-ethyl-dA is formed in DNA exposed to acetaldehyde. The two postlabeling assays for N<sup>6</sup>-ethyl-dA and for N<sup>2</sup>-ethyl-dG demonstrated similar sensitivities, approximately one adduct per 10<sup>8</sup> normal nucleotides. The recovery of N<sup>6</sup>-ethyl-dA was somewhat lower, but it had a later retention time, which reduced the background noise. The level of N<sup>6</sup>-ethyl-dA was, after treatment of the DNA in vitro with acetaldehyde, considerably lower than that of N<sup>2</sup>-ethyl-dG.

## 10 RESULTS AND DISCUSSION

### 10.1 ANIMAL STUDIES WITH ACRYLAMIDE

Glycidamide adducts to N7-guanine and N3-adenine have been detected in many studies with animals exposed to acrylamide (Beland, et al., 2013; Doerge, da Costa, et al., 2005; Gamboa da Costa, et al., 2003; Watzek et al., 2012). In most cases LC-MS/MS has been used, but in one case radiolabeled acrylamide was used (Segerback, et al., 1995). Based on these studies, it was expected that N<sup>6</sup>-GA-dA would be found, but it could not be detected in any of the DNA samples from livers of acrylamide-treated mice (Paper I). It had been estimated that the levels would be within the range of the sensitivity of the assay. The reasons for not detecting this adduct could be (1) that the expected level of N1-GA-dA was overestimated in double-stranded DNA (N1-dA adducts are formed at 10- to 20-fold higher yields in single-stranded compared to double-stranded DNA (Selzer & Elfarra, 1999)); (2) related to DNA repair (in earlier animal studies, the time between exposure and sacrifice was shorter — only a few hours compared to 48 hours in this experiment — allowing for DNA repair (Gamboa da Costa, et al., 2003)); (3) related to the route of exposure (oral versus intraperitoneal.) or (4) the result of methodological differences.

### 10.2 BACKGROUND LEVELS OF ADDUCTS

Background levels of N<sup>2</sup>-ethyl-dG were not detected in DNA in vitro without reduction (Paper II), which is consistent with other findings (Fang & Vaca, 1995; Wang, et al., 2006). After treating control DNA with a reducing agent most other laboratories report detectable levels of N<sup>2</sup>-ethyl-dG in DNA in vitro or in mammalian cells (Chen et al., 2007; Hori et al., 2012; Vaca, Nilsson, Fang, & Grafstrom, 1998; Wang, et al., 2006), but this was not the case with the LC-MS/MS- or postlabeling assay used in this thesis (Papers II and IV). This could be due to different sensitivities of the assay or related to the origin of the DNA and the isolation procedure. Other researchers have also demonstrated that rodent tissue DNA without any exposure to acetaldehyde had detectable levels of N<sup>2</sup>-ethyl-dG (after treatment with a reducing agent) (Matsuda, et al., 1999; Nagayoshi, et al., 2009; Wang, et al., 2006). The source of the adduct remains unknown but could be due to a methodological artifact (although this was tested and ruled out in one study (Wang, et al., 2006), or to endogenously formed acetaldehyde (Jacobsen, 1950).

### 10.3 ANALYSIS OF N<sup>2</sup>-ETHYL-DG IN HUMAN TISSUE DNA

The levels of N<sup>2</sup>-ethyl-dG in human lung DNA did not differ between smokers and non-smokers, and these levels were approximately 13 per 10<sup>8</sup> normal nucleotides (Paper II). Human lung DNA was previously analyzed using postlabeling in one study, and a mean adduct level of 1.8 per 10<sup>8</sup> normal nucleotides was reported (Yang, Coles,

Delongchamp, Lang, & Kadlubar, 2002). Even in investigations in which, in principle, the same MS method was used for DNA from the same source (leukocytes), levels of N<sup>2</sup>-ethyl-dG differed substantially between laboratories (Balbo, et al., 2012b; Chen, et al., 2007; Matsuda, et al., 2006; Yukawa, et al., 2012). This could be because the source of the samples differed, or due to the effect of different time-frames from exposure to analysis with regard to the instability of the adduct (Paper IV). However, it is known that for other types of DNA lesions, there are substantial differences between laboratories, even if the same assay is used on identical samples (Ersson et al., 2013; Phillips & Castegnaro, 1999).

The levels reported for human leukocytes range from approximately one adduct per 10<sup>7</sup> normal nucleotides and upwards, which should be within the detection limit of the postlabeling assays used in this project.

The human biomarker studies in which DNA was treated with a reducing agent and N<sup>2</sup>-ethyl-dG was analyzed show that levels in leukocyte DNA vary from 3 to 200 per 10<sup>8</sup> normal nucleotides (Balbo et al., 2008; Balbo, et al., 2012b; Chen, et al., 2007; Matsuda, et al., 2006; Yukawa, et al., 2012), with most studies reporting levels of 10-40 per 10<sup>8</sup>. A very similar average level (40 per 10<sup>8</sup>) was detected in our study (Paper III). These levels are high compared to the reported levels of DNA adducts of other chemicals in human populations (Bartsch, 1996; Schoket, 1999) and indicate that there is substantial exposure to acetaldehyde (endogenous and/or dietary), or some other chemical that results in a DNA adduct which after reduction will form N<sup>2</sup>-ethyl-dG.

Moderate alcohol consumption (social drinking) by healthy men (Paper III) was not shown to result in statistically significant increases in levels of N<sup>2</sup>-ethyl-dG detected in leukocyte DNA, perhaps because these levels were already relatively high before consuming alcohol, which is similar to what was reported in another study (Balbo, et al., 2008). In comparison, analysis of the same biomarker in DNA from the oral mucosa showed strong effects due to alcohol consumption (Balbo, et al., 2012a). Therefore, it is possible that leukocyte DNA may not be a good target to analyze for this biomarker. Furthermore, the high background of this adduct in leukocyte DNA may hamper its application in biomarker studies of low exposures to acetaldehyde.

## 10.4 ADDUCT STABILITIES

N<sup>1</sup>-alkyl-dA will spontaneously convert to N<sup>6</sup>-alkyl-dA in so called Dimroth rearrangement (Plna, et al., 1999). This reaction will also take place for N<sup>1</sup>-GA-dA, but the rate is unknown (Gamboa da Costa, et al., 2003). In the postlabeling assay N<sup>1</sup>-GA-dA is forced to convert to N<sup>6</sup>-GA-dA by treatment with base, and both N<sup>1</sup>- and N<sup>6</sup>-GA-dA will be detected as the chemically stable N<sup>6</sup>-GA-dA adduct.

N<sup>6</sup>-Ethylidene-dA and N<sup>2</sup>-ethylidene-dG are reduced to N<sup>6</sup>-ethyl-dA and N<sup>2</sup>-ethyl-dG, respectively, in order to stabilize the adduct. Without prior stabilization, the reported background levels of N<sup>2</sup>-ethyl-dG (for DNA from various sources) are below detection limit (Matsuda et al., 2007; Nagayoshi, et al., 2009; Wang, et al., 2006). N<sup>2</sup>-ethyl-dG

adducts could not be detected in any of the DNA samples without prior reduction with NaBH<sub>3</sub>CN (Paper II) in contrast to some previous reports (Fang & Vaca, 1997; Wang, et al., 2006; Yang, et al., 2002).

We found that the loss of N<sup>2</sup>-ethylidene-dG (Paper IV) was considerably more rapid than reported in previous studies (Hori, et al., 2012; Wang, et al., 2006). In our study, we analyzed more samples over a longer time period and found that there was a considerable increase in adduct levels between 0 and 4 h. Between the peak level at 4 h and 8 h, there was a 50% loss of these adducts. This was not seen in the study by Hori and coworkers, as they only analyzed samples at 0, 24 and 48 h after exposure, whereas the increase and rapid loss took place between 0 h and 24 h. Taking this into consideration, our findings are similar to those reported in the other studies.

## 10.5 USEFULNESS OF ADDUCT MEASUREMENTS

Measuring adducts is a useful approach for determining the biological impact on the organism and as a biomarker of exposure in humans. In contrast, measuring the level of external exposure will normally not take into account factors like uptake, distribution, metabolism, gender, genetic variations, underlying disease, DNA repair and individual variations in those processes. For example, comparing the amount of alcohol consumed by two individuals does not provide much information about the genotoxic effects, not only because acetaldehyde is of greater interest than ethanol but also because the amounts and rate of acetaldehyde produced is affected by bodyweight, gender, metabolism, underlying disease and genetic variations resulting in differences in ADH and ALDH activity. This is also the case for glycidamide, as acrylamide affects the organism not only directly but also via the metabolite glycidamide.

In order to increase the usefulness of DNA adducts in biomarker studies of living human donors, it is important to establish the relationship between the adduct levels in the surrogate tissue (blood sample) and the target tissue (liver, brain, lung, etc.). Such relationships have so far only been shown in a few studies (Godschalk, et al., 2000) (Talaska, Au, Ward, Randerath, & Legator, 1987). Moreover, it is important to determine the chemical stability and rate of DNA repair of the adduct, i.e. analysis of very short-lived DNA adducts are more difficult to apply in human studies. Additionally, the relationship between exposure and adduct levels needs to be established in order to determine acceptable levels of exposure for a given carcinogen. In animal studies of acetaldehyde, for example, this is relatively simple, whereas humans are subjected to multiple sources of exposure through air, drink and food, both at work and at home. Because of these multiple sources of exposure and because people do not always accurately remember or admit to actual consumption of food or drink, assessing the precise levels of exposure is difficult. In addition, when the dose-response relationship is non-linear, underestimation of risk is possible; for example, a high level of occupational exposure may hold a similar risk as that of a lower level of environmental exposure. Once these uncertainties are addressed and an adduct is validated, the adduct may be used as a biomarker of exposure. However, very few



biomarkers have been validated to this degree, with aflatoxin biomarkers as a notable exception (Ross et al., 1992).

Validation of biomarkers is important to order to ensure that the measurements of the exposure are reliable and valid. To validate biomarkers it is necessary to use epidemiologic study designs (Vineis & Perera, 2000). A common design is the case-control study where adduct levels are analyzed and compared in samples taken from cancer cases and matched controls. In the more complicated prospective nested case-control study, an area where a particular type of cancer is endemic is chosen and biological samples are collected from thousands of people living in the area of interest. When a sufficient number of cancer cases have happened (which may take decades) the samples from cancer cases and chosen controls are analyzed. The cancer rates are calculated and compared between individuals positive for the biomarker and individuals negative for the biomarker.

The major components of biomarker variability that affect the design of epidemiological studies are variability between subjects, within subjects and variability due to measurement errors. Publication bias may also be important because statistically significant outcomes, generally, are more likely to be published than non-significant studies (Stern & Simes, 1997).

## **10.6 BENEFITS AND LIMITATIONS OF THE $^{32}\text{P}$ -POSTLABELING METHOD**

In comparison to MS, the main benefit of the  $^{32}\text{P}$ -postlabeling method used in this thesis was its sensitivity, as it enabled the detection of very small changes while requiring small sample sizes. For in vivo studies, this is of importance, as the samples from living donors are by nature limited in amount. This postlabeling method also allows for the detection of small changes to the DNA, making it possible to detect effects early, before the effects on a large population could be observed. In addition, LC can detect adducts without discrimination, which makes it possible to study the effects of multiple exposures, and LC is cheap compared to MS.

The main limitation of the  $^{32}\text{P}$ -postlabeling method is that it is time consuming, currently rendering it of little use for large-scale studies. However, if this limitation could be overcome, this approach may be useful to accurately measure the accumulated impact of, for instance, acetaldehyde on a larger population and compare the internal dose to the observed cancer incidence. As a result, there would be better information available to determine acceptable levels of exposure from, for example, dietary sources such as yogurt.

## 11 CONCLUSION AND FUTURE PERSPECTIVES

In the scope of this study, highly sensitive  $^{32}\text{P}$ -postlabeling methods for analysis of the acrylamide-derived N1-GA-dA, and the acetaldehyde-derived N<sup>2</sup>-ethyl-dG, were developed. Both adducts were successfully analyzed in DNA and cells and the methods could be further used for studies of adduct levels after different exposures to acrylamide or acetaldehyde.

- The postlabeling assay was used for the first time to detect the DNA adduct N1-GA-dA (analyzed as N<sup>6</sup>-GA-dA) from glycidamide. However, this adduct could not be detected in liver DNA of mice exposed to acrylamide. One first attempt to try to find out why could be to use other experimental conditions, different sources of acrylamide, different routes of exposure, longer exposure times or use other detection methods to compare adduct levels.
- The DNA adduct N<sup>6</sup>-ethylidene-dA (analyzed as N<sup>6</sup>-ethyl-dA) was for the first time detected in DNA treated with acetaldehyde. Further investigations are needed to optimize the method and to see if the adduct is spontaneously formed in vivo. Regarding N<sup>2</sup>-ethylidene-dG, the stability of the adduct is an interesting and important task to study further as it may be an important factor when investigating adduct levels after consumption of alcohol or other exposures to acetaldehyde.

A new LC-MS/MS method in conjunction with online-column switching valve technology was developed for the detection of N<sup>2</sup>-ethyl-dG in cannabis cigarette smoke and in human lung DNA and was also used in the human intervention study.

- For the cannabis and cigarette study, further investigations of the N<sup>2</sup>-ethyl-dG-levels in samples from smokers and nonsmokers would be interesting to shed light on the fact that no significant differences were found, even though it is known that tobacco smoke contains acetaldehyde.
- Our human study involving male non-smoking individuals from a homogeneous Caucasian population showed no significant difference in N<sup>2</sup>-ethyl-dG levels in leukocyte DNA after social drinking. The inter-individual variations were substantial even though the polymorphisms known to decrease aldehyde dehydrogenase function occur at a low frequency in this population. Further studies in the same population would be interesting to determine the reason for these variations.

During the last decade or so there has been a substantial development in sensitivities of MS-based methods and for polar compounds, like DNA adducts, the introduction of LC, instead of GC, has made the methods easier to use (no derivatization needed). With these improvements the LC-MS/MS-based methods are approaching the postlabeling assay in sensitivity and they are most likely the assays of the future for adduct measurements.

Our risk for cancer may increase with combinations of particular DNA adducts. One challenging field is to combine studies of different adducts with other factors involved with cancer risk, such as inflammation. Another challenging field is to reduce DNA damage to lower the incidence of cancer if it is not possible to lower the exposure to a chemical carcinogen, such as cooking carcinogens.

A recent concept is to measure the “exposome”, all the exposures of an individual in a lifetime and how those exposures relate to health (Wild, 2005). In the adduct field this could be translated into “adductomics” an area which was initiated by Dr Matsuda and co-workers when he used MS for measuring a number of different adducts at the same time in one DNA sample (Kanaly et al., 2006). This area has since then been further developed by Dr Rappaport and co-workers (Rappaport, Li, Grigoryan, Funk, & Williams, 2012). In short adductomics means that one is not measuring a single adduct, but investigating the total load of adducts to that DNA sample. Such methods would be of great value, particularly if applied to DNA stored in biobanks. For instance, if cancer incidence has increased in the population, it would be possible to compare the stored DNA from the cancer cases to matched controls and analyze the load of DNA damage to see if any conclusions can be drawn from similarities or differences.

In Finland, a heated debate about the risks of acetaldehyde has had ample media coverage the last few years. Worldwide media coverage after the reports on acrylamide (2002) has led to questions that have been asked so many times that WHO has issued “FAQ – acrylamide in food”. EU member states are requested to perform yearly monitoring of acrylamide levels in food and EFSA’s Panel on Contaminants in the Food Chain (CONTAM Panel) is drafting a risk assessment, to be published in 2014.

It would be easier to make an accurate risk assessment if one would be able to measure the actual levels of exposure rather than making educated guesses. Biomarkers of exposure can fill part of the gap between guesswork and knowledge.

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